



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

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**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.** *The Journal of Immunology*, 2015, 194: 4215–4221.

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 ago-

nists. Consistent with the finding that human mMDSC express TLRs 2, 7, and 8 (but not 9), stimulation with the TLR1/2 agonist Pam<sub>3</sub>CSK<sub>4</sub> (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), 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  $-80^{\circ}$  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<sup>+</sup>, Lin<sup>−</sup> (CD3/19/57<sup>−</sup>), HLA-DR<sup>−</sup>, CD11b<sup>+</sup>, and CD14<sup>hi</sup>. Syngeneic CD4<sup>+</sup> T cells were isolated from PBMC by negative selection using the naive CD4<sup>+</sup> T cell isolation kit II from Miltenyi Biotec (Auburn, CA) as recommended by the manufacturer.

### T cell proliferation assay

CD4<sup>+</sup> T cells were purified using MACS, labeled with 1  $\mu$ M CFSE, and stimulated with anti-CD3/28–coated beads at a bead/cell ratio of 1:1. FACS-purified mMDSC plus R848 (3  $\mu$ g/ml), PAM3 (1  $\mu$ g/ml), and/or

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The online version of this article contains supplemental material.

Abbreviations used in this article: aRNA, amplified RNA; CD200R, CD200 glycoprotein receptor; MDSC, myeloid-derived suppressor cell; MFI, mean fluorescence intensity; mMDSC, monocytic MDSC; ODN, oligodeoxynucleotide; PAM3, Pam<sub>3</sub>CSK<sub>4</sub>.

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  $\mu$ g PAM3 or 3  $\mu$ g 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  $\mu$ g/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 for 6 h at a 1:40 ratio. The cells were then stained with FI-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 (Amersham Biosciences, Piscataway, NJ). A reference human sample (Stratagene) was processed in parallel and labeled with Cy3. For the coupling reaction, 10  $\mu$ l aRNA (2–4  $\mu$ g) in 0.1 M bicarbonate buffer (pH 8.7) was added to Cy3 or Cy5 in DMSO for 2 h in a final volume of 20  $\mu$ l. Unreacted Cy dye was quenched with 18  $\mu$ l 4 M hydroxylamine and labeled aRNA isolated using an RNeasy MinElute kit (Qiagen).

Human ODN microarrays were produced by Microarrays (Huntsville, AL). Cy3-labeled reference and Cy5-labeled sample aRNAs (15  $\mu$ l each) were combined, denatured by heating for 2 min at 98°C, and mixed with 18  $\mu$ l hybridization solution at 42°C (Ambion, Austin, TX). Microarrays were overlaid with this solution and hybridized for 18 h at 42°C using an actively mixing MAUI hybridization system (BioMicro Systems, Salt Lake City, UT). The arrays were washed after hybridization, dried, and scanned using an Axon scanner equipped with GenePix software 5.1 (Axon Instruments, Foster City, CA). Data were uploaded to the mAdb (a collaboration of the Center for Information Technology/Bioinformatics and Molecular Analysis Section and National Cancer Institute/Center for Cancer Research at the National Institutes of Health; <http://nciarray.nci.nih.gov/>) and formatted.

Raw microarray data from four independent donors were processed as previously described (13). The gene expression profile of treated cells was compared with baseline values of untreated cells from the same donor. Genes that were upregulated by >5-fold in all donors were identified.

#### Accession codes

Microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through accession number GSE57032.

#### Statistical analysis

A two-sided unpaired Student *t* test was used to analyze cellular responses. A *p* value <0.05 was considered to be statistically significant.

## Results

### Human mMDSC suppress T cell proliferation

Normal healthy volunteers were leukapheresed and mMDSC isolated by FACS sorting based on their expression of CD14, CD11b, and CD33 coupled with the absence of HLA-DR and the lineage markers CD3, CD19, and CD57 (Fig. 1A). mMDSC constituted  $0.4 \pm 0.3\%$  of PBMC in normal donors.

