TLR7-based cancer immunotherapy decreases intratumoral myeloid-derived suppressor cells and blocks their immunosuppressive function

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ABSTRACT
Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells with the capacity to inhibit immunological responses. During cancer progression, MDSC are recruited to the tumor sites and secondary lymphoid organs, leading to the suppression of the antitumor function of NK and T cells. Here, we show that the TLR7/8 agonist resiquimod (R848) has a direct effect on MDSC populations in tumor-bearing mice. Systemic application of R848 led to a rapid reduction in both intratumoral and circulating MDSC. The subpopulation of monocytic MDSC (m-MDSC) was the most affected by R848 treatment with an up to 5-fold decrease in the tumor. We found that TLR7 stimulation in tumor-bearing mice led to a maturation and differentiation of MDSC with upregulation of the surface molecules CD11c, F4/80, MHC-I, and MHC-II. MDSC treated with R848 lost their immunosuppressive function and acquired instead an antigen-presenting phenotype with the capability to induce specific T-cell proliferation. Importantly, we found that MDSC co-injected s.c. with CT26 tumor cells lost their ability to support tumor growth after pretreatment with R848. Our results demonstrate that treatment of tumor-bearing mice with a TLR7/8 agonist acts directly on MDSC to induce their maturation and leads them to acquire a non-suppressive status. Considering the obstacles posed by MDSC for cancer immunotherapy, targeting these cells by a TLR7/8 agonist may improve immune responses against cancer.

INTRODUCTION
The tumor microenvironment crucially contributes to cancer progression by supporting proliferation of tumor cells and at the same time suppressing antitumor immune responses. Myeloid-derived suppressor cells (MDSC) form a heterogeneous population of immature myeloid cells within this microenvironment that are instrumental for tumor-associated immune suppression. During cancer development, MDSC numbers increase at the tumor site as well as in secondary lymphoid organs. Reports have shown a correlation between MDSC frequency and tumor progression both in mice and in cancer patients.

MDSC are immature cells that are characterized by the co-expression of the granulocyte marker Gr1 and the myeloid cell marker CD11b. Under physiological conditions, Gr1+ CD11b+ cells undergo maturation and differentiate into macrophages, dendritic cells, and granulocytes. In tumor-bearing animals, circulating factors released by the tumor block the differentiation of Gr1+ CD11b+ cells, leading to the accumulation of MDSC in the tumor and lymphoid organs. This cell population impairs host immunity by several different mechanisms, including degradation of amino acids essential for T-cell proliferation and activation, secretion of IL-10 which promotes regulatory T-cell expansion, ROS and TGFβ production which inhibit NK-cell functions or production and nitric oxide that targets the T-cell receptor of CD8+ T cells. MDSC thus represent a substantial obstacle to successful cancer immunotherapy. Different strategies have been investigated to target MDSC for the treatment of cancer, such as the inhibition of MDSC suppressive functions, or the depletion of MDSC by inducing their apoptosis or by promoting their differentiation into mature cells that are non-suppressive.

The Toll-like receptor 7 (TLR7) is a sensor for single-stranded viral RNA that is present in the endosomal membrane of specialized immune cells including monocytes, macrophages, and dendritic cells. Targeting TLR7 by synthetic agonists such as resiquimod (R848), imiquimod, or 3M-052 as single therapy or as adjuvant induces a potent activation of both the innate and the adaptive immune systems. In tumor-bearing hosts, TLR7 stimulation leads to an activation of antitumoral immunity that can improve disease outcome in several cancer models. A recent report has shown that TLR7 stimulation can have beneficial effects on MDSC in vitro by inducing the maturation of these cells; however, in vivo data supporting this observation as well as the impact of TLR-driven MDSC maturation on immunotherapy are missing. In the present study, we...
show that treatment of tumor-bearing mice with the TLR7 agonist R848 drastically decreased MDSC numbers in tumors and in secondary lymphoid organs. Furthermore, MDSC from R848-treated mice showed a block in their inhibitory function and a modification of their phenotype toward a mature antigen-presenting cell (APC) phenotype. Together, these results show that MDSC can be efficiently targeted by TLR7 agonists to promote antitumor immunity.

