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Enhanced suppressive capacity of tumor-infiltrating myeloid-derived suppressor cells compared with their peripheral counterparts

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Although the main site of action for myeloid-derived suppressor cells (MDSCs) is most likely the tumor microenvironment, so far the study of these cells has been largely restricted to spleen-derived MDSCs. In this study, we compared the suppressive capacity of splenic and tumor-derived MDSCs in different subcutaneous mouse tumor models. We investigated which suppressive mechanisms were involved. Finally, we investigated whether MDSCs and regulatory T cells (T_{reg}) cooperate in the suppression of T-cell responses. In all models, splenic granulocytic MDSCs (grMDSC) strongly suppress $CD4^+$ T-cell proliferation while the suppressive effect on $CD8^+$ T cells is less pronounced. Splenic monocytic MDSCs (moMDSC) have a lower suppressive capacity, compared to grMDSC, on both $CD4^+$ and $CD8^+$ T-cell proliferation. Both grMDSC and moMDSC isolated from the tumor have a much stronger suppressive activity compared to MDSCs isolated from the spleen of tumor-bearing mice, associated with a higher NO_2^- production by the tumor-derived moMDSC and arginase activity for both subsets. The expression of CD80 is also elevated on tumor-derived grMDSC compared with their peripheral counterparts. Direct contact with tumor cells is required for the upregulation of CD80 and $CD80^+$ MDSCs are more suppressive than $CD80^-$ MDSCs. Coculture of T_{reg} and MDSCs leads to a stronger suppression of $CD8^+$ T-cell proliferation compared to the suppression observed by T_{reg} or MDSCs alone. Thus, we showed that tumor-infiltrating MDSCs possess a stronger suppressive capacity than their peripheral counterparts and that various suppressive mechanisms account for this difference.

Tumor Immunology

Despite the fact that the immune system has the ability to recognize and kill tumor cells by cytotoxic T-lymphocytes (CTLs), based on the expression of various tumor-associated antigens (TAAs), immunotherapy rarely induces objective tumor regression. As the majority of TAAs are self antigens, a major impediment of immunotherapeutic strategies is the failure to break tolerance.[1] Nevertheless, even when potent tumor-specific CTLs can be generated (*e.g.*, against mutated antigens), several inhibitory mechanisms have been identified that are responsible for actively quenching antitumor immune responses.[2] Defective T-cell function is a critical factor in immune escape in cancers. Thus, it has become clear that inhibitory cell types such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T_{reg}) are crucially involved in dampening effector T-cell functions, both in mouse models of cancer and in cancer patients.[3–5]

MDSCs represent a heterogeneous population of immature myeloid cells, including monocytes, macrophages, dendritic cells (DCs) and granulocytes that have been prevented to fully differentiate into functionally competent cells under the influence of different tumor-derived factors. These cells express different surface markers, depending on the tumor type, the factors released by the tumor and their anatomical location. Features that are common to all MDSCs include their myeloid lineage origin, their immature phenotype and, most importantly, their ability to strongly suppress T-cell responses.[6–9]

In mice, the markers CD11b and Gr-1 define this immunosuppressive cell population. Antibodies that specifically recognize Gr-1 bind to two antigens, Ly6G and Ly6C.[10] The use of epitope specific antibodies together with morphological

Key words: tumor immunology, myeloid-derived suppressor cells, CD80, immune suppression, regulatory T cell

Abbreviations: APCs: antigen presenting cells; CTLA-4: cytotoxic T-lymphocyte antigen-4; CTLs: cytotoxic T-lymphocytes; DCs: dendritic cells; grMDSC: granulocytic myeloid-derived suppressor cell; IDO: indoleamine-2,3-dioxygenase; MDSCs: myeloid-derived suppressor cells; moMDSC: monocytic myeloid-derived suppressor cell; TAAs: tumor-associated antigens; T_{reg}: regulatory T cells

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What's new?

Attempts to wield the body's immune system against cancer often fail. One reason is the suppression of T cells by myeloid-derived suppressor cells (MDSCs). This study investigated exactly how MDSCs thwart T cells. They found that MDSCs isolated from the solid tumor were far more potent against T cells than those from the spleen, and that they express more CD80. Furthermore, when MDSCs were cultured together with regulatory T cells, that improved their ability to suppress T cells. These findings suggest possible ways to counter the immunosuppressive tumor microenvironment.

analysis has led to the identification of two functionally distinct subtypes of MDSCs: $CD11b^{+}Ly6G^{+}Ly6C^{low}$ MDSCs, which are morphologically similar to polymorphonuclear granulocytes whereas $CD11b^{+}Ly6G^{-}Ly6C^{high}$ MDSCs have a monocytic phenotype. Both subsets are able to suppress T-cell proliferation, although they use different mechanisms to exert their function.[3,11,12]

In several studies, it has been shown that direct cell-cell contact between MDSCs and T cells is required for their suppressive activity, although the mechanisms involved have not been fully elucidated.[8,13–15] Interaction between receptors and their ligands expressed on both T cells and professional antigen presenting cells (APCs) are crucial for the delivery of either inhibitory or stimulatory signals and will determine the outcome of an immune response.[16] Many of these molecules belong to the CD28-B7 and TNFR/TNF superfamily. B7-1 (CD80), expressed on APCs, can bind both a stimulatory receptor, CD28 and an inhibitory receptor CD152 (CTLA-4, cytotoxic T-lymphocyte antigen-4) on T cells. Interaction between CD80 and CD28 provides important costimulatory signals required for the proper activation of naïve T cells. Shortly after, CTLA-4 expression is increased in order to avoid overstimulation of the immune system. CTLA-4 is also highly and constitutively expressed on T_{reg} and low levels of CD80 are required for T_{reg} survival.[17–20] The importance of the CD80-CD28/CTLA-4 pathway in the suppressive activity of MDSCs has so far not been established.[21–24] Moreover, CD80 can also bind to PD-L1, which results in the delivery of inhibitory signals to T cells. The complexity of these ligand-receptor interactions hampers the study of the role of the CD80-CD28/CTLA-4 pathway in the suppressive function of MDSCs.[25]

In this study, we show that tumor-infiltrating MDSCs possess a stronger suppressive capacity for both $CD8^{+}$ and $CD4^{+}$ T cells than their peripheral counterparts isolated from the spleen in different tumor models, which is associated with a higher production of NO_2^{-} by the tumor-derived moMDSC and a higher arginase activity displayed by both subsets of these tumor-derived MDSCs. Phenotypical analysis of the MDSCs revealed that MDSCs isolated from the tumor microenvironment express higher levels of CD80 compared with splenic MDSCs. Transwell assays showed that direct contact between MDSCs and tumor cells is necessary for CD80 upregulation on MDSCs, suggesting that CD80 might play an important role in the suppressive activity of MDSCs. Coculturing of MDSCs and T_{reg} resulted in a stronger suppression of T-cell proliferation, compared with the suppressive effect of either of these cell types alone.

Material and Methods

Mice

Female, 6- to 12-week-old C57BL/6 mice were purchased from Harlan (Horst, the Netherlands) or Charles River (L'Arbresle Cedex, France). Animals were treated according to the European guidelines for animal experimentation. All experiments were reviewed and approved by the ethical committee for use of laboratory animals of the Vrije Universiteit Brussel.

Tumor cell lines

The mouse melanoma cell line MO4 (kindly provided by K. Rock, University of Massachusetts Medical Center) and the T cell lymphoma E.G7-OVA (obtained from the American Type Culture Collection) were cultured at 37°C in a humidified 5% CO_2 atmosphere in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) supplemented with 5% fetal clone I (FCI), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino acids. The Lewis Lung Carcinoma (LLC; obtained from the American Type Culture Collection) cell line is maintained in Dulbecco's Modified Eagle Medium (DMEM), (Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. No full authentication was carried out. Cell lines were tested for their known characteristics including expression of antigens and MHC molecules by reverse transcriptase PCR or flow cytometry. Their *in vitro* and *in vivo* growth characteristics were closely monitored.

Preparation of a single-cell suspension from spleen and tumor from tumor-bearing mice

All mice were anesthetized by inhalation of isoflurane and inoculated with 5×10^5 tumor cells administered by subcutaneous injection in the lower back. Mice were sacrificed 14 days after tumor implantation and spleens and tumors were isolated. Single-cell suspensions prepared from splenocytes were treated with Tris-buffered ammonium chloride to remove red blood cells. Single-cell suspensions from tumor tissue were prepared using the GentleMACS single cell isolation protocol (Miltenyi Biotec). Briefly, tumors were isolated

and minced into small pieces followed by a mechanical dissociation step using the GentleMACS dissociator. Samples were then incubated for 40 min at 37°C with the following enzymes: collagenase I (10,000 U/ml) and dispase II (32 mg/ml). After a last mechanical disruption step, the digested tumors were harvested, filtered (over a 70 μM nylon filter, BD Falcon) and red blood cells were lysed by using Tris-buffered ammonium chloride.