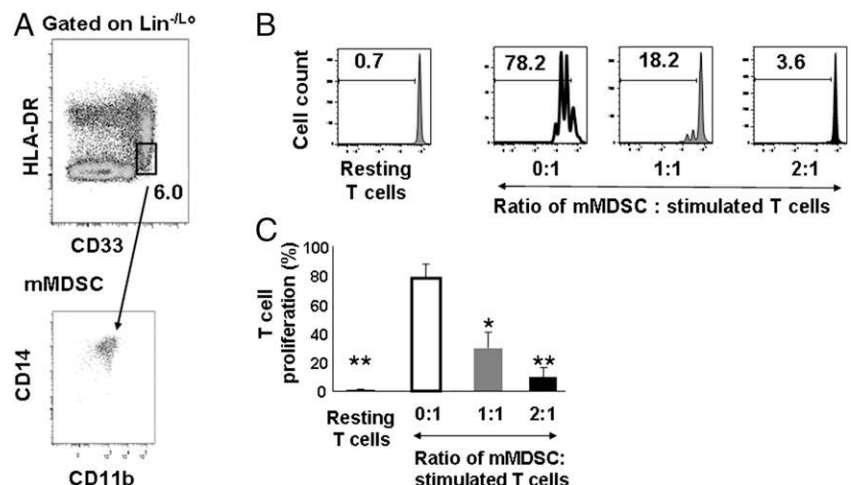
To examine the functional activity of these purified mMDSC, their interaction with CD4<sup>+</sup> T cells was examined. Syngeneic CD4<sup>+</sup> T cells were labeled with CFSE and stimulated to proliferate with anti-CD3/anti-CD28-coated beads. Adding mMDSC to these activated T cells resulted in a dose-dependent inhibition of proliferation (*p* < 0.05, Fig. 1B, 1C) (12, 14).

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

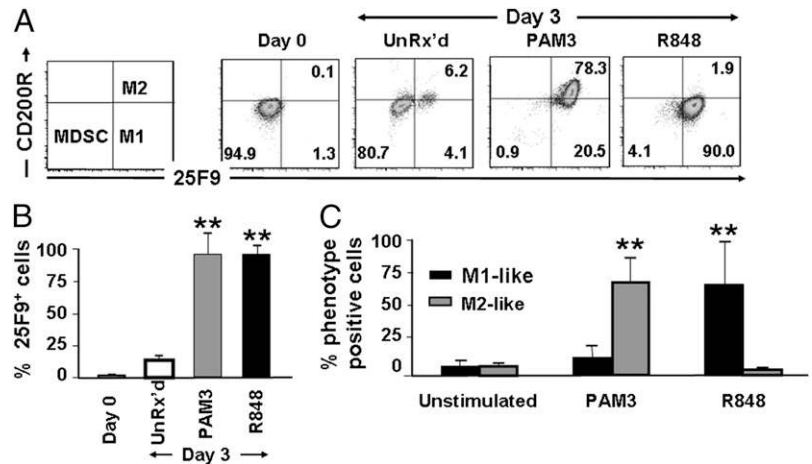
Previous studies showed that stimulating murine mMDSC with a TLR9 agonist prevented tumor growth (12). This led us to examine the effect of treating human mMDSC with various TLR agonists targeting TLRs 1, 2, 3, 4, 7, 8, and 9. Cell yields after 3 d showed the greatest increase in cultures containing 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<sup>+</sup> macrophages expressed the two M2-

**FIGURE 1.** mMDSC from normal volunteers suppress T cell proliferation. (A) mMDSC were identified based on their pattern of surface marker expression: Lin<sup>-</sup>, HLA-DR<sup>-</sup>, CD33<sup>+</sup>, CD14<sup>hi</sup>, and CD11b<sup>+</sup>. (B and C) mMDSC were FACS purified whereas syngeneic CD4<sup>+</sup> T cells were purified from the same donor sample by MACS. T cells (10<sup>5</sup>) were labeled with CFSE, stimulated with anti-CD3/28-coated beads, and cultured with 1–2  $\times$  10<sup>5</sup> mMDSC. T cell proliferation was examined on day 3. (B) Representative example and (C) combined results (mean  $\pm$  SD) of nine independently studied donors are shown. \**p* < 0.05, \*\**p* < 0.01 versus anti-CD3/28-treated T cells alone.



