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## CpG Blocks Immunosuppression by Myeloid-Derived Suppressor Cells in Tumor-Bearing Mice

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**Cancer Therapy: Preclinical**

See commentary p. 1645

**CpG Blocks Immunosuppression by Myeloid-Derived Suppressor Cells in Tumor-Bearing Mice**

Christine Zoglmeier, Helen Bauer, Daniel Nörenberg, Georg Wedekind, Philipp Bittner, Nadja Sandholzer, Moritz Rapp, David Anz, Stefan Endres, and Carole Bourquin

**Abstract**

**Purpose:** The Toll-like receptor (TLR) 9 ligand CpG has been used successfully for the immunotherapy of cancer. Chronic CpG application in tumor-free hosts leads, however, to the expansion of myeloid-derived suppressor cells (MDSC), which can cause T-cell suppression and may thus hamper the development of an effective immune response. Here, we investigated the effect of TLR9 activation on the function of MDSC in tumor-bearing mice.

**Experimental Design:** We investigated the effect of CpG treatment on the number, phenotype, and function of MDSC in mice bearing subcutaneous C26 tumors and in CEA424-Tag mice bearing autochthonous gastric tumors.

**Results:** CpG treatment blocks the suppressive activity of MDSC on T-cell proliferation in both tumor models. Inhibition of MDSC function by CpG was particularly pronounced for a highly suppressive Ly6G<sup>hi</sup> polymorphonuclear subset of MDSC. We further show that TLR9 activation by CpG promotes maturation and differentiation of MDSC and strongly decreases the proportion of Ly6G<sup>hi</sup> MDSC in both tumor-bearing and tumor-free mice. We demonstrate that IFN- $\alpha$  produced by plasmacytoid dendritic cells upon CpG stimulation is a key effector for the induction of MDSC maturation *in vitro* and show that treatment of mice with recombinant IFN- $\alpha$  is sufficient to block MDSC suppressivity.

**Conclusions:** We show here for the first time that TLR9 activation inhibits the regulatory function of MDSC in tumor-bearing mice and define a role for the antitumoral cytokine IFN- $\alpha$  in this process. *Clin Cancer Res*; 17(7); 1765–75. ©2011 AACR.

**Introduction**

Neoplastic growth leads to the expansion of a population of immature myeloid cells termed myeloid-derived suppressor cells (MDSC) in a wide range of murine tumor models and human cancers (1, 2). MDSC accumulate systemically due to a tumor-induced maturity block preventing differentiation of myeloid precursor cells into antigen-presenting cells (3). They strongly suppress effector T-cell proliferation *in vitro* and are thought to contribute to tumor-related immunosuppression *in vivo* by inducing T- and NK-cell dysfunction (4, 5). Murine MDSC are defined by the coexpression of the granulocyte differentiation antigen Gr1 (constituted by the 2 epitopes

Ly6G and Ly6C) and the  $\alpha 1\beta M$  integrin CD11b (1). MDSC represent a heterogeneous cell population, further subdivided by the differential expression of the Ly6G and Ly6C epitopes (6–8). Depending on the tumor model, additional surface markers (4, 9, 10) and the employment of different immunosuppressive mechanisms have been described. Reported mechanisms of T-cell inhibition through MDSC include depletion of the essential amino acids arginine (11) and cysteine (12) and the production of reactive oxygen and nitrogen species mediated by the expression of the arginine-metabolizing enzymes arginase I and inducible nitric oxide synthase (iNOS; refs. 13, 14).

We have demonstrated previously that activation of the innate immune system with the Toll-like receptor (TLR) 9 ligand CpG evokes efficient antitumor immune responses in mice bearing subcutaneous C26 tumors (15–17), despite the presence of large numbers of highly immunosuppressive MDSC in this model (8, 9). We hypothesized that TLR9 activation must therefore overcome MDSC-related immunosuppression, either by affecting MDSC numbers or MDSC function. However, chronic activation with CpG using a slow-release delivery system was reported to lead to immunosuppression in tumor-free mice (18). It was proposed that the observed immunosuppression may be mediated by the expansion of Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC-like

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### Translational Relevance

Myeloid-derived suppressor cells (MDSC) accumulate in tumor-bearing hosts and cancer patients. They play a major role in tumor-related immunosuppression and hamper successful immunotherapy approaches.

We show that short-term activation with the Toll-like receptor (TLR) 9 ligand CpG, which is clinically used as a vaccine adjuvant, blocks the suppressive function of MDSC in tumor-bearing mice. CpG treatment promotes the differentiation of MDSC and restores the disturbed balance of mature and immature myeloid cells through the induction of IFN- $\alpha$ .

Our results, confirmed in a subcutaneous and an autochthonous murine model, underline the benefit of short-term CpG treatment in cancer patients, because it restores immune responsiveness and improves the prospects for successful antitumor vaccination. In addition, we provide a rationale for the clinical application of IFN- $\alpha$  in cancer patients, by showing for the first time that this cytokine promotes maturation and abrogates suppressivity of MDSC.

immunosuppressive cells, arguing against the use of CpG in antitumor vaccines.

To address this issue, we investigated the impact of CpG treatment on the number, phenotype, and function of MDSC both in tumor-free mice and in mice bearing subcutaneous C26 tumors or autochthonous gastric tumors.

### Materials and Methods

#### Mice and cell lines

Female Balb/c mice or C57BL/6 mice (8–12 weeks old) were obtained from Harlan-Winkelmann (CEA424-Tag mice, ref. 19; C57BL/6 background, kindly provided by W. Zimmermann, LIFE Center, Ludwig-Maximilian University of Munich, Munich, Germany) were bred heterozygously from transgenic male mice. IFN-I receptor (IFNAR)-deficient mice on C57BL/6 background were kindly provided by Dr. Z. Waibler (Paul-Ehrlich Institute, Langen, Germany). All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The B16 melanoma cell line and the C26 colon carcinoma cell line were obtained from CLS.

#### Flow cytometry

Single cell suspensions were stained with fluorochrome-conjugated monoclonal antibodies (clones shown in brackets) against mouse Gr1 (RB6-8C5), Ly6G (1A8), Ly6C (AL-21), CD11b (M1/70), CD11c (HL3), F4/80 (CI:A3-1), CD80 (16-10A1), CD86 (GL1), MHCII (NIMR-4), Sca1 (D7), CD3 (145-2C11), and CD4 (RM4-5; BD Biosciences, BioLegend or Southern Biotech). Tumor cell suspensions were obtained by digesting dissected tumor tissues with collagenase D (1 mg/mL) and DNase

I (0.05 mg/mL; both Sigma-Aldrich) for 40 minutes at 37°C and subsequent passage through a 70- $\mu$ m pore cell mesh. Analysis was performed using a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

