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Effect of TLR Agonists on the Differentiation and Function of Human Monocytic Myeloid-Derived Suppressor Cells

Jing Wang,*1,2 Yuko Shirota,*1,3 Defne Bayik,* Hidekazu Shirota,*4 Debra Tross,* James L. Gulley,† Lauren V. Wood,‡ Jay A. Berzofsky,‡ and Dennis M. Klinman*†

Tumors persist by occupying immunosuppressive microenvironments that inhibit the activity of tumoricidal T and NK cells. Monocytic myeloid-derived suppressor cells (mMDSC) are an important component of this immunosuppressive milieu. We find that the suppressive activity of mMDSC isolated from cancer patients can be reversed by treatment with TLR7/8 agonists, which induce human mMDSC to differentiate into tumoricidal M1-like macrophages. In contrast, agonists targeting TLR1/2 cause mMDSC to mature into immunosuppressive M2-like macrophages. These two populations of macrophage are phenotypically and functionally discrete and differ in gene expression profile. The ability of TLR7/8 agonists to reverse mMDSC-mediated immune suppression suggests that they might be useful adjuncts for tumor immunotherapy.

Cancers survive by creating an immunosuppressive microenvironment that inhibits the activity of cytotoxic T and NK cells (1, 2). Myeloid-derived suppressor cells (MDSC) constitute most of these tumor-infiltrating leukocytes and are key contributors to the immunosuppressive milieu that protects tumors from elimination. MDSC arise in the bone marrow from myeloid progenitors (3, 4) and expand in patients with cancer. Although both granulocytic and monocytic MDSC (mMDSC) inhibit T and NK cell responses, mMDSC are more suppressive on a per cell basis (5–7) and promote the generation and expansion of regulatory T cells that further interfere with antitumor immunity (8). In clinical trials, agents that block the activity of mMDSC reduce Treg frequency and improve the efficacy of cancer immunotherapy (9–11). These observations support efforts to identify strategies that can be used in the clinic to inhibit mMDSC-mediated immune suppression.

Murine mMDSC express TLR9 and respond to stimulation by the TLR9 agonist CpG oligodeoxynucleotide (ODN) by differentiating into tumoricidal macrophages (12). In vivo administration of CpG ODN prevents the growth of murine tumors, an outcome linked to increased activity by tumoricidal T cells (12). These findings led us to examine whether the maturation and function of human mMDSC might also be altered by TLR agonists. Consistent with the finding that human mMDSC express TLRs 2, 7, and 8 (but not 9), stimulation with the TLR1/2 agonist Pam3CSK4 (PAM3) induced them to differentiate into immunosuppressive M2-like macrophages that expressed high levels of CD11b. In contrast, stimulation via TLR7/8 caused these mMDSC to differentiate into tumoricidal M1-like macrophages with low CD11b expression. Microarray analysis identified genes that were upregulated during the process of mMDSC differentiation and additional genes uniquely associated with the generation of M1-like macrophages. Because TLR7/8 agonists induce mMDSC from patients with cancer to lose their immunosuppressive capability and differentiate into tumoricidal M1-like macrophages, we propose their use as adjuncts during tumor immunotherapy.

Materials and Methods

Reagents
R848 and PAM3 were purchased from InvivoGen (San Diego, CA). The Live/Dead cell stain kit was purchased from Invitrogen (Eugene, OR). 3M-052 and CL-075 were gifts of Dr. John Vasilakos (3M Drug Delivery Systems, St. Paul, MN). Immunostimulatory CpG ODN was synthesized at the Core Facility of the Center for Biologics Evaluation and Research of the Food and Drug Administration (Bethesda, MD). All Abs used to purify and stain human MDSC were obtained from BD Biosciences (Franklin Lakes, NJ) except for anti-CD200 glycoprotein receptor (CD200R) (CD200R), which was obtained from R&D Systems (Minneapolis, MN).

Cell preparation
Leukaphereses, buffy coats, and PBMC were obtained from patients and healthy volunteers who gave written informed consent to participate in an Institutional Review Board–approved study for the collection of blood samples for in vitro research use (National Institutes of Health, Bethesda, MD). In some cases, PBMC were frozen and stored at −80°C until use. These samples were thawed, washed, and resuspended in RPMI 1640 containing 10% FBS. Fresh or previously frozen PBMC were isolated over a Ficoll-Hypaque gradient, stained with fluorochrome-conjugated Abs against CD33, CD3, CD19, CD57, HLA-DR, CD11b, and/or CD14 and then FACS sorted to isolate mMDSC as defined by the following characteristics: CD33+Lin− (CD3/19/57−), HLA-DR−, CD11b+, and CD14−. Syngeneic CD4+ T cells were isolated from PBMC by negative selection using the naive CD4+ T cell isolation kit II from Miltenyi Biotec (Auburn, CA) as recommended by the manufacturer.