Results

**TLR7-based immunotherapy decreases MDSC numbers in tumor-bearing mice**

To investigate the impact of systemic TLR7 stimulation on MDSC numbers and distribution, tumor-bearing mice were treated with the TLR7 agonist R848. Balb/c mice bearing substantially sized subcutaneous CT26 colon carcinoma-derived tumors (average size 60 mm²) received two injections of R848 subcutaneously on the opposite site of the tumor at a 24-h interval. Organs were harvested for flow cytometry analysis of MDSC 18 h after the second injection. It is well established that MDSC can be subdivided into two populations according to their expression of Gr1 and CD11b. Polymorphonuclear or granulocytic MDSC (g-MDSC) are defined by Gr1<sup>hi</sup> and CD11b<sup>+</sup> whereas monocytic MDSC (m-MDSC) are characterized by Gr1<sup>med</sup> and CD11b<sup>+</sup> and are considered to be, on a per cell basis, the most immunosuppressive population. We found that in mice treated with R848, the number of intratumoral m-MDSC was strongly decreased compared to control-injected mice, even at this early time point after treatment (Fig. 1). As the m-MDSC subpopulation represents the majority of MDSC in CT26 tumors, this decrease also led to a strong reduction in total intratumoral MDSC. An important reduction in m-MDSC was also observed in blood (3-fold decrease) and in spleen (5-fold decrease) of R848-treated mice, indicating that the effect of R848 on MDSC is not limited to the tumor itself but also affects systemic MDSC. Unlike m-MDSC, the g-MDSC population showed a tendency to be increased after R848 treatment, although this was not statistically significant.

In the bone marrow we assessed total MDSC rather than MDSC subpopulations, as these were not easily distinguished. Total MDSC numbers were also significantly reduced in the bone marrow of R848-treated mice. We also investigated the

![Figure 1. TLR7 stimulation decreases the number of MDSC in CT26 tumor-bearing mice. Flow cytometry analysis of MDSC subpopulations in different organs from CT26 tumor-bearing mice that were injected twice at a 24-h interval with 25 μg of R848 or with PBS and sacrificed 18 h after the last injection. Dot plots are representative of one mouse per group and graphs show the mean of 5 mice/group ± SEM. Data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; Student’s t-test.](image-url)
effect of R848 on MDSC in orthotopic tumors in the 4T1 breast cancer model. As for CT26 tumors, we observed a decrease in intratumoral m-MDSC, although this was not significant (Fig. S1). These mice presented an important MDSC accumulation in the spleen that was strongly decreased by TLR7 stimulation. Thus, we show that treatment of tumor-bearing mice with a TLR7 ligand leads to a rapid decrease in the number of m-MDSC, both intratumorally and systemically.

**TLR7-based immunotherapy induces the maturation and differentiation of MDSC in tumor-bearing mice**

In order to further characterize the effect of TLR7-based immunotherapy on MDSC, we analyzed the phenotype of splenic MDSC from R848-treated tumor-bearing mice. MDSC are defined by an immature status with low expression of myeloid differentiation markers such as F4/80 or CD11c, as well as low expression of maturation markers such as MHC-I and MHC-II and the costimulatory molecule CD80. We examined the expression levels of these cell-surface markers on splenic MDSC from CT26 tumor-bearing mice 18 h after the last of two R848 injections. We observed a clear upregulation of the differentiation markers F4/80 and CD11c, as well as of the maturation markers MHC-I, MHC-II, and CD80 on MDSC in response to TLR7 stimulation (Fig. 2A and B). Treatment with a TLR7 agonist thus clearly drives the differentiation and maturation of MDSC in vivo.

**Direct TLR7 stimulation of MDSC induces their maturation and blocks their suppressive activity**

We have previously shown that CpG, a TLR9 ligand, does not act directly on MDSC but only indirectly via cytokines produced by dendritic cells. To investigate whether TLR7 stimulation directly affects MDSC, we assessed the impact of R848 stimulation on in vitro differentiated MDSC with respect to their differentiation and maturation. Bone marrow cells were cultured in the presence of GM-CSF and IL-6 as previously described. After 4 d, most cells showed double expression of Gr1 and CD11b and strongly suppressed T-cell proliferation. These bone marrow-derived MDSC (BM-MDSC) were stimulated in culture with R848 for 48 h. As seen previously with MDSC following TLR7 treatment in vivo, we observed an increase in differentiation markers (F4/80 and CD11c) and maturation markers (MHC-I and MHC-II) on BM-MDSC upon in vitro R848 stimulation (Fig. 3A). We also performed in vitro R848 stimulation of primary MDSC isolated from the spleen of tumor-bearing mice, and observed a similar strong upregulation of differentiation and maturation markers (Fig. 3B). Thus, TLR7 stimulation directly activates the MDSC population, which has been shown to express TLR7.