#### Isolation of MDSC

Total MDSC were purified from splenocyte suspensions by magnetic cell sorting (EasySep System, StemCell Technologies) using biotinylated anti-Gr1 antibodies following the instructions of the manufacturer. In tumor-bearing mice, approximately 98% of Gr1<sup>+</sup> cells were CD11b<sup>+</sup> and purity was typically greater than 95%. In tumor-free mice, 10% to 15% of Gr1<sup>+</sup> cells were CD11b negative. For *in vitro* activation assays, untouched MDSC were isolated by negative sorting to avoid stimulation using a custom selection kit containing anti-CD3, CD5, CD19, CD45R/B220, and Ter119 antibodies (StemCell Technologies). Purity was typically above 90%. For the separation of Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC, a 3-step sequential purification protocol was established. Briefly, splenocytes were depleted of T cells, B cells, and dendritic cells (DC) using biotinylated anti-CD3 antibodies followed by magnetic selection with anti-biotin, anti-CD19, and anti-CD11c microbeads (Miltenyi Biotec). For isolation of Ly6G<sup>hi</sup> MDSC, CD3<sup>-</sup>B220<sup>-</sup>CD11c<sup>-</sup> splenocytes were positively selected using an anti-Ly6G Microbead Kit (Miltenyi Biotec). In a second purification step, the Ly6G-depleted fraction was positively sorted using biotinylated anti-Gr1-biotin and anti-biotin microbeads to recover the remaining Ly6G<sup>lo</sup>Gr1<sup>+</sup> MDSC. Ly6G eluates typically contained greater than 95% Ly6G<sup>hi</sup> cells and Gr1 eluates greater than 90% CD11b<sup>+</sup> cells.

#### Generation of plasmacytoid DC

After erythrocyte lysis, bone marrow cells were incubated in RPMI supplemented with 1 mmol/L sodium pyruvate, 2 mmol/L nonessential amino acids, (1  $\times$  10<sup>-4</sup>%) 2-mercaptoethanol (complete RPMI) and 20 ng/mL recombinant Flt3-Ligand (Tebu-Bio) for 8 days. Plasmacytoid DCs were purified by magnetic bead separation using anti-B220 (CD45R) microbeads (BD Biosciences) with a purity of greater than 90% B220<sup>+</sup> cells.

#### *In vitro* maturation

Plasmacytoid DC cultures (1  $\times$  10<sup>6</sup> cells per well in 24-well plates) were stimulated with 6  $\mu$ g/mL CpG for 2 hours, washed 3 times with complete RPMI medium, and incubated for an additional 24 hours. Conditioned supernatants were collected and stored at -80°C until use. A total of 1  $\times$  10<sup>5</sup> purified MDSC per well were seeded onto 96-well plates and incubated in triplicate with undiluted conditioned supernatants, recombinant IFN- $\alpha$  (300 IU per well; Miltenyi), or CpG1826 (6  $\mu$ g/mL). In some conditions, neutralizing antibodies against IFN- $\alpha$  (2  $\mu$ g/mL; PBL Interferon Source), IL-2 (20  $\mu$ g/mL), IL-6 (5  $\mu$ g/mL), IL-10 (5  $\mu$ g/mL), or IL-12 (20  $\mu$ g/mL) were added (all R&D Systems). After 40 hours, expression of surface markers on MDSC was determined by flow cytometry.

### T-cell proliferation and ELISA

For evaluation of MDSC suppressivity,  $1 \times 10^5$  splenocytes or  $7.5 \times 10^4$  splenic T cells (DynaMouse Negative T Cell Isolation Kit, Invitrogen) from naive mice were cultured with different ratios of MDSC in flat-bottom 96-well plates in complete RPMI medium. T-cell proliferation was stimulated by the addition of anti-CD3/anti-CD28-coated microbeads (Invitrogen) at a bead-to-cell ratio of 1:75. Cells were cultured for 72 hours and 5-bromo-2'-deoxyuridine (BrdU; Roche Diagnostics) was added for the last 12 hours of culture. Proliferation was measured using a chemiluminescence-based BrdU detection assay (Roche Diagnostics) according to the manufacturer's protocol. Interleukin (IL)-2 and IFN- $\gamma$  were measured after 72 hours in culture supernatants using BD OptEIA Kits (BD Biosciences). Relative proliferation or cytokine production was calculated relative to stimulated responder cells cultured without MDSC. All assays were performed in triplicate.

### Tumor induction and *in vivo* treatment

For tumor induction,  $2.5 \times 10^5$  C26 cells suspended in 100  $\mu$ L PBS were injected s.c. into the flank of Balb/c mice or  $10^6$  B16 cells were injected s.c. into the flank of C57BL/6 and IFNAR-deficient mice. For s.c. tumors, tumor area is expressed as the product of perpendicular diameters of individual tumors. The volume of CEA424-TAg-derived gastric tumors was calculated as the product of 3 perpendicular diameters. Treatment was initiated following establishment of tumors (tumor area > 25 mm<sup>2</sup> or age of CEA424-TAg mice > 85 days) unless indicated otherwise. Mice received s.c. injections of 100  $\mu$ g phosphorothioate (PTO)-modified CpG1826 from Coley Pharmaceutical Group (5'-TCC ATG ACG TTC CTG ACG TT-3') or i.p. injections of  $5 \times 10^4$  IU recombinant IFN- $\alpha$  from Miltenyi Biotech or 250  $\mu$ g poly(I:C) from Invitrogen.

### Statistical analysis

Statistical significance was determined by unpaired Student's *t* test using GraphPad Prism 5 (GraphPad Software). Comparison of multiple groups was performed using 1-way ANOVA with pair-wise Bonferroni posttests. Tumor sizes were analyzed using the Mann-Whitney *U* test. All data are expressed as mean  $\pm$  SEM. A 2-tailed value of *P* < 0.05 was considered significant.

## Results

### CpG therapy leads to expansion of Gr1<sup>+</sup>CD11b<sup>+</sup> cells in tumor-free mice but does not increase their suppressive function

Cells with a Gr1<sup>+</sup>CD11b<sup>+</sup> phenotype, which are normal myeloid cell precursors, are present in low numbers in tumor-free mice (<4% of spleen cells). To examine the effect of immune stimulation with CpG oligonucleotides (CpG) on the number and function of these cells, we treated naive mice with s.c. injections of CpG and subsequently analyzed the proportion of these Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC-like cells in spleen and bone marrow. CpG stimula-