T cell proliferation assay
CD4+ T cells were purified using MACS, labeled with 1 μM CFSE, and stimulated with anti-CD3/28–coated beads at a bead/cell ratio of 1:1. FACs-purified mMDSC plus R848 (3 μg/ml), PAM3 (1 μg/ml), and/or...
anti-CD11b were added for 3 d. Cell division as determined by CFSE content was determined using an LSR II (BD Biosciences).

**Surface marker expression by mMDSC**

FACS-purified mMDSC were cultured with 1 μg PAM3 or 3 μg R848 for 3 d and stained with fluorescence-conjugated Abs against 25F9, CD200R, CD206, CD80, CD86, and/or CD11b for 30 min on ice. Cells were washed, resuspended in PBS/0.1% BSA plus sodium azide, and analyzed using the LSR II.

**Detection of intracytoplasmic and secreted cytokines**

FACS-purified mMDSC were cultured for 72 h with PAM3 or R848 as described above. PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml) (Sigma-Aldrich, St. Louis, MO) were added during the final 5 h of culture. Cells were then treated with permeabilization solution (BD Pharmingen, Franklin Lakes, NJ) and stained with Abs specific for IL-6, IL-12, and/or IL-10. The frequency of internally stained mMDSC was determined by LSR II.

**Cytotoxicity function assay**

MDSC were FACS sorted from PBMC of healthy donors and cultured for 3 d with R848 or PAM3 as described above. A549 tumor cells were then mixed with the MDSC, 6 h at a 1:40 ratio. The cells were then stained with Fl-conjugated anti-EGFR Ab and fluorescent-reactive dye for 30 min on ice. Cells were washed, resuspended in PBS/0.1% BSA plus sodium azide, and lysed tumor cells were identified using an LSR II.

**Microarray analysis of gene expression**

Total RNA was extracted from FACS-purified mMDSC using the RNeasy mini kit (Qiagen) as previously described (13). The RNA was reverse transcribed into cDNA and transcribed in vitro using T7 RNA polymerase into antisense amplified RNA (aRNA) using the Amino Allyl MessageAmp II aRNA kit (Ambion/Life Technologies, Grand Island, NY). aRNA from mMDSC samples was labeled with Cy5 monoreactive dye (Amershams Biosciences, Piscataway, NJ). aRNA from mMDSC samples was labeled with Cy5 monoreactive dye (Amershams Biosciences, Piscataway, NJ). aRNA from mMDSC samples was labeled with Cy5 monoreactive dye (Amershams Biosciences, Piscataway, NJ).

**Effect of TLR agonists on the phenotype of human mMDSC**

Previous studies showed that stimulating murine mMDSC with a TLR9 agonist prevented tumor growth (12). This led us to examine the effect of treating human mMDSC with various TLR agonists targeting TLRs 1, 2, 3, 4, 7, 8, and 9. Cell yields after 3 d showed the greatest increase in cultures containing the TLR1/2 agonist PAM3 or the TLR7/8 agonist R848. Eighty to 90% of the viable cells in these cultures upregulated expression of 25F9, a surface marker identifying mature macrophages (p < 0.01, Fig. 2A, 2B). In the absence of stimulation, <20% of human mMDSC survived and <10% of those typically upregulated 25F9 expression (Fig. 2A, 2B). Subsequent experiments focused on clarifying the effects of PAM3 and R848 on human mMDSC.

Macrophages are categorized into classically activated M1-like or alternatively activated M2-like subsets (15). Although both M1- and M2-like macrophages express 25F9, those of the M2 subset can also express the CD200R and the mannose receptor CD206 (16, 17). When human mMDSC were cultured with PAM3, >70% of the resulting 25F9+ macrophages expressed the two M2-
associated surface markers, CD200R and CD206 (Fig. 2A, 2C, Supplemental Fig. 1). In contrast, 70% of the cells cultured with R848 upregulated 25F9 but failed to express these M2-associated surface markers and thus were phenotypically M1-like. The same effect was observed when mMDSC were cultured with the selective TLR7 agonist 3M-055 or the TLR8 selective agonist CL-075 (Supplemental Fig. 2). In the absence of stimulation, only a small fraction (generally <10%) of mMDSC survived or expressed 25F9. Those displayed a balanced M1/M2 phenotypic ratio (Fig. 2A, 2C, Supplemental Fig. 2).