**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  $\mu\text{g/ml}$ ) or R848 (3  $\mu\text{g/ml}$ ). 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<sup>+</sup> cells (mean  $\pm$  SD) of nine independently studied donors. (C) The percentage of cultured cells bearing an M1-like (25F9<sup>+</sup>/CD200R<sup>-</sup>) versus M2-like (25F9<sup>+</sup>/CD200R<sup>+</sup>) phenotype was determined by independently analyzing 12 donors/group (mean  $\pm$  SD). \*\**p* < 0.01 versus unstimulated cultures.



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*

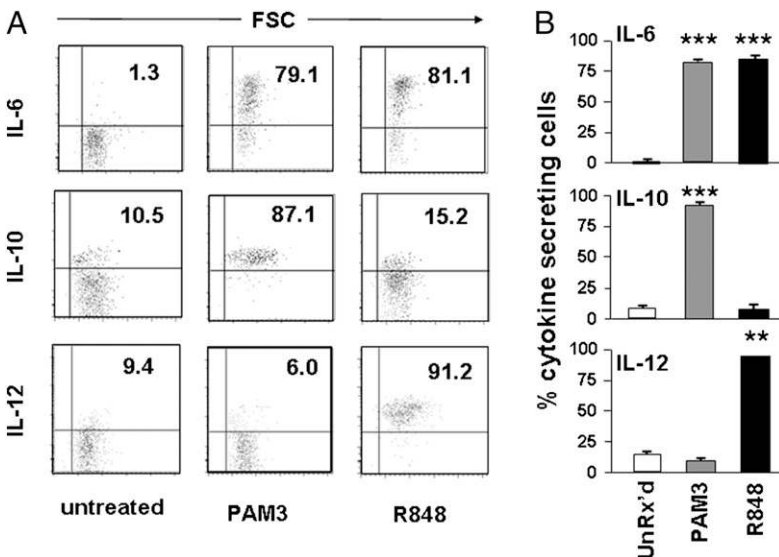
Previous studies established that M1 macrophages protect the host from infection and support tumor destruction in vivo (18–23). Classical M1-like macrophages are characterized by their ability to present Ag, support the development of type I polarized immune responses, and produce proinflammatory cytokines (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,  $\approx$ 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 but not IL-10 (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*

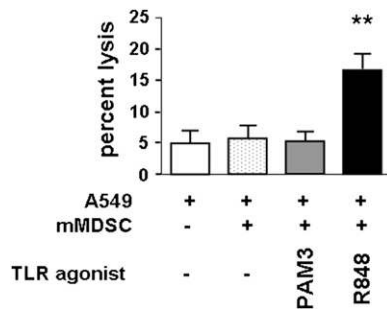
Two assays were used to assess the function of cells generated after human mMDSC were stimulated with PAM3 or R848. In the first, their ability to kill A549 tumor targets was evaluated. mMDSC incubated with PAM3 did not acquire the ability to lyse tumor targets, consistent with their M2-like character (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<sup>+</sup> 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 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  $\pm$  SD of samples from four independently analyzed donors per group. \*\**p* < 0.01, \*\*\**p* < 0.001 versus unstimulated cells.





**FIGURE 4.** Effect of TLR stimulation on tumoricidal activity. mMDSC were purified as described in Fig. 1 and stimulated with TLR ligands for 3 d as described in Fig. 2. Labeled A549 tumor cells were added to these cultures at an E:T ratio of 40:1, and their viability was determined after 6 h. Data represent the means  $\pm$  SD of samples from four independently analyzed donors per group. \*\* $p < 0.01$  versus control mMDSC.