Purification of MDSCs from spleen and tumor

Different subsets of MDSCs were purified from spleens and tumors of tumor-bearing mice. First, the $CD11b^+$ cell fraction was enriched by MACS sorting using CD11b MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. These enriched $CD11b^+$ cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b, allophycocyanin (APC)-labeled anti-Ly6G and phycoerythrin (PE)-Cy7-conjugated anti-Ly6C. Subsets of MDSCs were sorted to a purity of $>$ 90% using a BD FACSAria III cell sorter (BD Biosciences).

Suppression assay

For the analysis of the suppressive function of the purified subpopulations of MDSCs, freshly isolated splenocytes, obtained from healthy mice, were labeled with 5 μM CellTrace™ Violet (Invitrogen) and seeded in 96-well plates at 2×10^5 cells/well. Purified MDSCs were then added at different ratios, ranging from 1:1 to 1:8 (MDSC:splenocytes). T-cell proliferation was induced by anti-CD3/CD28 beads (Invitrogen) in the presence of IL-2 (100 U/ml, Chiron). In some experiments neutralizing antibodies against CD80 (clone 16-10A1, 10 μg/ml, BioXCell), CTLA-4 (clone 9H10, 10 μg/ml, BioXCell) or isotype control (hamster IgG, 10 μg/ml, BioXCell) were added at the beginning of the cultures. After 3 days, proliferation of $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry by staining with peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-mouse CD3 (BioLegend), Alexa fluor 700-conjugated anti-mouse CD4 (BioLegend) and APC-H7-conjugated CD8 (BD Biosciences). Data were collected using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FACSDiva (BD Biosciences) or FlowJo (Tree Star) software. The percentage of T-cell suppression was calculated using the following formula:

$$\%\,\text{Suppression} = \left[1 - \frac{\%\,\text{proliferation with MDSCs}}{\%\,\text{proliferation without MDSCs}}\right] \times 100$$

Nitrite/NO Production

Different subpopulations of MDSCs were isolated from the spleen or tumor of tumor-bearing mice using FACS, as described above, and 10^5 cells were seeded in a 96-well plate in the presence or absence of 1 μg/ml LPS from Escherichia coli serotype 055: B5 (Sigma-Aldrich). Supernatants were collected after 48 hours and nitrite concentration was determined using the Griess Reagent system according to the manufacturer's instructions (Promega).

Arginase activity

Different subpopulations of MDSCs were isolated from the spleen or tumor of tumor-bearing mice using FACS, as described above, and 10^5 cells were seeded in a 96-well plate in the presence or absence of 1 μg/ml LPS. Cell lysates were prepared by incubating the cell pellets in 100 μl lysis buffer (10 mM Tris.HCL (pH 7.4) containing 0.4% Triton X-100 and protease inhibitors) for 10 min at 4°C. Lysates were then centrifuged at 14,000 rpm at 4°C for 10 min and supernatants were used for arginase assay. Arginase activity was measured using the Quantichrom™ Arginase Assay Kit (BioAssay Systems) according to the manufacturer's instructions.

ELISA

The concentration of interferon-gamma (IFN-γ), tumor-necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2) in culture supernatants was quantified using commercially available ELISA kits (all from eBioscience) according to the manufacturer's instructions. The optical density was measured at 450 nm using a Thermomax microplate reader.

Coculture of MDSCs and tumor cells

Purified $CD11b^+Ly6C^{int}Ly6G^+$ MDSCs (grMDSC) were cultured either in complete medium, in tumor-conditioned medium (TCM), in direct contact with E.G7-OVA tumor cells or in a Transwell plate (24-well cell culture, inserts 0.4 μM polycarbonate membrane plate, Costar), where the MDSCs were cultured in the upper chamber and the E.G7-OVA tumor cells in the lower compartment. Three days later, cells were harvested and the expression of CD80 (16-10A1, biotinylated, prepared in house) was determined by flow cytometry.

Purification of T_{reg} from spleen

T_{reg} were purified from the spleen of E.G7-OVA tumor-bearing mice. First, the $CD4^+$ T-cell fraction was enriched by MACS sorting using CD4 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. These enriched $CD4^+$ T cells were stained with PERCP-Cy5.5-conjugated anti-CD4 and phycoerythrin (PE)-labelled anti-CD25 antibodies. T_{reg} were sorted as $CD4^+CD25^{high}$ cells using a BD FACSAria III cell sorter (BD Bioscience) to a purity of $>$ 90%.

Coculture of T_{reg} and MDSCs

For the analysis of the suppressive function of the purified subpopulations of MDSCs and T_{reg}, freshly isolated splenocytes, obtained from healthy mice, were labeled with 5 μM CellTrace™ Violet (Invitrogen) and seeded in 96-well plates at 2×10^5 cells/well. Purified MDSCs and T_{reg} were then

added at a 1:1:4 ratio (MDSC:T_{reg}:splenocytes). T-cell proliferation was induced by anti-CD3/CD28 beads (Invitrogen) in the presence of IL-2 (100 U/ml). After 3 days, proliferation of T cells was analyzed by flow cytometry.

Statistical analysis

A one-way ANOVA followed by a Bonferroni multiple comparison test was conducted to evaluate statistical significance between multiple groups. For the comparison of two groups, Student's t-test was carried out. Sample size and number of repetitions for each experiment are indicated in the figure legends. All statistical analyses were performed using GraphPad Prism 5.

Results

Granulocytic MDSCs possess a higher suppressive capacity than monocytic MDSCs

The differential suppressive capacity of granulocytic ($CD11b^+Ly6G^+Ly6C^{int}$, grMDSC) and monocytic ($CD11b^+Ly6G^-Ly6C^{high}$, moMDSC) MDSCs on $CD4^+$ versus $CD8^+$ T cells has so far not been studied in detail. Therefore we cocultered splenocytes derived from healthy animals with grMDSC or moMDSC sorted from spleens of E.G7-OVA tumor-bearing mice (Supporting Information Fig. S1) and analyzed their effect on the proliferation of $CD4^+$ and $CD8^+$ T cells, respectively.

Overall, grMDSC were found to be more suppressive than moMDSC, both on the proliferation of $CD4^+$ and $CD8^+$