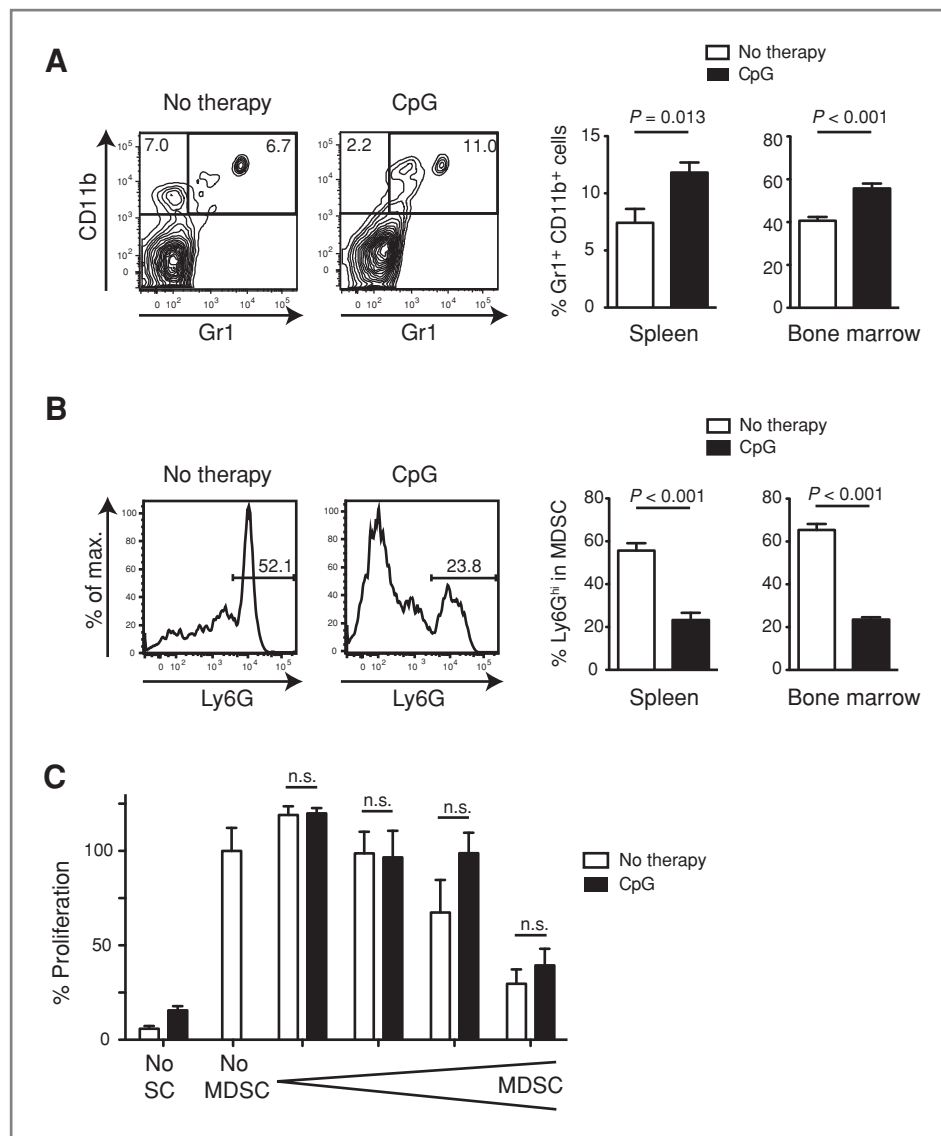
tion led to an increase in Gr1<sup>+</sup> cells within the CD11b<sup>+</sup> cell population, resulting in a higher proportion of MDSC-like cells in the spleen of CpG-treated mice, as reported previously (Fig. 1A; ref. 18). This was paralleled by an increase in the proportion of MDSC-like cells in the bone marrow of CpG-treated mice, suggesting that splenic expansion was not caused by increased mobilization from the bone marrow. Since the differential expression of the Ly6G epitope has been used to distinguish between MDSC subpopulations (7, 20), we investigated the expression level of this marker on Gr1<sup>+</sup>CD11b<sup>+</sup> cells following CpG treatment. Interestingly, the upregulation of Gr1 was associated with a decrease in Ly6G expression by MDSC-like cells, resulting in a more than 2-fold reduction in the proportion of Ly6G<sup>hi</sup> MDSC-like cells after CpG treatment in both spleen and bone marrow (Fig. 1B).

We then examined the suppressive function of splenic MDSC-like cells following CpG stimulation. Purified Gr1<sup>+</sup>CD11b<sup>+</sup> cells from both untreated and CpG-treated mice showed a moderately suppressive effect on the proliferation of T cells from naive mice activated with anti-CD3/CD28 antibodies (Fig. 1C). There was no change in suppressivity following CpG treatment. We thus see no evidence for activation of Gr1<sup>+</sup>CD11b<sup>+</sup> cells toward highly suppressive MDSC following *in vivo* immune stimulation through TLR9.

### CpG therapy inhibits the suppressive function of MDSC in tumor-bearing mice

We next investigated the impact of CpG treatment on MDSC in mice bearing established C26 tumors. We selected a CpG treatment protocol leading to a significant reduction of tumor size without complete rejection (Fig. 2A). Following CpG therapy, splenic MDSC were analyzed by flow cytometry. As expected, the proportion of Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC was increased in untreated C26 tumor-bearing mice compared with tumor-free mice. There was, however, no further increase in the percentage of MDSC following short-term CpG treatment (Fig. 2B, left). In fact, when using longer treatment protocols, we observed a reduction in MDSC numbers (data not shown). As seen in tumor-free mice, we observed a reduction in the proportion of Ly6G<sup>hi</sup> MDSC following CpG treatment (Fig. 2B, right). Next, we examined the effect of CpG therapy on the suppressive function of MDSC in C26 tumor-bearing mice. Unexpectedly, MDSC derived from CpG-treated tumor-bearing mice were significantly less suppressive on the proliferation of activated T cells than MDSC isolated from untreated tumor-bearing mice (Fig. 2C). Furthermore, MDSC from CpG-treated animals suppressed IL-2 production by activated T cells less strongly than MDSC from untreated mice.

To determine whether the decrease in MDSC suppressivity following CpG treatment could also be observed in mice bearing spontaneously developing tumors, we examined the effect of CpG treatment in CEA424-TAg transgenic mice. These mice develop spontaneous gastric tumors from the age of about 40 days (19). As observed in C26 tumor-bearing mice, the proportion of splenic MDSC was



**Figure 1.** CpG therapy leads to expansion of MDSC-like cells in tumor-free mice but does not increase their suppressive function. Balb/c mice received 3 s.c. injections of CpG at 3-day intervals. The percentage of (A) Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC-like cells (gated on live cells) and (B) Ly6G<sup>hi</sup> MDSC-like cells (gated on Gr1<sup>+</sup>CD11b<sup>+</sup> cells) in spleen and bone marrow of naïve or CpG-treated mice was determined by flow cytometry 2 days after the last treatment ( $n = 5$  per group). Plots and histograms show splenic MDSC-like cells. C, MDSC-like cells were isolated from spleens of naïve and CpG-treated mice and suppressivity was assessed in a T-cell proliferation assay using anti-CD3/CD28-stimulated splenocytes as responder cells (MDSC-to-splenocyte ratio: 1:16, 1:8, 1:4, and 1:2). Controls show anti-CD3/CD28-stimulated MDSC without splenocytes (no SC) and activated splenocytes without MDSC (no MDSC). n.s., not significant ( $P > 0.05$ ).