Cytokine production by mMDSC cultured with TLR agonists

Previous studies established that M1 macrophages protect the host from infection and support tumor destruction in vivo (18–23). Classical M1-like macrophages are characterized by their ability to present Ag, support the development of type I polarized immune responses, and produce proinflammatory cytokines (including IL-12). In contrast, M2-like macrophages have been shown to produce immunosuppressive factors (such as IL-10), to support Th2 immunity, and to support tumor growth (24, 25). The cytokine profile of macrophages generated when human mMDSC were triggered via their TLRs was therefore analyzed. After 3 d in culture with PAM3, 90% of the cells produced IL-10 but not IL-12 (consistent with an M2 profile) whereas the cells generated in the presence of R848 produced IL-12 (consistent with an M1 profile; Fig. 3, Supplemental Fig. 3). Nearly all of the cells cultured in the presence of either PAM3 or R848 produced IL-6.

Functional activity of mMDSC cultured with TLR agonists

Two assays were used to assess the function of cells generated after human mMDSC were stimulated with PAM3 or R848. In the first, their ability to kill A549 tumor targets was evaluated. mMDSC incubated with PAM3 did not acquire the ability to lyse tumor targets, consistent with their M2-like character (Fig. 4). In contrast, mMDSC cultured with R848, 3M-052, or CL-075 gained the ability to lyse A549 tumor cells (p < 0.01, Fig. 4, Supplemental Fig. 3A).

The second assay examined their ability to inhibit T cell proliferation. Syngeneic CD4+ T cells and mMDSC were copurified from leukapheresis samples. The T cells were stimulated to proliferate by the addition of anti-CD3/28–coated beads. This proliferation was inhibited by freshly isolated mMDSC (Fig. 5). The same outcome was observed when mMDSC cultured for 3 d with PAM3 were added: the M2-like macrophages generated in vitro suppressed T cell proliferation. In contrast, mMDSC cultured with R848, 3M-052, or CL-075 lost their ability to inhibit T cell proliferation and thus behaved similar to M1-like macrophages (Fig. 5, Supplemental Fig. 3B). This outcome could not be attributed to any direct effect of PAM3 or R848 on T cells, as anti-CD3/CD28–stimulated T cells proliferated normally in cultures supplemented with these TLR agonists but lacking mMDSC.

FIGURE 2. R848 and PAM3 induce mMDSC to differentiate into macrophages. mMDSC were purified from normal donors as described in Fig. 1 and stimulated in vitro with PAM3 (1 μg/ml) or R848 (3 μg/ml). 25F9 and CD200R expression was examined on day 3. (A) Representative example showing changes in surface marker expression over time. (B) Change in the percentage of 25F9+ cells (mean ± SD) of nine independently studied donors. (C) The percentage of cultured cells bearing an M1-like (25F9+/CD200R−) versus M2-like (25F9+/CD200R+) phenotype was determined by independently analyzing 12 donors/group (mean ± SD). **p < 0.01 versus unstimulated cultures.

FIGURE 3. Effect of TLR stimulation on cytokine production by mMDSC. mMDSC were purified as described in Fig. 1 and stimulated with PAM3 or R848 as described in Fig. 2. The cells were cultured for 1–3 d with brefeldin A being added during the final 5 h. The cells were then permeabilized and stained with Abs specific for IL-6, IL-10, or IL-12. The frequency of mMDSC containing intracytoplasmic cytokine was determined by LSR II. (A) Representative example of cytokine production by cells stimulated with R848 or PAM3. (B) Mean ± SD of samples from four independently analyzed donors per group. ***p < 0.01, ****p < 0.001 versus unstimulated cells.
Expression of CD11b is associated with differences in the suppressive activity of mMDSC cultured with R848 versus PAM3

The above findings establish that both PAM3 and R848 could induce mMDSC to mature into 25F9+ macrophages but that the phenotype and functional activity of mMDSC incubated with PAM3 differed from those exposed to R848. Insight into the mechanism underlying these differences was provided by studies of CD11b. CD11b is a b2 integrin expressed by macrophages that plays a critical role in the formation of cell–cell contacts required to suppress T cell activity. Virtually all of the M2-like macrophages generated after 3 d of culture with PAM3 expressed high levels of CD11b+ (Fig. 6A; mean fluorescence intensity [MFI], 4180 ± 636). This contrasted with the M1-like macrophages generated by R848 whose expression of CD11b was markedly lower (Fig. 6A; MFI, 1465 ± 193, p < 0.02). The relevance of these findings was clarified by adding neutralizing anti-CD11b Ab to cultures of TCR-stimulated T cells plus syngeneic mMDSC. In the absence of neutralizing Ab, the mMDSC efficiently inhibited T cell proliferation (Fig. 6B, 6C). In the presence of anti-CD11b, this suppressive activity was significantly reduced.