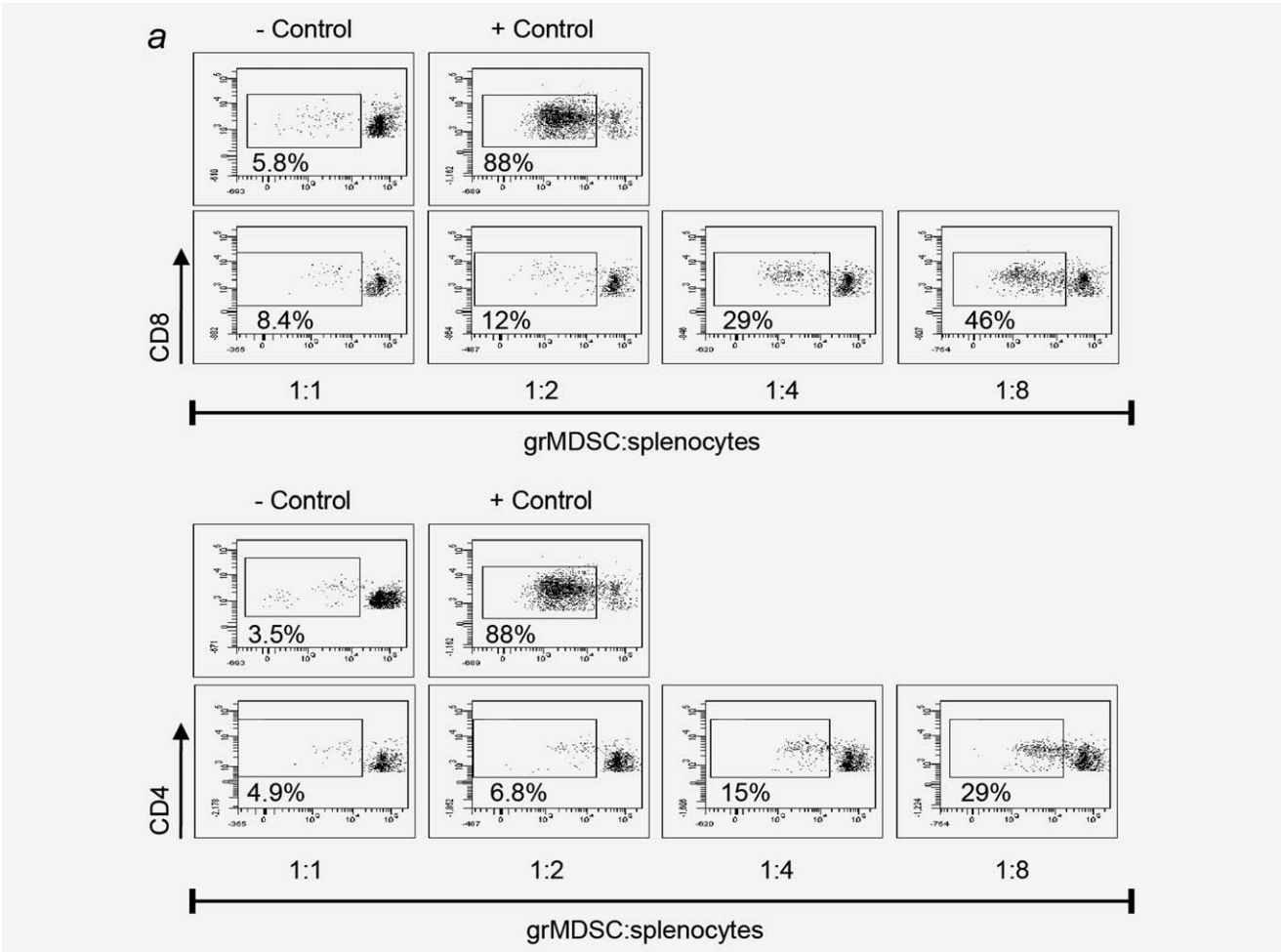

Figure 1. MDSCs isolated from the spleen of E.G7-OVA tumor-bearing mice suppress proliferation and cytokine production of both $CD4^+$ and $CD8^+$ T cells. (*a*) Sorted grMDSC were cultured at different ratios [ranging from 1:1 to 1:8 (MDSCs:splenocytes)] with 2×10^5 CellTrace Violet labeled splenocytes from healthy mice in the presence of anti-CD3/CD28 beads for 3 days after which proliferation of $CD8^+$ T cells (upper panel) and $CD4^+$ T cells (lower panel) was determined. Controls included T cells cultured in the absence of MDSCs with and without T-cell stimulation. One representative FACS profile is shown. (*b*) Overview of the percentage proliferation of $CD8^+$ (upper panel) and $CD4^+$ T cells (lower panel) in the presence of grMDSC or moMDSC (*c*) and the percentage of suppression by grMDSC (*b*) or moMDSC (*c*). Results of 5 independent experiments are presented as mean ± SEM; *, statistically significant differences from values of T-cell proliferation in the absence of MDSCs ($p < 0.05$) NS, no statistically significant differences from values of T-cell proliferation in the absence of MDSCs. (*d*) IFN-γ, TNF-α and IL-2 production by splenocytes was determined after 3 days of culture with different ratios [ranging from 1:1 to 1:8 (MDSCs:splenocytes)] of either grMDSC or moMDSC (*e*). Results of 3 independent experiments are shown as mean ± SEM. UD, undetectable because values were below the detection limit of the assay.

T cells (Figs. 1*a*–1*c*). Whereas grMDSC were still suppressive at ratios of 1:8 (MDSC: splenocytes), moMDSC already showed markedly reduced suppression starting from 1:4 ratios. For both grMDSC and moMDSC, the suppressive effect was more pronounced on $CD4^+$ T cells than on $CD8^+$ T cells. This was most obvious for moMDSC, where suppression of $CD8^+$ T cells was almost completely lost at 1:2 ratios whereas $CD4^+$ T-cell proliferation was still significantly suppressed (Fig. 1*c*).

To extend these findings, we analyzed the suppressive capacity of MDSCs from two other tumor models, the melanoma model MO4 and LLC and found that although the relative levels of suppression differed, similar trends as for E.G7-OVA could be observed (Supporting Information Fig. S2).

The suppressive effect of MDSCs on T cells is not limited to a reduction in T-cell proliferation, but also affects other T-cell effector functions such as cytokine production. Therefore, we analyzed the effect of grMDSC and moMDSC on IFN-γ, TNF-α and IL-2 secretion by splenocytes. Addition of different ratios of grMDSC led to a marked, dose-dependent reduction in IFN- γ, TNF-α and IL-2 production by splenocytes (Fig. 1*d*). However, the addition of different ratios of moMDSC did not influence the secretion of either IFN-γ nor TNF-α, although the secretion of IL-2 was decreased in a dose-dependent manner (Fig. 1*e*). These cytokine profiles confirmed the higher suppressive activity of grMDSC.

Tumor-derived MDSCs possess a stronger suppressive activity compared with their peripheral counterparts, which is associated with a higher NO_2^- production and arginase activity

In the vast majority of studies about MDSCs in tumor-bearing mice, analysis of MDSC function is limited to cells isolated from the spleen.[12,22] As the main effect of MDSCs *in*

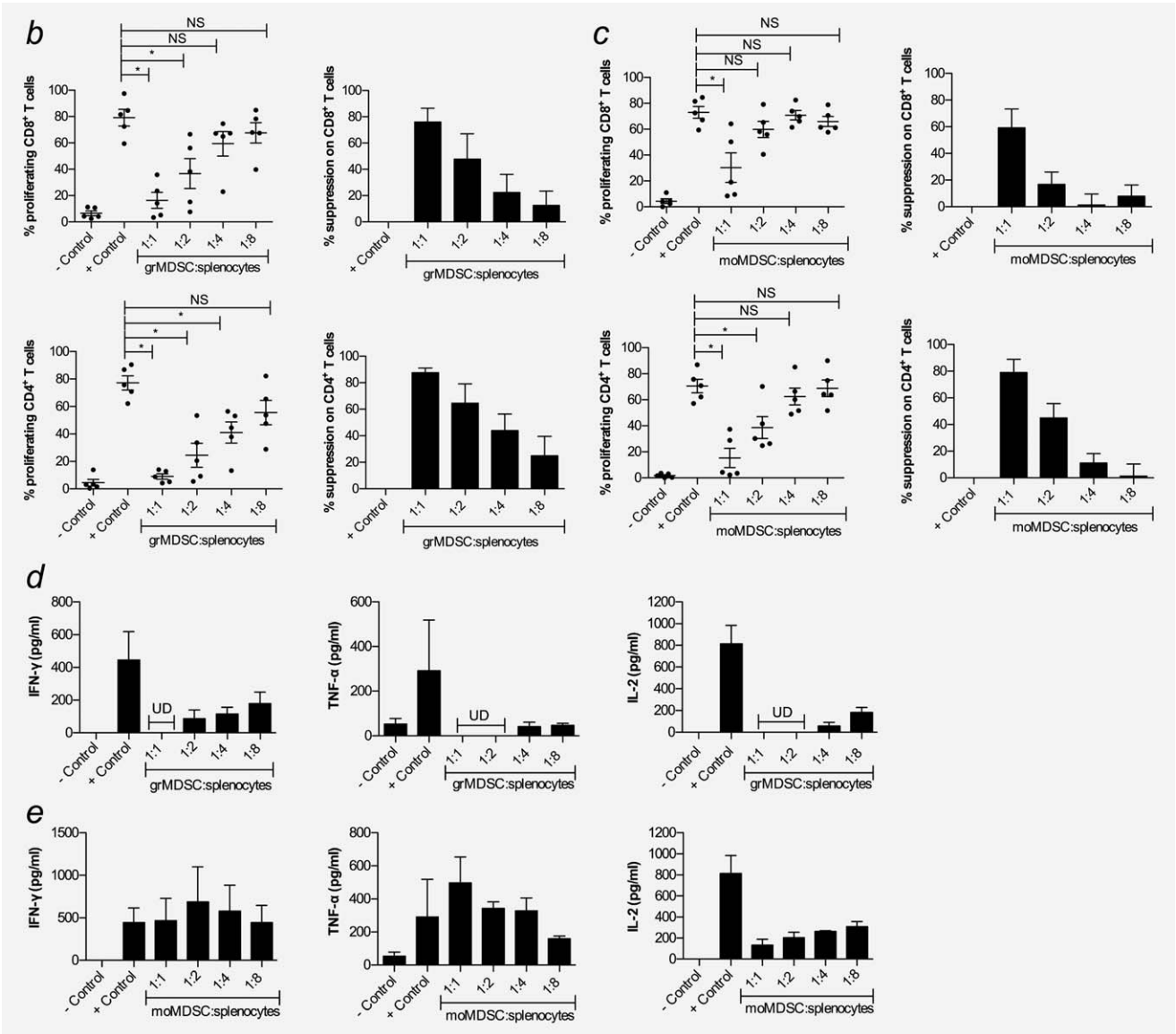

Figure 1. (Continued)

vivo most likely takes place in the tumor microenvironment and not in the periphery, we decided to compare the suppressive capacity of MDSCs derived from the tumor and the spleen, respectively.