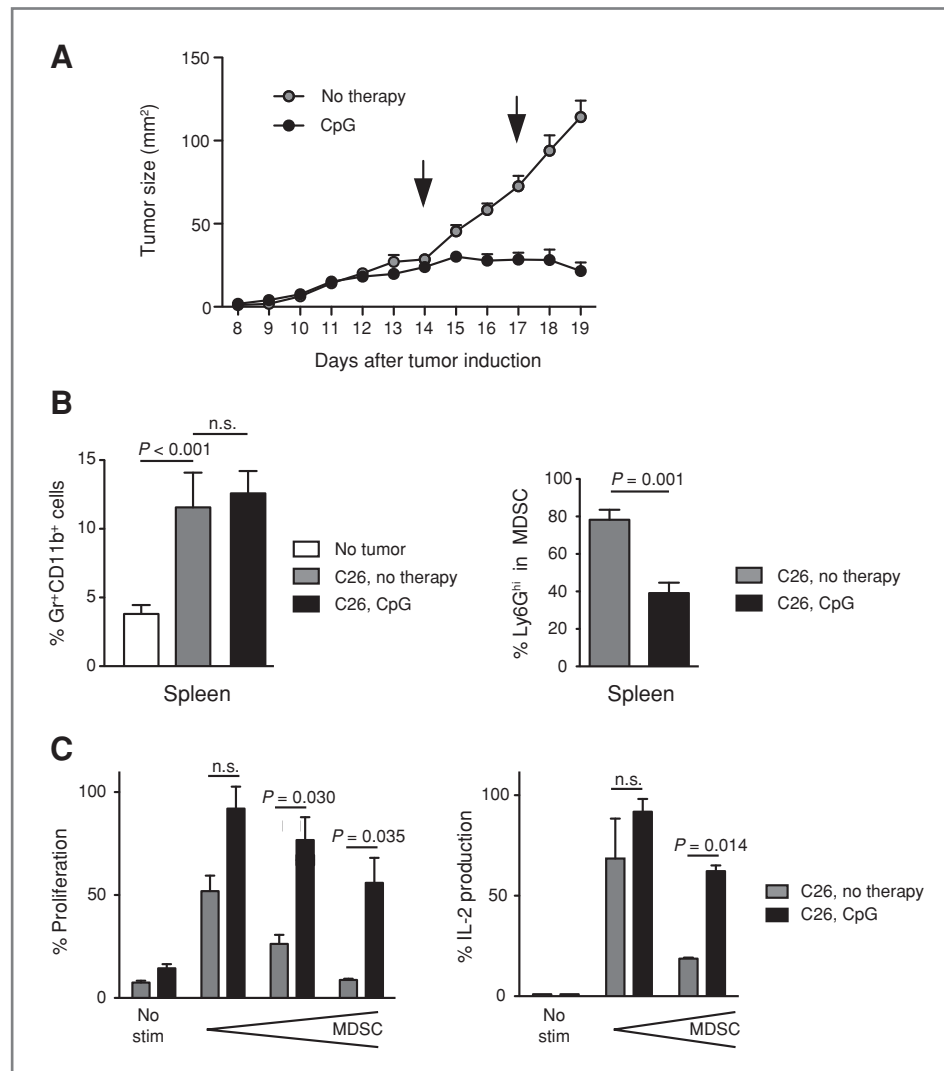
increased in 90- to 100-day-old CEA424-TAg mice, but CpG treatment did not lead to further expansion of MDSC (Fig. 3A). Furthermore, although there was no significant regression of the autochthonous gastric tumors, CpG therapy reduced MDSC suppressivity on both proliferation and IL-2 production of activated T cells (Fig. 3B and C). Thus, immune activation through TLR9 blocks the suppressive function of MDSC in mice bearing both induced and spontaneous tumors.

#### CpG therapy inhibits the highly suppressive Ly6G<sup>hi</sup> MDSC subset

Since CpG treatment decreases the proportion of Ly6G<sup>hi</sup> MDSC in favor of the Ly6G<sup>lo</sup> fraction, we developed a magnetic bead-based technique to isolate Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC to study the effect of CpG treatment on these subpopulations. With this procedure, Ly6G<sup>hi</sup> and

Ly6G<sup>lo</sup> MDSC populations could be isolated with high purity (Fig. 4A and see the "Materials and Methods" section). By histologic evaluation, purified Ly6G<sup>hi</sup> MDSC were exclusively polymorphonuclear, whereas the nuclear morphology of Ly6G<sup>lo</sup> MDSC was more heterogeneous, comprising both monocyte-like and polymorphonuclear cells (data not shown). To determine whether the purified MDSC subpopulations retained their suppressive function, we isolated Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC from C26 tumor-bearing mice and compared them in a functional assay to MDSC-like cells from tumor-free mice. Both tumor-derived MDSC subpopulations were significantly more suppressive than the respective subpopulations from tumor-free mice (Fig. 4B). We consistently observed throughout all experiments that the Ly6G<sup>hi</sup> MDSC fraction was more suppressive than the Ly6G<sup>lo</sup> fraction.

**Figure 2.** CpG treatment inhibits the suppressive function of MDSC in C26 tumor-bearing mice. Mice bearing established C26 tumors (tumor area > 25 mm<sup>2</sup>) were treated with CpG on day 14 and 17 after tumor induction (*n* = 5 per group). A, CpG treatment significantly reduced tumor size (*P* < 0.05 from day 15). B, the proportion of total splenic MDSC and Ly6G<sup>hi</sup> MDSC was assessed as in Figure 1. C, splenic Gr1<sup>+</sup> MDSC were isolated from untreated or CpG-treated tumor-bearing mice. Suppressivity was tested in a proliferation assay using splenocytes as responder cells (MDSC:SC ratio = 1:10, 1:5, and 1:2.5) or by measuring IL-2 production from purified T cells (MDSC:T cell ratio = 1:4 and 1:2). No stim, MDSC:responder cell ratio 1:2.5 without anti-CD3/CD28 stimulation.