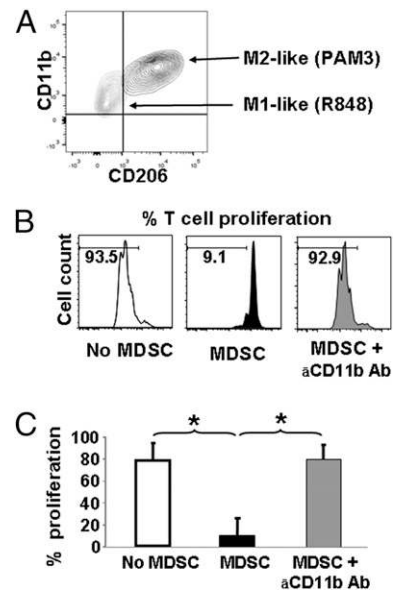
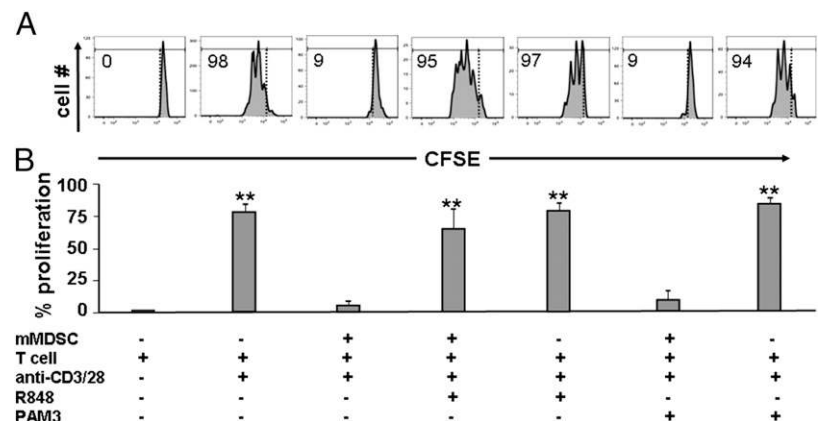
*Expression of CD11b is associated with differences in the suppressive activity of mMDSC cultured with R848 versus PAM3*

The above findings establish that both PAM3 and R848 could induce mMDSC to mature into 25F9<sup>+</sup> 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  $\beta_2$  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<sup>+</sup> (Fig. 6A; mean fluorescence intensity [MFI], 4180  $\pm$  636). This contrasted with the M1-like macrophages generated by R848 whose expression of CD11b was markedly lower (Fig. 6A; MFI, 1465  $\pm$  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

**FIGURE 5.** Effect of TLR stimulation on the ability of mMDSC to inhibit T cell proliferation. mMDSC and CD4<sup>+</sup> T cells were purified as described in Fig. 1. mMDSC ( $2 \times 10^5$ ) were cultured with  $10^5$  syngeneic CFSE-labeled CD4<sup>+</sup> T cells in the presence of anti-CD3/28-coated beads, 3  $\mu$ g/ml R848, or 1  $\mu$ g/ml PAM3. T cell proliferation was examined on day 3. (A) Representative example of the effect of R848 and PAM3 on the ability of mMDSC to inhibit T cell proliferation. (B) The percentage of T cells proliferating (mean  $\pm$  SD) was determined independently in four to eight donors per treatment group. \*\* $p < 0.01$  versus mMDSC-suppressed cultures.