For this purpose, mice were inoculated with E.G7-OVA tumors and after 14 days, we isolated grMDSC and moMDSC from both spleens and tumors. Both tumor-derived grMDSC and moMDSC were found to be suppressive

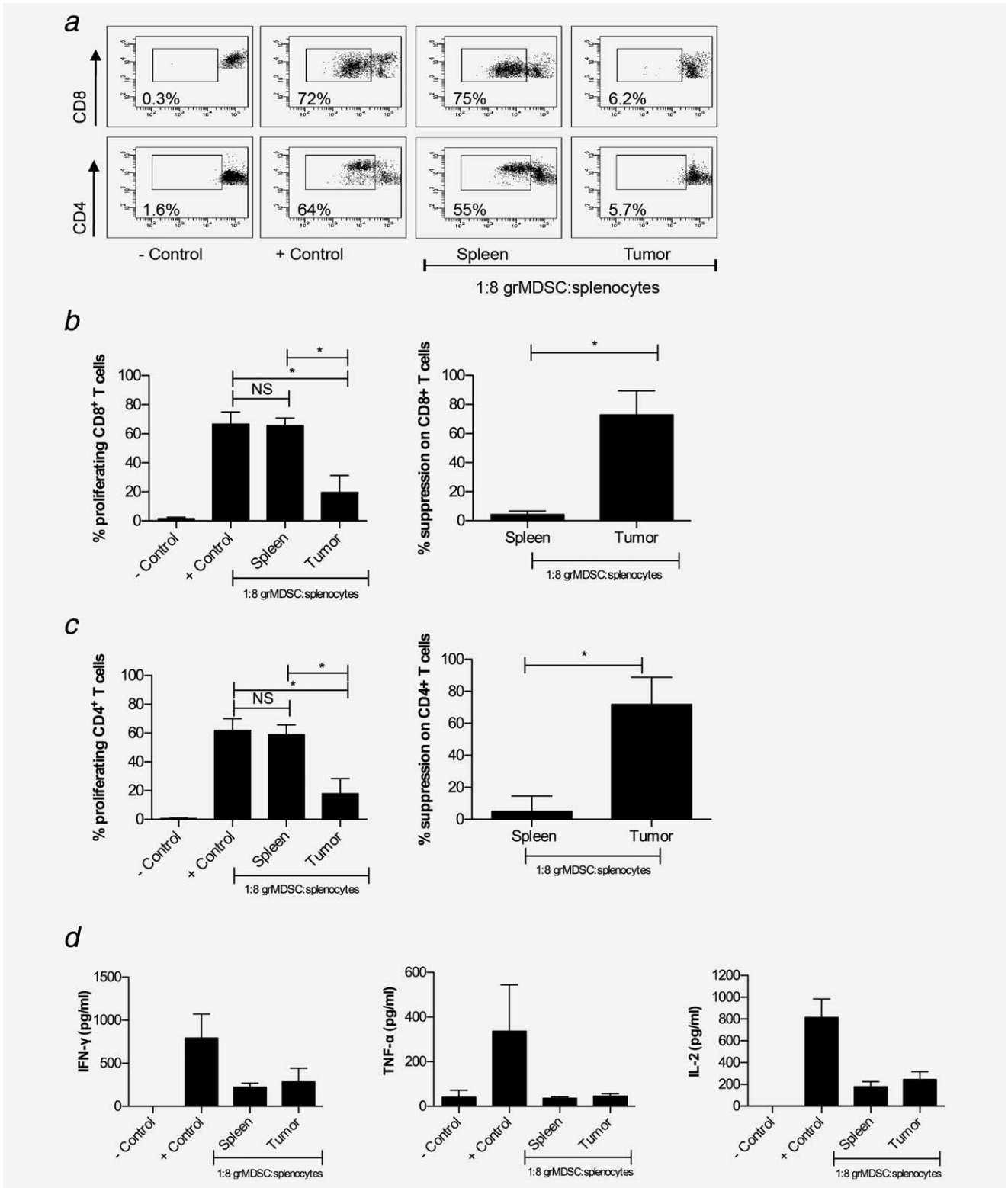

Figure 2.

for $CD4^+$ and $CD8^+$ T-cell proliferation. When comparing the suppressive capacity of MDSCs isolated from either the spleen or the tumor, we observed that tumor-derived grMDSC possess a stronger suppressive capacity on the proliferation of both $CD4^+$ and $CD8^+$ T cells (Fig. 2*a*). At a 1:8 ratio the mean suppression exerted by grMDSC isolated from the tumor was 72% on $CD4^+$ T cells and 73% on $CD8^+$ T cells (Figs. 2*b* and 2*c*), whereas the mean suppression by splenic MDSCs was only 5 and 4% on $CD4^+$ T cells and $CD8^+$ T cells, respectively (Figs. 2*b* and 2*c*). However, when we compared the cytokine secretion

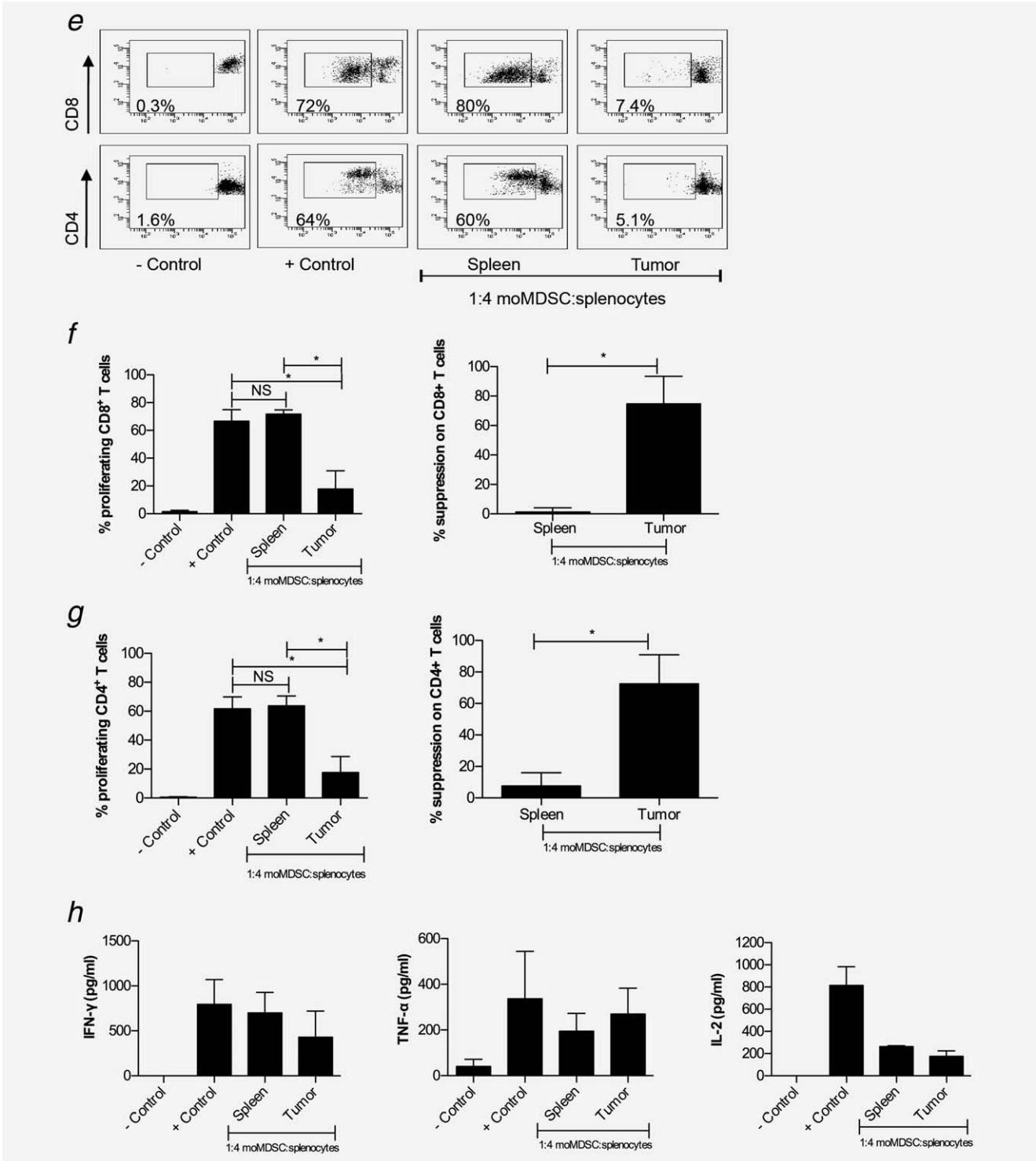

Figure 2.