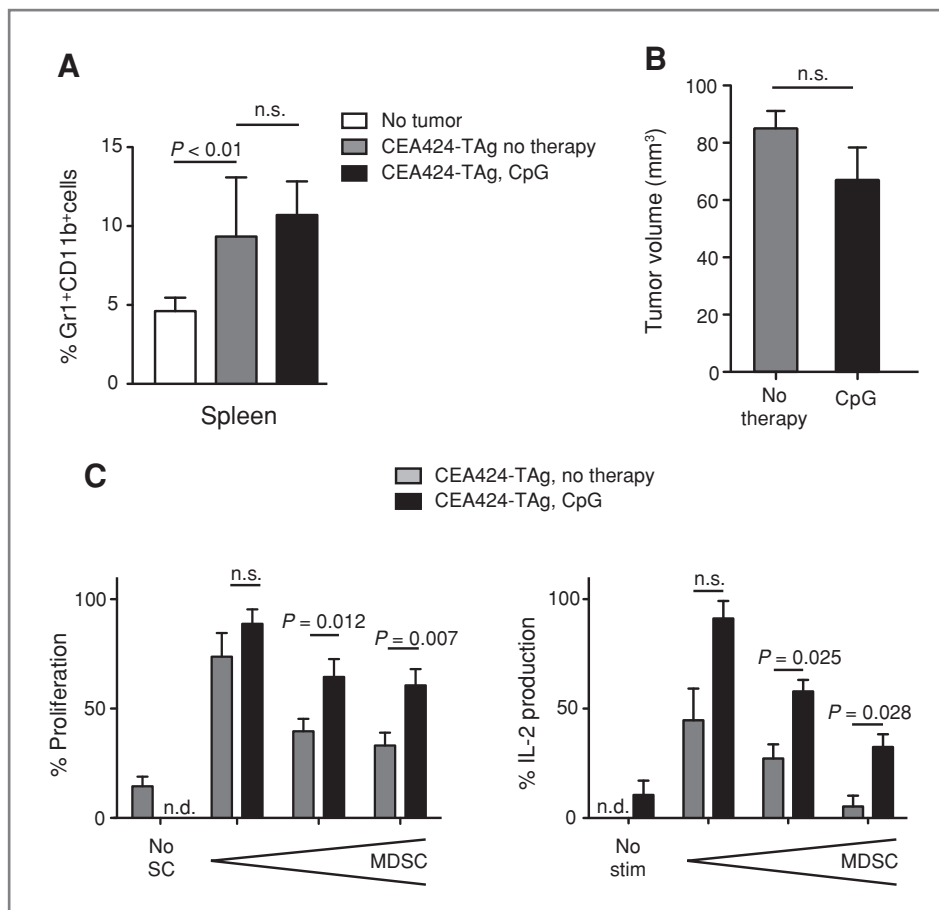


In a next step, we evaluated the effect of CpG treatment on the function of Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC subpopulations from C26 tumor-bearing mice. Suppressivity of isolated Ly6G<sup>hi</sup> MDSC on T-cell proliferation and on the production of the antitumoral cytokine IFN- $\gamma$  was inhibited by CpG therapy (Fig. 4C). For the Ly6G<sup>lo</sup> subpopulation, suppressivity was also reduced by CpG treatment, although the effect was not significant. Thus, CpG treatment inhibits the suppressive function of Ly6G<sup>hi</sup> MDSC and, in addition, decreases the proportion of these cells in tumor-bearing mice.

#### CpG induces the maturation and differentiation of MDSC

One of the defining traits of MDSC is their state of immaturity, characterized by low expression of markers associated with myeloid cell differentiation (3, 21). We confirmed that the macrophage differentiation marker F4/80, the DC maturation markers MHCII and CD11c,

and the costimulatory molecule CD80 are expressed at lower levels by splenic Gr1<sup>+</sup>CD11b<sup>+</sup> cells from C26 tumor-bearing mice than by the corresponding cells from tumor-free mice (Fig. 5A). In addition, we observed that the Ly6 protein family members Sca1 (Ly6A/E) and Ly6C are also expressed at lower levels by MDSC from tumor-bearing mice. When C26 tumor-bearing mice were treated with CpG, these maturation and differentiation markers were upregulated on MDSC to similar or even higher levels than those seen in tumor-free mice (Fig. 5B). The same effect was observed in CpG-treated CEA424-TAG mice (data not shown). Most of the investigated markers were upregulated on both the Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC subpopulations (Supplementary Fig. S1). Thus, CpG treatment provides a strong stimulus to promote MDSC maturation and differentiation in tumor-bearing mice and restores expression levels of myeloid cell markers to those expressed by Gr1<sup>+</sup>CD11b<sup>+</sup> cells in tumor-free mice.



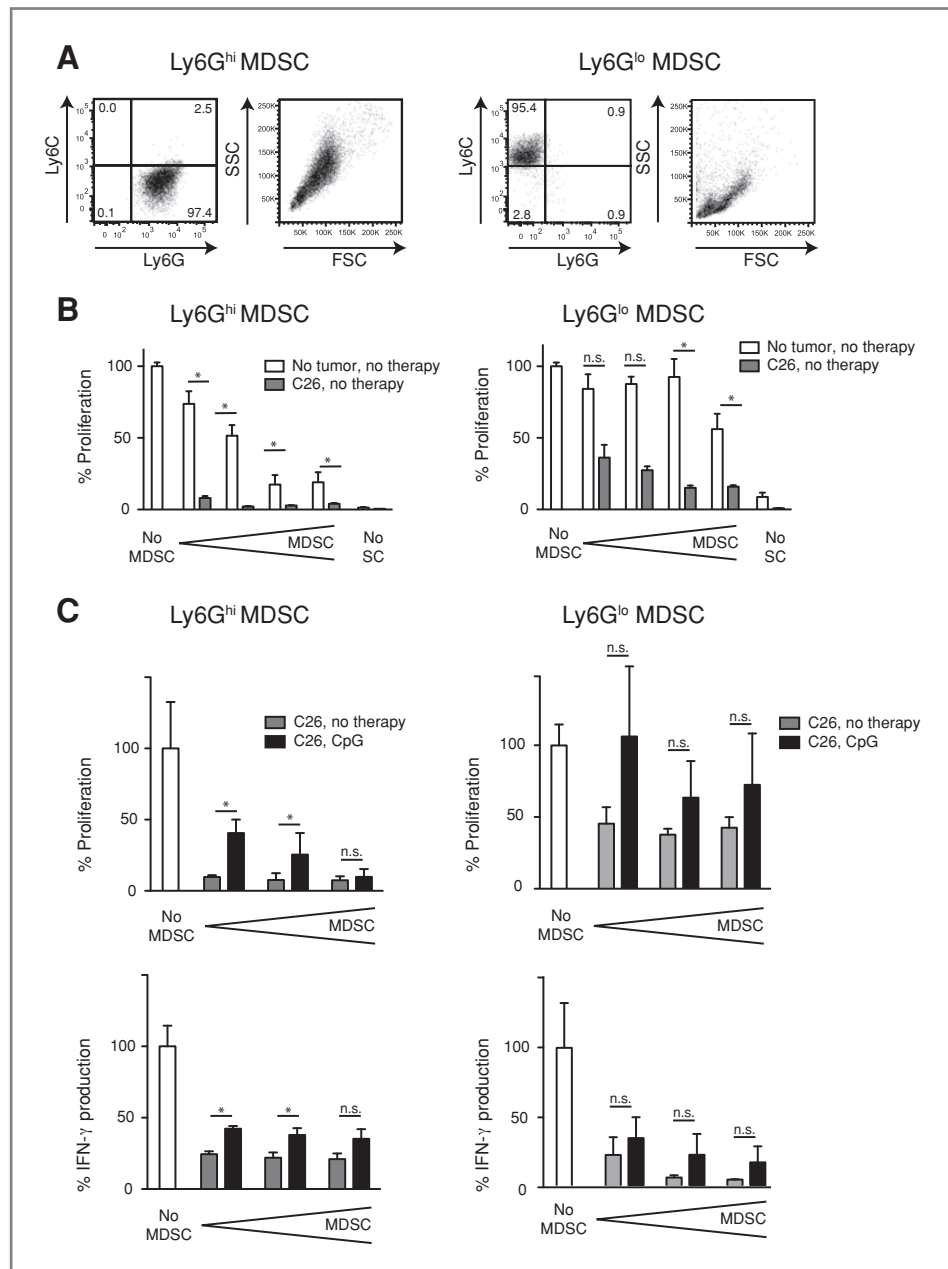
**Figure 3.** CpG treatment inhibits the suppressive function of MDSC in CEA424-TAg mice bearing spontaneous gastric tumors. CEA424-TAg mice aged 90 to 100 days received 3 s.c. injections of CpG at 3-day intervals. A, two days after the last treatment the proportion of splenic MDSC was analyzed in untreated tumor-free wild-type mice ( $n = 7$ ), untreated CEA424-TAg mice ( $n = 10$ ), and CpG-treated CEA424-TAg mice ( $n = 8$ ) and (B) size of gastric tumors was determined. C, MDSC suppression of T-cell proliferation and IL-2 production was determined (MDSC:SC ratio = 1:16, 1:8, and 1:4). No SC, anti-CD3/CD28-activated MDSC without splenocytes; n.s., not significant ( $P > 0.05$ ); n.d., not done (due to lack of material).