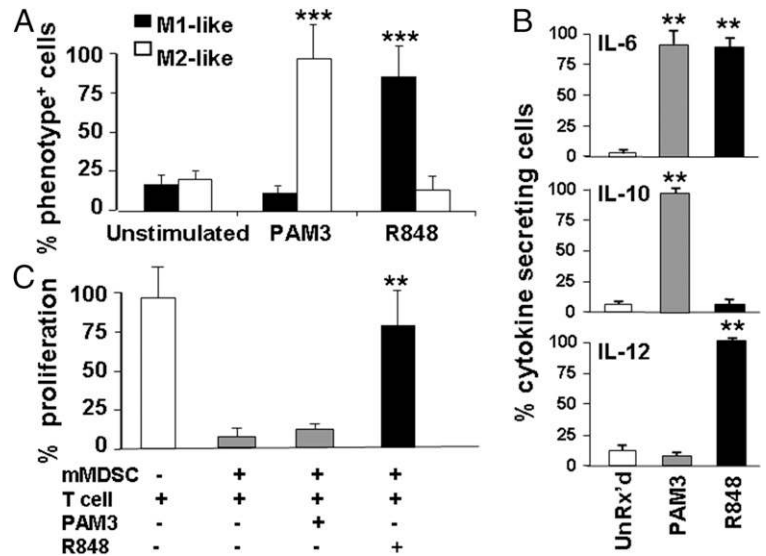
**FIGURE 6.** Effect of R848 and PAM3 on CD11b expression. mMDSC were FACS purified from PBMC and cultured for 3 d in the presence of R848 or PAM3 as described in Fig. 1. (A) Representative example of the level of CD11b expression by M1-like macrophages generated by R848 and by M2-like macrophages generated by PAM3. Similar results were observed in three independent experiments and the MFI of each treatment is described in *Results*. (B and C) mMDSC and CD4<sup>+</sup> T cells were purified as described Fig. 1. CFSE-labeled CD4 T cells ( $10^5$ ) were stimulated with anti-CD3/28-coated beads and cultured with  $10^5$  syngeneic mMDSC for 3 d in the presence of 10  $\mu$ g/ml neutralizing anti-CD11b Ab. A representative example of the effect of anti-CD11b on the inhibition of T cell proliferation mediated by the mMDSC is shown in (B). The mean  $\pm$  SD of this effect in four independently studied donors per group is shown in (C). \* $p < 0.05$  compared with stimulated T cells mixed with mMDSC.

( $p < 0.02$ ). 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<sup>+</sup>, CD200R<sup>+</sup> M2-like macrophages that secreted IL-10 and inhibited the proliferation of TCR-stimulated syngeneic T cells (Fig. 7). R848 treatment primarily generated 25F9<sup>+</sup>, CD200R<sup>-</sup> 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<sup>+</sup> 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 percentage of cells (mean ± SD) expressing an M1-like (25F9<sup>+</sup>/CD200R<sup>-</sup>) versus M2-like (25F9<sup>+</sup>/CD200R<sup>+</sup>) 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 CFSE-labeled CD4<sup>+</sup> 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.



or M2-like macrophages. Preliminary experiments revealed extensive variation in baseline mRNA levels among individual volunteers. To compensate for this variability, microarray profiles from TLR-stimulated samples were compared with unstimulated controls from the same donor. A gene was considered to be significantly upregulated when its level of expression rose >5-fold (exceeding the mean ± 3 SD of all upregulated genes) in all donors at any time during the period from 0.5 through 3.5 h poststimulation.

Results showed that >50% of the genes stimulated by R848 were never upregulated by PAM3 whereas >90% of the genes upregulated by PAM3 were also upregulated by R848 (Table I). Because PAM3 treatment generates M2-like macrophages, we hypothesized that the genes upregulated by both TLR agonists were associated with the differentiation of mMDSC into M2-like macrophages. Conversely, as R848 treatment generated M1-like macrophages, we hypothesized that genes uniquely upregulated by R848 influenced the differentiation of M1-like macrophages.