by splenocytes cultured in the presence of either tumor-derived or spleen-derived grMDSC, no differences in the secretion of IFN-γ, TNF-α or IL-2 were observed (Fig. 2*d*). Similar results were obtained for moMDSC (Fig. 2*e*) where at a 1:4 ratio the tumor-derived moMDSC showed a mean suppression of 72% on $CD4^+$ T cells and 75% on $CD8^+$ T cells whereas for spleen-derived MDSCs a mean suppression of 8 and 1% was observed on $CD4^+$ T cells and $CD8^+$ T cells, respectively (Figs. 2*f* and 2*g*). However, at a 1:8 ratio the moMDSC isolated from the spleen or from the tumor were found to be equally suppressive (data not shown). Similar observations were made for the moMDSC in the MO4 model (Supporting Information Fig. S3). Again no differences in the secretion of IFN-γ, TNF-α or IL-2 were observed between splenocytes cultured in the presence of tumor-derived or spleen-derived moMDSC (Fig. 2*h*). The suppressive activity of MDSCs is correlated with an increased production of nitrogen species and a higher activity of arginase. Therefore we compared the arginase activity and nitrite production between spleen- and tumor-derived MDSCs stimulated with LPS. Neither grMDSC nor moMDSC derived from the spleen were able to produce NO_2^- upon stimulation with LPS. Tumor-derived grMDSC do not produce detectable levels of NO_2^-. In contrast, the moMDSC isolated from the tumor produce high levels of NO_2^- when they are stimulated with LPS for 48 hours (Fig. 3*a*). Both in the spleen and tumor, the arginase activity of the moMDSC was higher compared with the grMDSC. When comparing spleen- and tumor-derived MDSCs, arginase activity was enhanced in both tumor-derived grMDSC and moMDSC (Fig. 3*b*).

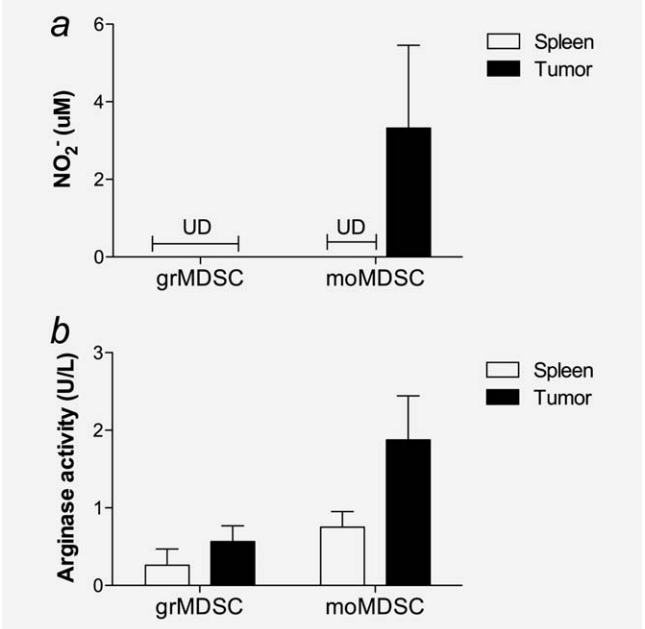

Figure 3. Tumor-derived moMDSC produce high levels of NO_2^- and possess an enhanced arginase activity compared to spleen-derived moMDSC. moMDSCs and grMDSCs were purified from the spleen and tumor of E.G7-OVA tumor-bearing mice and 10^5 cells were cultured in the presence of 1 μg/ml LPS for 48 hours. (*a*) NO_2^- production by grMDSC and moMDSC isolated from spleen or tumor was determined in the supernatants of the cultures using the Griess Reagent System. Results of 3 independent experiments are shown as mean ± SEM. UD, undetectable because values were below the detection limit of the assay. (*b*) Arginase activity of grMDSC and moMDSC isolated from spleen or tumor was determined on cell lysates using the QuantiChrom Arginase assay Kit. Results of 3 independent experiments are shown as mean ± SEM.

CD80 expression is increased on tumor-derived MDSCs

As we observed an enhanced suppressive capacity for MDSCs isolated from the tumor compared with the spleen, we tried to identify phenotypical differences between the tumor-derived and spleen-derived MDSCs that could be responsible for their distinct suppressive capacities. When we looked at CD80 expression on MDSCs, we consistently found an increased expression on tumor-derived grMDSC compared with the expression levels found on grMDSC isolated from the spleen, with a mean expression level of 55 and 19%, respectively (Fig. 4*a*).

Direct contact with tumor cells leads to an upregulation of CD80 on MDSCs

To determine whether the upregulation of CD80 was caused by secreted tumor-derived factors or by direct contact between tumor cells and MDSCs, grMDSC isolated from the spleen of E.G7-OVA tumor-bearing mice were cultured either with supernatant derived from E.G7-OVA cell cultures

Figure 2. MDSCs isolated from the tumor microenvironment possess a stronger suppressive capacity compared with their peripheral counterparts. grMDSC and moMDSC were purified from the spleen and tumor of E.G7-OVA tumor-bearing mice and cultured at different ratios [ranging from 1:1 to 1:8 (MDSCs:splenocytes)] with 2×10^5 CellTrace Violet labeled splenocytes from healthy mice in the presence of anti-CD3/CD28 beads for 3 days after which proliferation of $CD8^+$ and $CD4^+$ T cells was determined by flow cytometry. (*a*) Representative FACS plots showing the proliferation of $CD8^+$ (upper panel) and $CD4^+$ T cells (lower panel) in the presence of grMDSC isolated from the spleen or the tumor. (*b*) Percentage proliferation and percentage suppression by grMDSC on $CD8^+$ and $CD4^+$ (*c*) T cells. Five independent experiments were performed and results are presented as mean ± SEM. *, statistically significant differences from values of T-cell proliferation in the absence of MDSCs ($p < 0.05$). NS, no statistically significant differences from values of T-cell proliferation in the absence of MDSCs. (*d*) IFN-γ, TNF-α and IL-2 production by splenocytes was determined after 3 days of culture with grMDSC isolated either from the spleen or the tumor of E.G7-OVA tumor-bearing mice. Results of 3 independent experiments are shown as mean ± SEM. (*e*) Representative FACS plots showing the proliferation of $CD8^+$ (upper panel) and $CD4^+$ T cells (lower panel) in the presence of moMDSC isolated from the spleen or the tumor. (*f*) Percentage proliferation and percentage suppression by moMDSC on $CD8^+$ and $CD4^+$ (*g*) T cells. Five independent experiments were performed and results are presented as mean ± SEM. *, statistically significant differences from values of T-cell proliferation in the absence of MDSCs ($p < 0.05$). NS, no statistically significant differences from values of T-cell proliferation without the presence of MDSCs. H, IFN-γ, TNF-α and IL-2 production by splenocytes was determined after 3 days of culture with moMDSC isolated either from the spleen or the tumor of E.G7-OVA tumor-bearing mice. Results of 3 independent experiments are shown as mean ± SEM.