### CpG-induced maturation of MDSC is mediated by IFN- $\alpha$

To characterize the mechanisms leading to MDSC differentiation and maturation upon CpG treatment, we analyzed the expression of maturation markers on purified MDSC following stimulation with CpG. CpG stimulation of isolated MDSC did not, however, result in a significant upregulation of the differentiation markers Sca1 or F4/80 (Fig. 6A). Furthermore, no reduction in MDSC suppressivity was seen (Supplementary Fig. S2), suggesting that CpG cannot directly activate MDSC. In accordance with this hypothesis, we confirmed that the receptor for CpG TLR9 was expressed only at low levels on MDSC (Supplementary Fig. S3). DCs are one of the main cell populations that express TLR9 and are thus able to respond to CpG by rapid production of proinflammatory cytokines. We tested both myeloid and plasmacytoid DC subtypes for their ability to produce cytokines promoting MDSC differentiation following CpG stimulation. Culture of MDSC with supernatants from CpG-activated DCs led to an increase in MDSC expression of differentiation markers. The strongest increase in the expression of the markers Sca1 and F4/80 was seen when MDSC were cultured with supernatants from CpG-activated plasmacytoid DCs (Fig. 6A). A

key feature of plasmacytoid DC is the ability to secrete substantial amounts of type I IFN following TLR9 activation (22). Indeed, stimulation of isolated MDSC with recombinant IFN- $\alpha$  induced a significant upregulation of F4/80 (Fig. 6A) and Sca1 (not shown). The upregulation of these markers was increased upon addition of a cocktail of neutralizing antibodies against the proinflammatory cytokines IL-2, IL-6, IL-10, and IL-12, but was completely abolished when adding neutralizing antibodies against IFN- $\alpha$  to CpG-activated DC supernatants.

To investigate whether IFN- $\alpha$  can also promote MDSC maturation *in vivo*, we treated C26 tumor-bearing mice with recombinant IFN- $\alpha$  for 3 days. This short-term treatment had no effect on tumor size (data not shown). IFN- $\alpha$  stimulated maturation and differentiation of splenic and intratumoral MDSC (Fig. 6B), as seen with CpG therapy. Treatment of C26 tumor-bearing mice with the immunostimulatory RNA ligand poly(I:C), which induces high amounts of serum IFN- $\alpha$  (23, 24), resulted in phenotypic and functional changes in MDSC similar to those induced by CpG (Fig. 6C and Supplementary Fig. S4). Strikingly, the upregulation of Sca1 and other maturation markers observed upon poly(I:C) treatment was completely abolished in IFN-I receptor-deficient (IFNAR-KO) mice



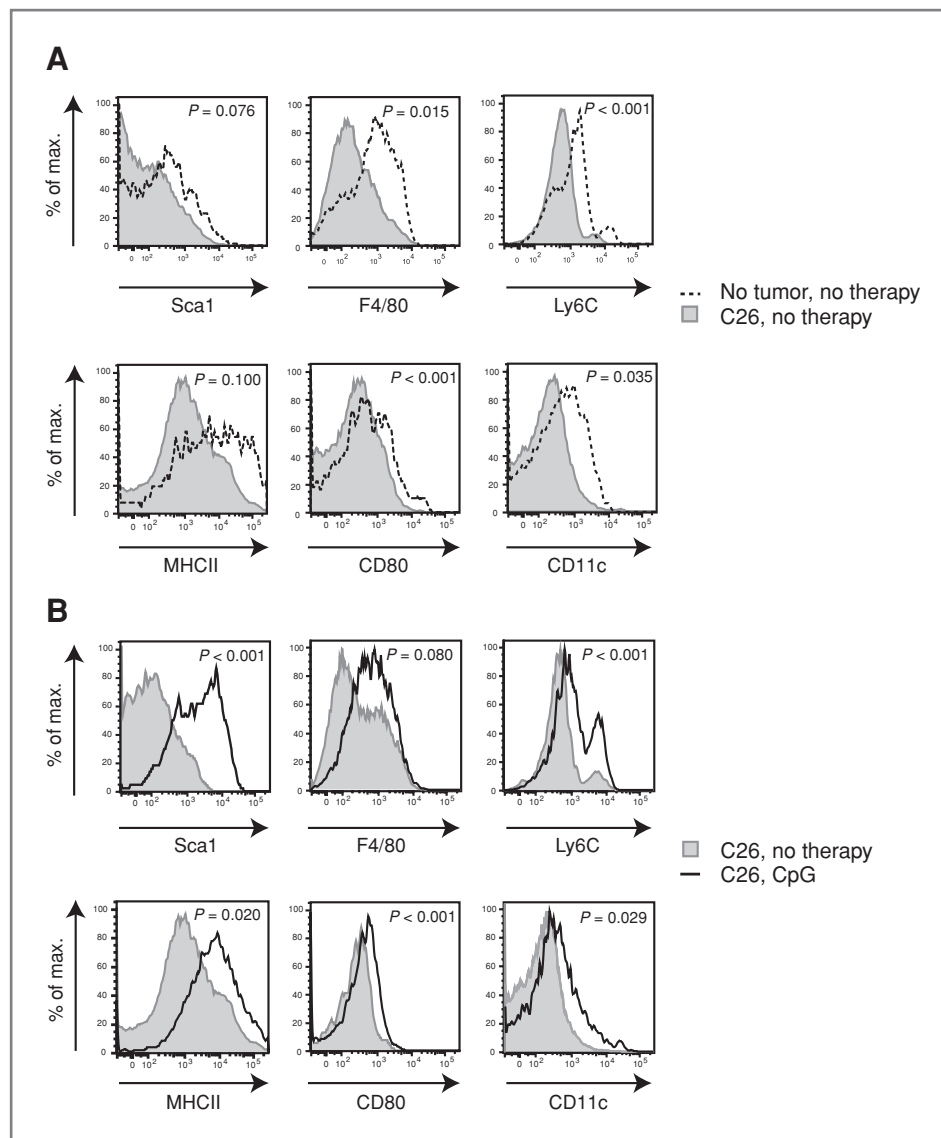
**Figure 4.** CpG therapy inhibits the highly suppressive Ly6G<sup>hi</sup> MDSC subset. Splenic Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC subpopulations were isolated by sequential MACS (magnetic activated cell sorting) separation. A, purity was verified by flow cytometry. Numbers indicate the percentage of Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC expressing Ly6G and Ly6C (gated on live cells). B, suppressivity of purified MDSC subpopulations derived from naive and C26 tumor-bearing mice on T-cell proliferation was assessed ( $2 \times 10^5$  SC, MDSC:SC ratio of 1:8, 1:4, 1:2, and 1:1). C, mice bearing established C26 tumors were treated with 3 CpG injections at 3-day intervals. Ly6G<sup>hi</sup> and Ly6G<sup>lo</sup> MDSC were purified 2 days after the last application. Suppressivity on T-cell proliferation and IFN- $\gamma$  production was examined as in B (MDSC:SC ratio: 1:8, 1:4, and 1:2). \*, significant ( $P < 0.05$ ) values; n.s., not significant ( $P > 0.05$ ); FSC, forward scatter; SSC, side scatter.