To examine this process of differentiation, mMDSC were incubated with PAM3 for 2 d, washed, and then cultured with R848 for a final day (Table II). Whereas most of the macrophages present after 2 d in culture with PAM3 expressed the M2-associated marker CD200R, exposure to R848 solely on day 3 yielded cultures in which most cells expressed an M1-like phenotype (25F9<sup>+</sup>/CD200R<sup>-</sup>) (Table II). Indeed, the frequency of macrophages with this M1-like phenotype in cultures treated for 2 d with PAM3 and 1 final day with R848 was statistically indistinguishable from that of mMDSC treated for all 3 d with R848. In contrast, treatment with R848 for 2 d induced nearly half of the mMDSC to differentiate into M1-like macrophages, and the frequency of these 25F9<sup>+</sup>/CD200R<sup>-</sup> macrophages was not changed by the addition of PAM3 on day 3 (Table II). These findings are consistent with the interpretation that genes induced by both PAM3 and R848 drive the differentiation of mMDSC into M2-like macrophages whereas the genes uniquely activated by R848 divert this differentiation toward the M1 lineage.

**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

Table I. Genes upregulated by PAM3 and/or R848

Only PAM3	Only R848	Both PAM3 and R848
IL8	BCL2	ARL5B
KBTD8	BCL2A1	BAG3
NSMAF	CA2	C13orf15
OLR1	CCL2	CCL20
	CFLAR	CD44
	EDN1	CD83
	EREG	CXCL1
	FFAR2	CXCL2
	FLJ37505	CXCL3
	GEM	DNAJA4
	KCNMA1	ELOVL6
	LOC338758	ETNK1
	LOC646329	F3
	LRRC50	IL1A
	MAP3K8	IL6
	NFKBIZ	IRG1
	NPR1	KRT16P2
	OR6K3	LOC399884
	PLAUR	LY6K
	PLLP	MIR155HG
	PMAIP1	PHLDA1
	PNRC1	PURG
	PPP1R15A	RRAD
	PTGS2	SERPINB2
	RECQL4	ST20
	REPS2	TNF
	RGS1	ZC3H12C
	RGS20	ZNF784
	RRP7A	
	RRP7B	
	TNFAIP3	
	TNFAIP6	
	TRIB3	
	ZNF544	

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.

Table II. Kinetics of TLR agonist-induced macrophage differentiation

Day 1	Day 2	Day 3	% M1	% M2
—	—	—	5 ± 2	6 ± 4
R848	R848	R848	83 ± 7	3 ± 1
PAM3	PAM3	PAM3	3 ± 1	89 ± 5
PAM3	PAM3	R848	74 ± 11	18 ± 4
R848	R848	PAM3	42 ± 5	24 ± 4
R848	R848	—	45 ± 6	8 ± 2
PAM3	PAM3	—	7 ± 3	27 ± 1

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) for 2 d. The cells were then washed and restimulated with the same or different TLR agonist for a final day. Data show the mean percentage ± SD of cells bearing an M1-like (25F9<sup>+</sup>/CD200R<sup>-</sup>) versus M2-like (25F9<sup>+</sup>/CD200R<sup>+</sup>) phenotype in independent studies of three donors. Results of treating cells for only 2 d are also shown.

tumors were injected with CpG ODN *in vivo*, infiltrating mMDSC again differentiated into macrophages, an outcome associated with tumor elimination (12). Unfortunately human mMDSC do not express TLR9 or respond to CpG ODN, limiting the clinical applicability of the murine findings. We therefore sought to determine whether other TLR agonists might reduce the immunosuppressive activity of human mMDSC (28). Consistent with the observation that human mMDSC express TLRs 2, 7 and 8, the TLR1/2 agonist PAM3 and the TLR7/8 agonist R848 induced human mMDSC to differentiate into IL-6-secreting 25F9<sup>+</sup> macrophages (Figs. 2, 3). This is consistent with an earlier finding that R848 caused human PBMC and CD34<sup>+</sup> bone marrow cells to differentiate along the myeloid lineage and produce Th1 cytokines (35–37).