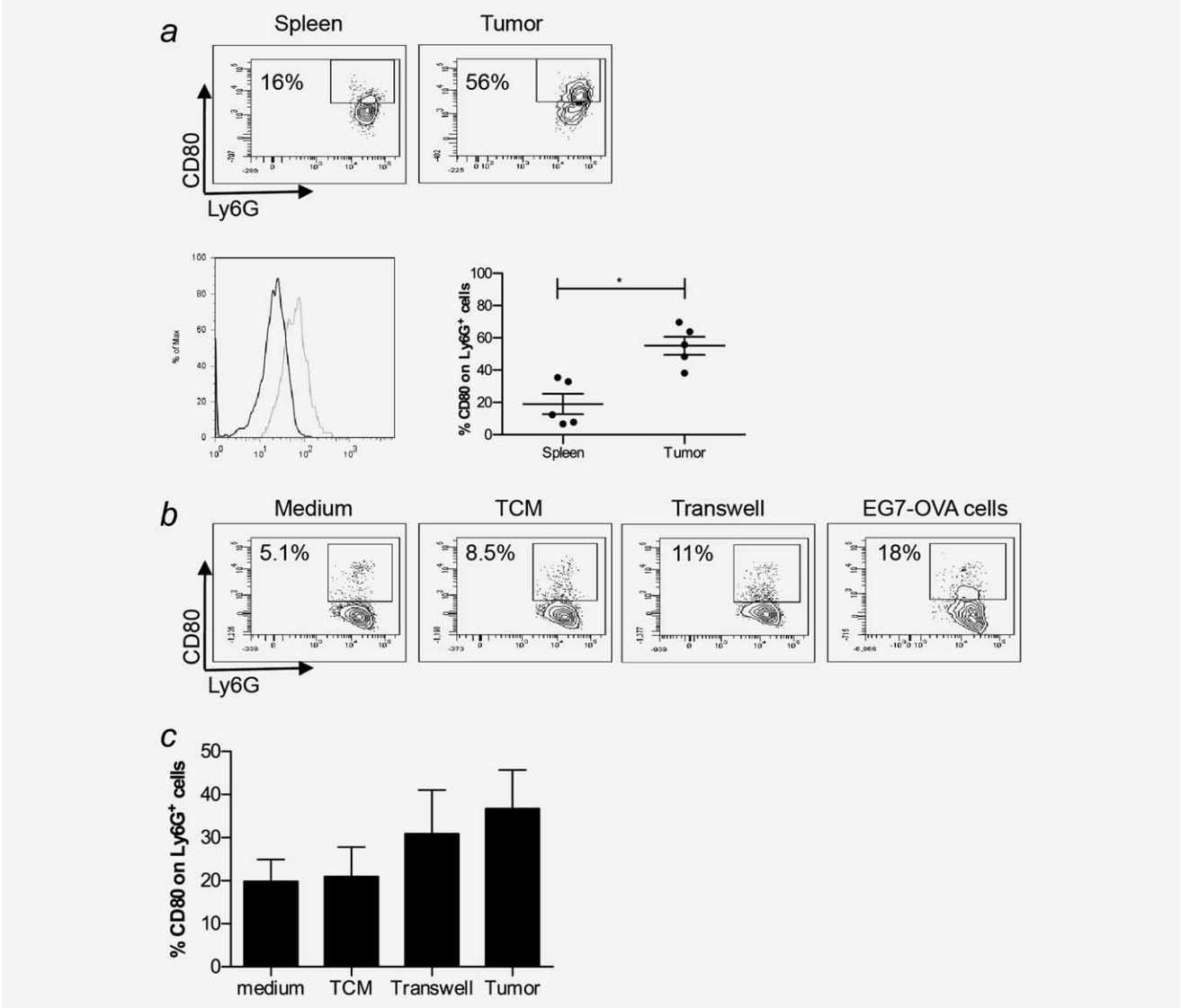

Figure 4. CD80 expression is higher on tumor-derived grMDSC and is upregulated by direct contact with tumor cells. Single cell suspensions of tumors and spleens were stained with antibodies against CD11b, Ly6G, Ly6C and CD80. Within the grMDSC fraction, CD80 expression was compared between cells isolated from the spleen and cells isolated from the tumor. (*a*) A representative FACS profile is shown. The histogram shows the CD80 expression on grMDSC isolated from the spleen (black line) or the tumor (gray line). Five independent experiments were performed and results are presented as mean ± SEM. *, statistically significant differences between the two groups ($p < 0.05$). (*b*) grMDSC derived from the spleen of E.G7-OVA tumor-bearing mice were cultured for 3 days in complete medium, TCM, in a transwell system or in direct contact with E.G7-OVA tumor cells after which expression of CD80 was determined. One representative FACS profile is shown. Results of 5 independent experiments are shown as mean ± SEM (*c*).

(TCM), in direct contact with E.G7-OVA cells or in an *in vitro* transwell assay. Only when they were cultured in direct contact with E.G7-OVA cells, did we observe an upregulation of CD80 on grMDSC (Figs. 4*b* and 4*c*).

CD80$^+$ MDSCs possess enhanced suppressive capacity

Since we observed a stronger suppression by grMDSC isolated from the tumor microenvironment, as well as a higher expression of CD80 on these cells, we wanted to determine whether CD80 plays a functional role in the suppressive activity of the grMDSC. Therefore, we first cocultured spleen-derived grMDSC in direct contact with E.G7-OVA tumor cells to upregulate CD80 expression on these cells. After 3 days of culture, we sorted the CD80$^+$ and the CD80$^-$ cell fraction and used these cells in a suppression assay. We observed an enhanced suppressive capacity for CD80$^+$ grMDSC compared with CD80$^-$ grMDSC, at a 1:10 ratio, on both CD4$^+$ and CD8$^+$ T-cell proliferation (Figs. 5*a* and 5*b*). When we compared the cytokine secretion, we found a decreased production of both IFN-γ and IL-2 when splenocytes were cultured in the presence of CD80$^+$ grMDSC, even at a 1:10 ratio.

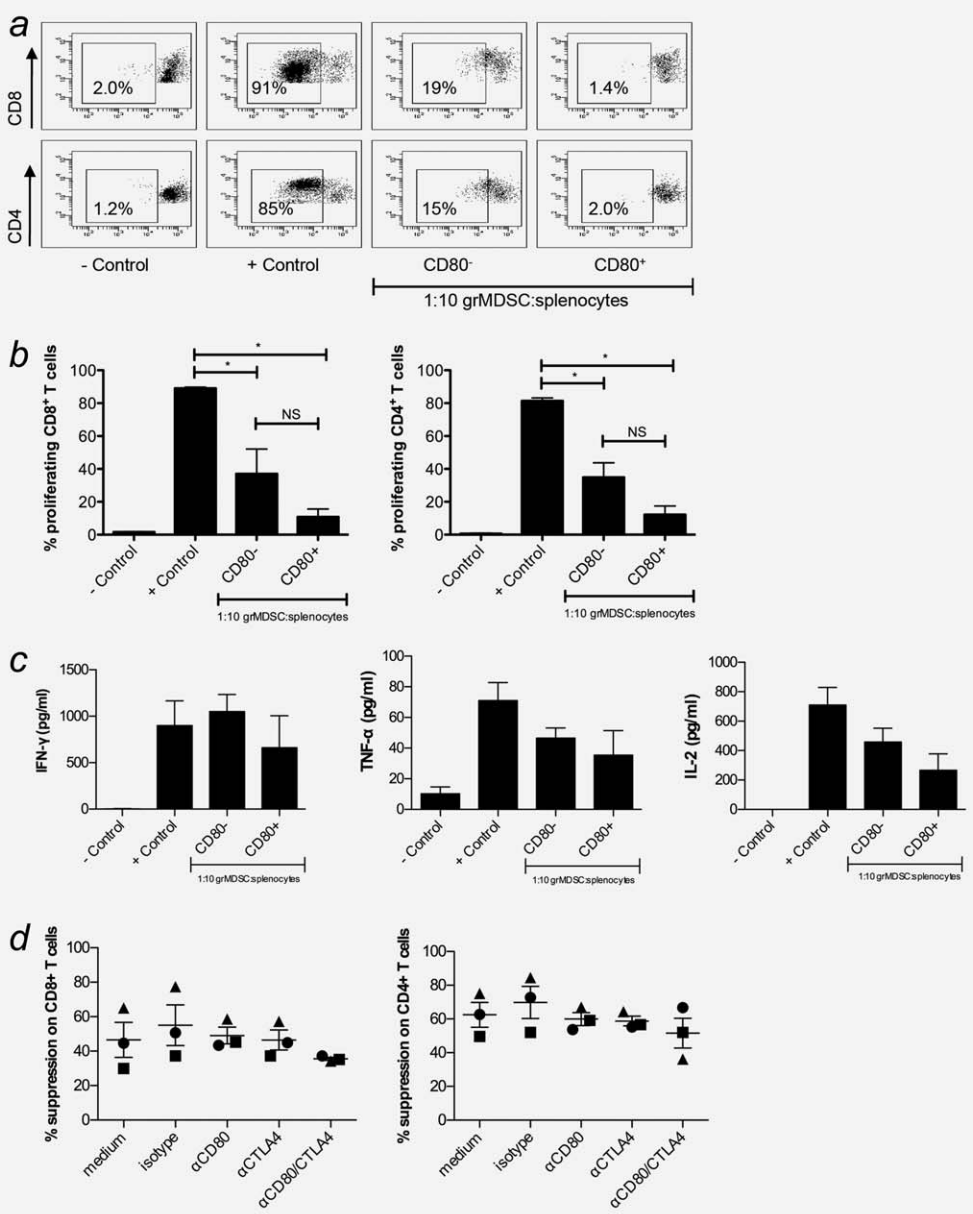

Figure 5. CD80 plays a role in the suppressive function of grMDSC. grMDSC were purified from the spleen of E.G7-OVA tumor-bearing mice and co-cultured in direct contact with E.G7-OVA tumor cells for 3 days. Afterwards cells were stained with an antibody against CD80 and the $CD80^+$ and $CD80^-$ cell fractions were sorted. These cells were subsequently used in a suppression assay and proliferation of $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry. (*a*) A representative FACS plot showing $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T-cell proliferation in the presence of $CD80^+$ or $CD80^-$ grMDSC. (*b*) Overview of the proliferation of $CD8^+$ (left panel) and $CD4^+$ T cells (right panel) cultured in the presence of either $CD80^+$ or $CD80^-$ grMDSC. Three independent experiments were performed and results are presented as the mean ± SEM of these experiments. (*c*) IFN-γ, TNF-α and IL-2 production by splenocytes was determined after 3 days of culture with $CD80^+$ or $CD80^-$ grMDSC. Results are presented as the mean ± SEM of three independent experiments. (*d*) Neutralizing antibodies against CD80 and/or CTLA-4 (10 μg/ml) were added at the beginning of the cocultures of T cells and grMDSC, at a 1:2 ratio (MDSC:splenocytes) and proliferation of CD8+ (left panel) and CD4+ T cells (right panel) was determined after 3 days. Results are presented as the mean ± SEM of three independent experiments.