(Fig. 6C and data not shown), indicating that type I IFN is critically involved in MDSC maturation following innate immune activation.

To examine whether IFN- $\alpha$  treatment also affected the function of MDSC, we isolated Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC from untreated and IFN- $\alpha$ -treated tumor-bearing mice and compared their suppressivity. Indeed, MDSC from IFN- $\alpha$ -treated mice were less suppressive than MDSC from untreated mice (Fig. 6D). Thus, the *in vivo* application of recombinant IFN- $\alpha$  is sufficient to induce the striking phenotypic and functional changes in MDSC seen with CpG therapy.

## Discussion

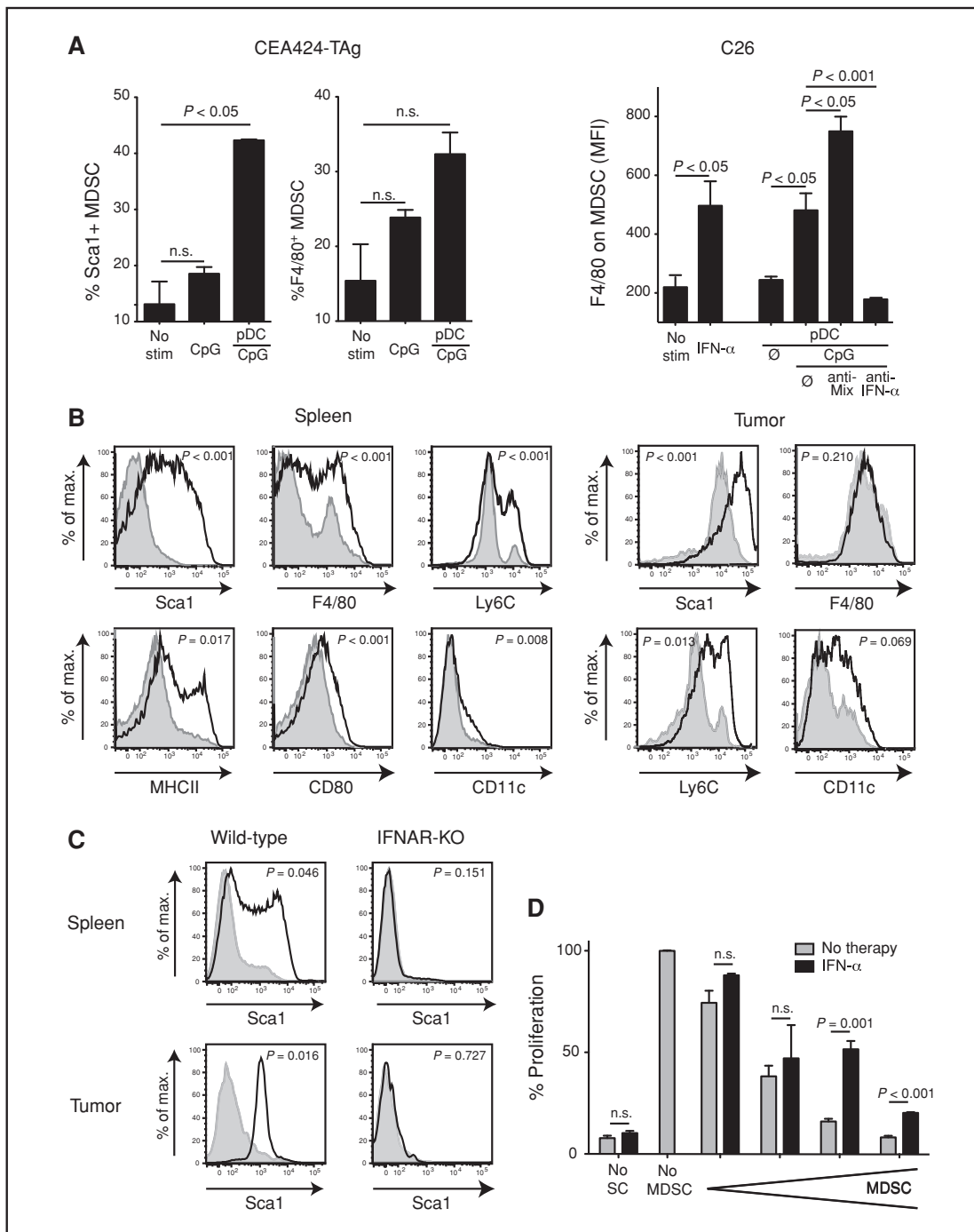
MDSC-mediated immunosuppression is observed in various inflammatory pathologies such as trauma, acute or chronic infections, sepsis, and cancer (1). To acquire their full immunosuppressive potential, MDSC require a first signal triggering their expansion, followed by a second stimulus to activate their suppressive function (1, 2). In polymicrobial sepsis, expansion of MDSC depends on TLR signaling through the adaptor molecule MyD88 (25). Expansion of MDSC-like cells can also be induced pharmacologically by chronic administration of single TLR



**Figure 5.** CpG induces maturation and differentiation of MDSC. A, the expression of different maturation and activation markers by splenic Gr1<sup>+</sup>CD11b<sup>+</sup> cells was analyzed in tumor-free and C26 tumor-bearing mice. Significances (*P*) indicate differences in the median marker expression between tumor-free and C26 tumor-bearing mice (*n* = 5 per group). Histograms show representative data from individual mice. B, Mice bearing established C26 tumors were treated with 3 s.c. injections of CpG every 3 days. Mice were sacrificed 2 days after the last treatment. Significances (*P*) indicate differences in the median marker expression between untreated and CpG-treated mice (*n* = 5 per group).

ligands, as shown for TLR2, TLR3, TLR4, and TLR9 (18). It was proposed that TLR stimulation also delivers the second signal required to activate MDSC suppressivity (1, 26, 27). Because TLR9 ligands are used in cancer vaccines, where MDSC activation would be deleterious for the clinical outcome, we investigated the effect of TLR9 stimulation on the suppressive function of MDSC. In tumor-free mice, we confirmed that CpG therapy leads to an expansion of MDSC-like cells, but we observed that CpG-expanded cells were not activated toward higher suppressivity. Thus, whereas expansion of MDSC-like cells in tumor-free mice can be triggered by several TLR ligands, activation of suppressivity may be limited to selected TLR ligands. In fact, evidence for an activation of MDSC suppressivity has so far only been provided for the TLR4 ligand lipopolysaccharide (LPS; refs. 26, 27), which may therefore hold a unique position among TLRs.