Effect of TLR agonists on mMDSC from cancer patients

mMDSC contribute to the suppressive milieu that protects human tumors from immune-mediated elimination. To examine the response of mMDSC from cancer patients to TLR stimulation, peripheral blood was collected from 22 individuals with colon, prostate, pancreatic, or liver cancer (Supplemental Table I). The frequency of mMDSC in these samples ranged from 0.5–9.2%, significantly exceeding the frequency found in normal volunteers (p < 0.02). The behavior of mMDSC from cancer patients cultured with TLR agonists was indistinguishable from that of normal controls. PAM3 induced these mMDSC to differentiate into 25F9+, CD200R+ M2-like macrophages that secreted IL-10 and inhibited the proliferation of TCR-stimulated syngeneic T cells (Fig. 7). R848 treatment primarily generated 25F9+, CD200R− M1-like macrophages that secreted IL-12 and could not suppress T cell proliferation (Fig. 7). mMDSC from patients with different tumor types responded similarly to TLR agonist treatment.

Changes in gene expression induced by TLR ligation

Microarrays were used to examine changes in gene expression that accompanied the differentiation of human mMDSC into either M1-
FIGURE 7. Effect of TLR stimulation on mMDSC isolated from cancer patients. mMDSC and CD4+ T cells were purified from patient PBMC as described in Fig. 1. The types of cancer studied included: liver (n = 8), pancreatic (n = 5), prostate (n = 4), and GI (n = 5). A description of patient characteristics is provided in Supplemental Table I. The purified cells were cultured in the presence of R848 or PAM3 as described in Fig. 2. (A) Cells were stained for surface expression of 25F9 and CD200R on day 3. The frequency of cells (mean ± SD) expressing an M1-like (25F9+/CD200R-) versus M2-like (25F9+/CD200R+) phenotype was determined independently in 22 patients. (B) The accumulation of intracytoplasmic cytokine was examined in 14 patients as described in Fig. 3. (C) mMDSC and syngeneic CSFE-labeled CD4+ T cells were treated as described in Fig. 5. The proliferation of T cells (mean ± SD) was determined independently in samples from four patients. **p < 0.01, ***p < 0.001 versus unstimulated cultures.

Table I. Genes upregulated by PAM3 and/or R848

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Discussion

MDSC facilitate the growth and survival of cancer cells by inhibiting the activity of tumoricidal NK and T cells and by secreting factors that support tumor proliferation (3, 4, 7). The importance of mMDSC is underscored by clinical findings showing that their frequency in the peripheral blood of cancer patients correlates with tumor progression and metastatic potential (26–30). Treatments that reduce mMDSC activity have been shown to improve tumor-specific immunity (9, 31–34). Current results demonstrate that agonists targeting TLR7 and TLR8 represent an effective and previously unrecognized means of reducing the immunosuppressive activity of human mMDSC.

Rodent mMDSC express TLR9. When treated in vitro with the TLR9 agonist CpG ODN, murine mMDSC differentiate into tumoricidal M1 macrophages (12). When large established murine mMDSC from four donors were purified and stimulated with PAM3 or R848 for 0, 30, 75, and 225 min. Results show those genes that were reproducibly upregulated (>5-fold increase versus unstimulated cells) in all donors during this period.
null


R848 and PAM3 induce mMDSC to differentiate into macrophages.

mMDSC were purified from normal donors as described in Fig 1 and stimulated in vitro with PAM3 (1 ug/ml) or R848 (3 ug/ml). 25F9 and CD206 expression were examined on day 3. A) Representative example showing changes in surface marker expression over time. B) Change in the percentage of 25F9+ cells (mean + SD) of 4 independently studied donors. **, p < .01; vs unstimulated cultures
Supplemental Figure 2

3M-055 and CL-075 induce mMDSC to differentiate into phenotypically M1-like macrophages.

mMDSC were purified from normal donors as described in Fig 1 and stimulated \textit{in vitro} with optimal concentrations (determined by preliminary dose-ranging studies) of 3M-055 (100 ng/ml) or CL-075 (200 ng/ml). 25F9 and CD200R expression was examined on day 3. Data are representative of results from 4 independently studied donors.
Effect of 3M055 and CL075 on mMDCS from cancer patients.

mMDSC and CD4+ T cells were purified from cancer patient samples as described in Fig 6. A) Purified mMDSC were cultured with 3 ug/ml R848, 100 ng/ml of 3M055 or 200 ng/ml of CL075 for 3 days as described in Fig 4. Labeled A549 tumor cells were added at a 1:40 ratio for the final 6 hr and the percent targeted for lysis determined. B) 2 x 10^5 mMDSC were cultured with 1 x 10^5 syngeneic CSFE-labeled CD4 T cells in the presence of anti-CD3/28 coated beads, 100 ng/ml of 3M055 or 200 ng/ml of CL075. T cell proliferation was examined on day 3. The percent of T cells proliferating (mean ± SD) was determined independently in samples from 4 patients/treatment group. Data represent the mean ± SD of samples from 4 independently analyzed donors/group. **, p < .01 vs control group.
Supplemental Table I: Characteristics of cancer patients

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