However, no differences were seen in the secretion of TNF-α by splenocytes cultured in the presence of either $CD80^+$ or $CD80^-$ grMDSC (Fig. 5*c*). To further elucidate the importance of CD80 in the immunosuppressive function of grMDSC, neutralizing antibodies against CD80 were added at the beginning of the cultures. Compared to the isotype control, the presence of a neutralizing antibody against CD80 partially inhibited the immunosuppressive activity of grMDSC. On $CD4^+$ T cells, a mean suppressive activity of 60% was found after the addition

of the neutralizing CD80 antibody compared with a mean suppression level of 70% in the presence of the isotype control. On CD8+ T cells, this effect was less pronounced (mean suppression of 55 and 49% with the isotype and the neutralizing antibody, respectively). Because antibody blockade suggests that CD80 plays a role in the suppressive function of grMDSC we wondered whether blockade of the CD80 ligand CTLA-4 might have a similar effect. For both the $CD4^+$ and $CD8^+$ T cells addition of a neutralizing antibody against CTLA-4 had a similar effect than the addition of a neutralizing antibody against CD80. However, addition of both neutralizing antibodies to the same culture leads to a stronger inhibition of the suppressive activity of grMDSC on both $CD4^+$ and $CD8^+$ T cells (mean suppression of 51 and 36%, respectively; Fig. 5*d*).

Coculturing of MDSCs and T_{reg} leads to a stronger suppression of $CD8^+$ T-cell proliferation compared with MDSCs or T_{reg} alone

Although both T_{reg} and MDSCs have been studied extensively, very little data is available on the interactions between T_{reg} and MDSCs. Therefore, we decided to evaluate whether T_{reg} and MDSCs can cooperate in suppressing effector T cells.

For this purpose, we sorted the $CD4^+CD25^{high}$ T cells to high purity (Supporting Information Fig. S4A) and used these cells in a suppression assay, as described above for the MDSCs. FoxP3 staining confirmed that the sorted $CD4^+CD25^{high}$ T cells were *bonafide* T_{reg} (Supporting Information Fig. S4B). We confirmed in our model that T_{reg} isolated from the spleen of EG7-OVA tumor-bearing mice can strongly suppress the proliferation of $CD8^+$ T cells with a mean suppression of 56% at a 1:2 ratio and at a 1:8 ratio, we still observed a median suppression of 23% (Supporting Information Fig. S5A,B). As was observed for MDSCs, the suppressive effect of T_{reg} on T cells is not limited to a reduction in T-cell proliferation, but also affects other T-cell effector functions such as cytokine production. Addition of different ratios of T_{reg} led to a marked, dose-dependent reduction in IFN-γ, TNF-α and IL-2 production by splenocytes (Supporting Information Fig. S5C).

Since we observed CD80 expression on the grMDSC, we wondered whether these cells could influence the suppressive activity of T_{reg}. When we cocultured grMDSC with T_{reg} cells, each at a 1:4 ratio, enhanced suppression of $CD8^+$ T-cell proliferation was observed (mean suppression of 79%) compared to the conditions where either grMDSC or T_{reg} were cultured with $CD8^+$ T cells (mean suppression of 40 and 44%, respectively; Figs. 6*a* and 6*b*). Secretion of IFN-γ, TNF-α and IL-2 was also decreased when splenocytes were cocultured with both T_{reg} and grMDSC compared to the conditions where splenocytes were cultured with either grMDSC or T_{reg} alone (Fig. 6*c*).

Discussion

An increasing number of studies is focusing on the role of MDSCs in the suppression of antitumor immune responses. However, the lack of a unified phenotypes makes it difficult to compare the results obtained in different laboratories. The vast majority of the studies performed defines MDSCs as $CD11b^+Gr\text{-}1^+$ cells, and does not distinguish between granulocytic and monocytic MDSCs, based on the expression of Ly6G and Ly6C, respectively. Moreover, although MDSCs are characterized by a potent ability to inhibit $CD8^+$ T-cell function, a lot of controversy exists about the suppressive activity of MDSCs on $CD4^+$ T-cell function.[15,26–29] In this study we looked at the effect of the different subsets of MDSCs in a polyclonal, non-antigen specific system and looked both at the proliferation and cytokine secretion by $CD4^+$ and $CD8^+$ T cells. We describe that grMDSC isolated from the spleen of tumor-bearing mice are more suppressive than moMDSC, both on $CD4^+$ and $CD8^+$ T cells. For both subsets, the suppressive effect is more pronounced on $CD4^+$ T cells than on $CD8^+$ T cells.

Although the main site of action for MDSCs is most likely the tumor microenvironment, so far the study of these cells has been largely restricted to spleen-derived MDSCs.[12,22] Only in a minority of studies MDSCs isolated from the tumor microenvironment have been characterized. In these studies, tumors are most commonly grown as ascites in order to facilitate the isolation of MDSCs from the tumor microenvironment[23,24,30] and to our knowledge so far only one study has been performed on the suppressive activity of MDSCs isolated from subcutaneously implanted tumors.[31] In this study, the authors showed that only MDSCs isolated from an acute or chronic (tumor-induced) inflammatory environment possess the immediate capacity to regulate antigen-specific $CD8^+$ T-cell proliferation in a short-term proliferation assay, while MDSCs isolated from the spleen did not have a suppressive function in this assay.[31] They showed that IFN-γ, produced during a standard proliferation assay of 3 days, converts precursor splenic $CD11b^+Gr\text{-}1^+$ into functional MDSCs.[31,32] In our study, we show that grMDSC and moMDSC isolated from a subcutaneously grown solid tumor indeed possess a stronger suppressive capacity on both $CD4^+$ and $CD8^+$ T cells than their peripheral counterparts in a 3 day proliferation assay. However, spleen-derived MDSCs could also suppress T-cell proliferation and cytokine secretion in our system. Moreover, we show that there is no production of IFN-γ when spleen-derived grMDSC were cocultured with splenocytes, indicating that at least for the granulocytic subset of MDSCs other mechanism are responsible for the suppression of T-cell proliferation by spleen-derived MDSCs. Conflicting results concerning the suppressive activity of spleen- and tumor-derived MDSCs could be explained by differences in the activation status of the responder cells, differences in the duration of the suppression assay, differences between antigen specific and non-antigen specific T-cell responses, differences in the subsets of MDSCs under