In tumor-bearing hosts, both the expansion and the activation of MDSC are triggered by endogenous factors released upon neoplastic growth (28). This leads to large numbers of highly suppressive, immature MDSC, which are thought to contribute to tumor-associated immunosuppression (29). In our study, we demonstrate for the first time that CpG treatment effectively blocks the suppressive activity of MDSC in C26 tumor-bearing mice. We further confirmed these findings in transgenic mice bearing autochthonous gastric tumors. While the CpG treatment protocol used here effectively reduced growth of s.c. C26 tumors, no reduction was seen for the autochthonous gastric tumors, suggesting that the effect of CpG treatment on MDSC function is at least partially independent of tumor size. Indeed, MDSC suppressivity was significantly reduced by short-term IFN- $\alpha$  treatment, which did not affect tumor size. We further investigated the effect of



**Figure 6.** CpG-induced maturation of MDSC is mediated by IFN- $\alpha$ . **A**, splenic MDSC were isolated from CEA424-TAg mice (left) and cultured for 40 hours in medium supplemented with CpG or IFN- $\alpha$  or with supernatants from CpG-activated plasmacytoid DC (pDC). The percentage of Sca1-positive MDSC or the median fluorescence intensity of F4/80 on MDSC was determined by flow cytometry. Splenic MDSC were isolated from C26 tumor-bearing mice (right) and incubated with recombinant IFN- $\alpha$  or conditioned supernatants from unstimulated or CpG-stimulated plasmacytoid DC. A cocktail of neutralizing antibodies against IL-2, IL-6, IL-10, and IL-12 (anti-Mix) or against IFN- $\alpha$  alone (anti-IFN- $\alpha$ ) was added to cultures with CpG-activated pDC supernatants. The median expression of F4/80 on MDSC was determined after 40 hours. **B**, mice bearing large C26 tumors (tumor area >100 mm<sup>2</sup>) were treated with 3 i.p. injections of recombinant IFN- $\alpha$ . Histograms show the changes in the expression of activation markers on splenic and intratumoral Gr1<sup>+</sup>CD11b<sup>+</sup> MDSC of representative individual mice (gray, no therapy; black line, IFN- $\alpha$ ). **C**, wild-type or IFN-I receptor-deficient (IFNAR-KO) mice bearing s.c. B16 tumors (tumor area approx. 50 mm<sup>2</sup>) were treated once with poly(I:C). Histograms show the changes in Sca1 expression on MDSC 2 days after treatment [gray, no therapy; black line, poly(I:C)]. **B** and **C**, significances (*P*) indicate differences in median expression between untreated and IFN- $\alpha$ -treated mice (*n* = 5 per group). **D**, isolated MDSC from C26 tumor-bearing mice treated with IFN- $\alpha$  were tested for suppressivity in a proliferation assay (MDSC:SC ratio = 1:16, 1:8, 1:4, and 1:2).

CpG and IFN- $\alpha$  treatment on 2 of the mechanisms by which MDSC inhibit T-cell proliferation. We observed a decrease in the production of reactive oxygen species and in arginase activity of MDSC following CpG and IFN- $\alpha$  treatment, although neither effect was significant (Supplementary Fig. S5). The decrease in MDSC suppressivity induced by CpG may thus be due to a combination of these suppressive mechanisms and other factors such as iNOS activity and cysteine depletion.

Inhibition of MDSC suppressivity by CpG treatment was associated with the induction of maturation and differentiation of MDSC. As the suppressive function of MDSC is closely linked to their immature state (30), the induction of differentiation is an essential step in restoring normal immune responses. Inhibition of suppressivity was most evident in a polymorphonuclear Ly6G<sup>hi</sup> subpopulation of MDSC that is preferentially expanded in a wide range of tumors (8).

Immune activation by TLR ligands *in vivo* leads to the production of many proinflammatory cytokines (31). A hallmark of stimulation through TLR7 and TLR9 is, however, the induction of high levels of IFN- $\alpha$  that are produced by a specialized population of DC, the plasmacytoid DC (32). We show here that IFN- $\alpha$ , a cytokine shown to have antitumoral activity in murine tumor models including C26 colon carcinoma (33, 34), is sufficient to induce MDSC maturation and block their suppressive function. Our findings suggest that IFN- $\alpha$  plays a major role in mediating the functional and phenotypic changes in MDSC following CpG therapy. In accordance with this hypothesis, we observed a reduction in MDSC suppressivity when treating tumor-bearing mice with the TLR3 and MDA-5 ligand poly(I:C), which induces high amounts of type I IFN (35), but not with the TLR4 ligand LPS (data not shown).

Among the surface proteins upregulated following CpG therapy are the Ly6 protein family members Sca1 (Ly6A/E) and Ly6C that have been used as activation markers for T lymphocytes (36, 37). We show for the first time that Sca1 and Ly6C are strongly upregulated by MDSC in response to CpG or IFN- $\alpha$  treatment and that they represent highly sensitive activation markers for this cell population. Furthermore, their upregulation inversely correlates with the suppressive activity of MDSC. Similarly, we observed upregulation of the macrophage marker F4/80 by MDSC following CpG treatment. Interestingly, within the tumor microenvironment, the increased expression of F4/80 by

Gr1<sup>+</sup>CD11b<sup>+</sup> cells indicates differentiation into tumor-associated macrophages, which are associated with T-cell suppression (38). In contrast, expression of F4/80 by splenic macrophages does not confer immunosuppressivity (38). We detected no upregulation of F4/80 by tumor-infiltrating MDSC upon IFN- $\alpha$  treatment, suggesting that there is no detrimental differentiation into tumor-associated macrophages. Generally, upregulation of maturation and differentiation markers following TLR and IFN- $\alpha$  treatment was also observed for intratumoral MDSC. However, the effects were less pronounced and there was more variability between individual animals, a fact that may reflect the decreased availability of CpG-induced cytokines within the tumor.

Taken together, our findings provide further evidence for the concept that overcoming the maturity block of MDSC is a highly promising approach to abrogate MDSC-related immunosuppression and to improve the success of cancer vaccines. Previously, successful approaches have been undertaken to promote MDSC maturation with all-trans retinoic acid (39) and with 5-aza-2-deoxycytidine (40). Here, we provide further evidence for the remarkably high plasticity of these cells by showing that CpG, through IFN- $\alpha$  induction, is highly efficient in promoting maturation and reducing suppressivity of MDSC. CpG treatment completely reverses MDSC subset composition, induces maturation, and inhibits suppressivity in 2 different tumor models. We thus describe a novel mechanism by which TLR9 ligands promote antitumor immune responses.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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