Although the signaling pathways triggered by TLRs 2, 7, and 8 are alike in proceeding via MyD88, NF-κB, and MAPK (38, 39), the behavior and phenotype of the macrophages generated by their ligation differed. mMDSC treated with PAM3 matured into “alternatively activated” M2-like macrophages similar to those found in the Th2-polarized environment that characterizes large tumors (40, 41). M2-like macrophages are characterized phenotypically by their expression of CD200R, CD163, and/or CD206 and functionally by their production of factors that support tumor growth and suppress tumor-specific immunity (including glucocorticoids, IL-4, IL-13, and IL-10) (17, 18, 42). As seen in Figs. 2–5 and Supplemental Fig. 1, the 25F9<sup>+</sup> macrophages generated when mMDSC were cultured with PAM3 expressed CD200R and/or CD206, produced IL-10 (but not IL-12), and inhibited the proliferation of TCR-activated T cells. In contrast, the macrophages generated from mMDSC cultured with R848 were M1-like in phenotype and function: they expressed 25F9 but not CD200R or CD206, secreted the proinflammatory cytokine IL-12 but not IL-10, and lost their ability to suppress T cell proliferation while gaining the ability to lyse tumor cells (Figs. 2–5, Supplemental Figs. 1–3).

Microarray analysis of mRNA isolated from TLR-stimulated mMDSC identified genes associated with 1) the general process of differentiation into macrophages and 2) the generation of M1-versus M2-like macrophages. We found that a common set of genes activated by both PAM3 and R848 supported the generation of M2-like macrophages from mMDSC (Table I). A distinct set of genes was upregulated by R848 but not PAM3 and was associated with the further differentiation of mMDSC into M1-like macrophages. The possibility of M2 macrophages being the “default” pathway is consistent with results obtained from mMDSC cultured sequentially with these TLR agonists. mMDSC treated with PAM3 for 2 d differentiated into M2-like macrophages. Adding R848 for the final day of culture diverted differentiation to yield predominantly M1-like macrophages (Table II). No such diversion was observed

when mMDSC were incubated first with R848 and then with PAM3. We are in the process of defining the contribution of specific genes and regulatory pathways to the differentiation of mMDSC into M1- or M2-like macrophages.

CD11b is a β<sub>2</sub> integrin that forms heterodimers with CD18 to generate Mac-1. Mac-1 mediates much of the ICAM binding activity characteristic of mature macrophages (43). Recent reports suggest that the ability of macrophages to recognize T cells and suppress their proliferation is dependent on the expression of CD11b (43, 44). Indeed, Pillay et al. (44) speculated that CD11b is central to the suppression of T cell function mediated by myeloid cells. We found that R848 did not increase the expression of CD11b by 25F9<sup>+</sup>/CD200R<sup>-</sup> M1-like macrophages, consistent with their loss of immunosuppressive activity (Fig. 6B). Similarly, the addition of neutralizing anti-CD11b Ab abrogated the ability of mMDSC to suppress T cell proliferation (Fig. 6C).

R848 was developed as a topical immune response modifier. When administered systemically, undesirable side effects were observed (including a profound depletion of circulating leukocytes) (45–48). We therefore examined the activity of novel TLR7/8 agonists designed for *in vivo* use and found to be safe when administered to mice (49, 50). 3M-055 and CL-075 are selective TLR7 and TLR8 agonists, respectively (48, 51). Phenotypic and functional studies showed that each of these agonists duplicated the ability of R848 to induce human mMDSC to mature into M1-like macrophages and thus might be of clinical utility (Supplemental Figs. 2, 3) (52).

mMDSC isolated from patients with liver, pancreatic, prostate, and GI cancers (Supplemental Table I) responded to stimulation by TLR1/2 and TLR7/8 agonists in a manner indistinguishable from that of normal volunteers (Fig. 7). Of particular relevance, patient cells treated with TLR7/8 agonists (including 3M-055 and CL-075) lost their immunosuppressive activity. This parallels the effect of CpG ODN on murine mMDSC, an activity associated with the elimination of large tumors in mice (12, 53, 54). Current findings thus support clinical testing of TLR7/8 agonists as adjuncts to tumor immunotherapy. Conversely, PAM3 may be useful in generating M2-like macrophages that could be useful in the treatment of autoimmune diseases (55, 56).

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## Disclosures

The authors have no financial conflicts of interest.

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