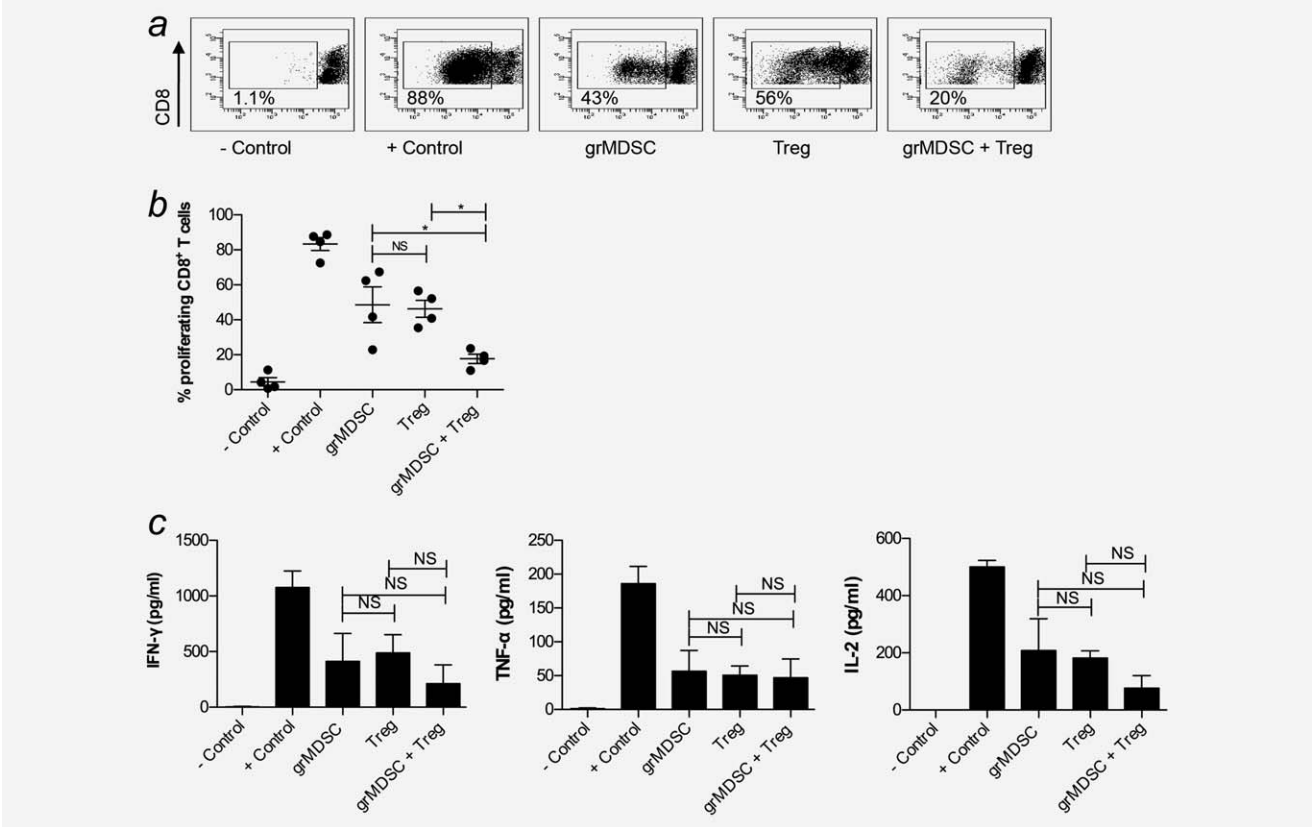

Figure 6. Inhibition of T-cell proliferation by coculture of grMDSCs and T_{reg}. T_{reg} and grMDSC were purified from the spleen of E.G7-OVA tumor-bearing mice and cocultured at a 1:1:4 ratio (MDSC: T_{reg}:splenocytes) with 2×10^5 CellTrace Violet labeled splenocytes from healthy mice in the presence of anti-CD3/CD28 beads for 3 days. (*a*) A representative FACS plot showing the proliferation of $CD8^+$ T cells after 3 days of coculture with grMDSC, T_{reg} or both cell types. (*b*) Overview of the proliferation of $CD8^+$ T cells and percentage of suppression by grMDSC, T_{reg} or both cell types. Four independent experiments were performed and results are presented as mean ±SEM. *, statistically significant differences ($p < 0.05$). NS, no statistically significant differences. (*c*) IFN-γ, TNF-α and IL-2 production by splenocytes was determined after 3 days of culture with grMDSC, T_{reg} or both cell types. Results are presented as the mean ±SEM of three independent experiments.

investigation and the use of different tumor models.[21,22,30–32] We show in this study that moMDSC isolated from the tumor microenvironment are able to produce high amounts of NO_2^- and show an increased arginase activity compared to the spleen-derived moMDSC, supporting their higher suppressive capacity. Neither spleen-, nor tumor-derived grMDSC produced NO_2^- although the tumor-derived grMDSC showed enhanced arginase activity compared to the spleen-derived grMDSC. These observations are in line with the observations made by Schlecker *et al.*, who showed higher nitrite production and arginase activity of tumor-derived moMDSC in the RMA-S model.[33] Unfortunately, we were not able to detect nitrite production in our coculturing experiments with tumor-derived moMDSC and T cells. This is in contrast to Youn *et al.* who showed that the suppressive activity of moMDSC is directly correlated with nitrite production.[22] Differences between antigen specific T-cell responses (model used by Youn *et al.*) and antigen non-specific T-cell responses (our model) can account for this difference.

In cancer patients, the study of MDSCs is largely restricted to the peripheral blood since tumor tissue cannot be readily obtained.[34–36] However, Gros *et al.*[37] showed in melanoma patients that $CD14^+HLA\text{-}DR^{low}$ cells obtained from the blood, but not from the tumor suppress T-cell proliferation, which is in sharp contrast with different studies performed in preclinical mouse models and with our observations.[30,31] Divergence between fast growing subcutaneously implanted tumors and the slow evolution of human tumors can account for this difference. Moreover, melanoma is a highly immunogenic tumor, which could influence the accumulation of MDSCs in this type of cancer. Although suppression of T-cell proliferation is the key mechanism by which MDSCs suppress immune responses, induction of T_{reg} and enhancement of angiogenesis, are also mechanisms by which MDSCs could promote tumor growth.[38–40] More detailed studies to determine how MDSCs are recruited and function at tumor sites are needed to assess the importance of this cell population in cancer patients.

The search for additional markers to further identify and discriminate between different subpopulations of MDSCs is still ongoing and a lot of conflicting data has been reported. In this context, Yang *et al.* demonstrated an upregulation of CD80 on $CD11b^+Gr1^+$ MDSCs derived from both spleen and ascites of mice intraperitoneally injected with ID8 ovarian carcinoma cells.[24] Haverkamp *et al.* observed lower levels of CD80 on MDSCs isolated from the inflammatory site, suggesting that MDSCs from the inflamed prostate are less mature compared with MDSCs isolated from the spleen.[31] In contrast, Youn *et al.* did not find CD80 expression on spleen-derived $CD11b^+Gr\text{-}1^+$ MDSCs from different subcutaneous tumor models. However, they did not look at the CD80 expression on tumor-derived MDSCs.[22] In line with Youn *et al.* we show in the E.G7-OVA model that MDSCs isolated from the spleen do not express CD80. However, grMDSC isolated from the tumor show a significant upregulation of CD80 in this tumor model. Furthermore, our *in vitro* data, showing that direct contact with tumor cells is required for the upregulation of CD80 on grMDSC, also support the *in vivo* data where we only find expression of CD80 on tumor-derived MDSCs. The difference between our observations and those made by Yang *et al.* could be explained by the fact that we used mice that received subcutaneous tumor inoculations, where the tendency to metastasize is much lower compared to intraperitoneally injected tumors, which might lead to a more localized expression of CD80 on MDSCs only within the tumor microenvironment. Youn *et al.* also performed their study in a model of subcutaneously implanted tumors, supporting this hypothesis.

Conflicting reports have been published concerning the outcome of CD80-dependent signaling in the regulation of T-cell function and the importance of the CD80-CD28/CTLA-4 pathway in the suppressive activity of MDCSs has so far not been established.[21–24] Yang *et al.* showed that $CD11b^+Gr\text{-}1^+$ MDSCs isolated from $CD80^{-/-}$ mice lose their suppressive activity. This is in sharp contrast with observations made by Tomihora *et al.*, who showed that $CD11b^+Gr\text{-}1^+$ cells found in ascites of epithelial ovarian cancer-bearing mice at advanced stages of disease are immunostimulatory rather than immunosuppressive and that they augment the proliferation of functional cytotoxic T-lymphocytes *via* signaling through the expression of the costimulatory molecule CD80.[41] Differences in disease stage or in sorting procedures could explain these observations. Our observations are in line with those made by Yang *et al.* since we show that $CD80^+$ grMDSC are more suppressive than $CD80^-$ grMDSC, indicating a role for CD80 in the suppressive function of grMDSC. However, in our model, the $CD80^-$ grMDSC are still suppressive, indicating that CD80 is not the only molecule required for the suppressive function of these cells.[24] Moreover, addition of neutralizing antibodies against CD80 and/or CTLA-4 did not completely inhibit the suppressive activity of grMDSC. This is in line with experiments performed by Poschke *et al.* on $CD14^+HLA\text{-}DR^{low}$ MDSCs isolated from the blood of melanoma patients, where they showed that blocking CD80 did not completely reverse the suppression of T-cell proliferation by the MDSCs, indeed indicating that other receptors might play an important role.[42] Further research is needed to determine the importance of other receptors, including PD-L1, in the suppressive function of MDSCs in different tumor models.[23]

Although both T_{reg} and MDSCs have been studied extensively, very little data is available on the interactions between T_{reg} and MDSCs. It is well established that MDSCs can indirectly influence T-cell function by inducing the accumulation of T_{reg}, which in turn hamper antitumor immune responses.[38,43] Recently, a functional crosstalk, through the PD-L1 pathway, between T_{reg} and MDSCs has been described in ret melanomas.[44] Moreover, Yang *et al.* showed that CD80, expressed on $CD11b^+Gr\text{-}1^+$ MDSCs, induces antigen specific immune suppression by modulating the activity of T_{reg}.[24] In the present study we describe that coculturing of T_{reg} and grMDSC leads to a stronger suppression of T-cell function, both on the proliferation as well as on the cytokine secretion of these cells, compared to culture of either of the cell types alone. These results indicate that there is a functional crosstalk between MDSCs and T_{reg}. One possible mechanism for this crosstalk is that binding of CD80 and CTLA-4 may induce the expression of indoleamine-2,3-dioxygenase (IDO) by MDSCs. IDO is involved in the metabolism of tryptophan and tryptophan metabolites suppress T-cell responses *in vitro* and *in vivo*.[45,46] Taken together these results suggest the existence of a crosstalk between T_{reg} and MDSCs. Therefore, combinatorial targeting of both T_{reg} and MDSCs may induce potent antitumor immune responses.

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