We next examined whether TLR7 stimulation affects the immunosuppressive function of MDSC, which interfere with T-cell activity and inhibit T-cell proliferation. BM-MDSC were stimulated with R848 for 48 h and co-cultured with T-cells activated to proliferate with anti-CD3/anti-CD28-coated beads. Control PBS-treated BM-MDSC clearly inhibited T-cell proliferation in a dose-dependent manner (Fig. 3C), whereas R848-stimulated BM-MDSC consistently showed a decreased capacity to inhibit T-cell proliferation. Splenic MDSC isolated from CT26 tumor-bearing mice also suppressed T-cell proliferation, whereas ex vivo stimulation of the splenic MDSC with R848 strongly reduced their immunosuppressive ability (Fig. 3D). Taken together, we demonstrate that R848 acts directly on MDSC, leading to the differentiation and maturation of these cells and to a loss of their suppressive function.

**R848-stimulated MDSC acquire the ability to present antigen and to prime T-cell responses**

Since TLR7-stimulated MDSC upregulate the expression of maturation markers and of lineage markers associated with APC, we examined whether these MDSC also gained the ability to present antigen and to prime T-cell responses. We performed an antigen-presentation assay with R848-stimulated MDSC exposed to ovalbumin (OVA), and co-cultured these MDSC with OVA-specific CD4+ T cells from OT-II TCR transgenic mice. We then assessed whether these cells induced proliferation of the T cells. Interestingly, R848-stimulated MDSC acquired the ability to induce T-cell proliferation in an antigen-specific manner (Fig. 4). Thus, direct TLR7 stimulation leads to transdifferentiation of MDSC into mature APC.

![Figure 2. TLR7 stimulation induces the maturation and differentiation of MDSC in tumor-bearing mice. Expression of F4/80, CD80, MHC-I, CD11c, and MHC-II was analyzed by flow cytometry on splenic MDSC from tumor-bearing mice treated with R848 as in Fig. 1. (A) representative histograms of R848-treated (black line) and PBS-treated mice (light shading). (B) Mean fluorescence intensity (MFI) (5 mice/group). Data are representative of three independent experiments, mean ± SEM are shown in (B). **p < 0.01; Student’s t-test.](image-url)
R848 stimulation abolishes the MDSC supporting function on tumor growth

It is well established that the presence of MDSC in the tumor microenvironment supports tumor growth. In order to observe the effect of R848 treatment on MDSC for tumor-supporting functions, we co-injected subcutaneously CT26 tumor cells together with BM-MDSC, pretreated with R848 or untreated, and measured tumor growth. As expected, we observed that co-injection of untreated MDSC significantly promoted CT26 tumor growth. In contrast, tumor cells co-injected with R848-treated MDSC showed the same tumor growth rate as tumor cells injected in the absence of MDSC. Thus, treatment of MDSC by R848 abolished their supporting function for tumor growth (Fig. 5).

Discussion

Toll-like receptor agonists are promising therapeutic agents in the context of cancer. The TLR7 agonist imiquimod is indeed already used as standard care for the topical treatment of some primary skin tumors. We and others have shown that the systemic use of TLR7 agonists also blocks tumor growth in mice, an effect that is at least in part due to the enhancement of the antitumoral function of CD8 T cells and NK cells as well as to the inhibition of Treg function. Indeed, repeated treatment of CT26 tumor-bearing mice with the TLR7 ligand R848 inhibits tumor progression (Fig. S2). In the present study, we analyzed the effect of R848 treatment on MDSC populations after a single cycle of two injections, in order to detect early effects of TLR7 stimulation. We show that administration of R848 drastically decreased the number of intratumoral MDSC in CT26 tumor-bearing mice. Although all MDSC have immunosuppressive activity, intratumoral MDSC are more suppressive than peripheral or splenic MDSC, and a reduction in their numbers is thus highly predictive for treatment outcome. We also show that TLR7 activation significantly decreased the number of MDSC in the bone marrow, which functions as a reservoir for these cells. We made similar observations in the 4T1 orthotopic model of breast cancer, suggesting that these results are not limited to a single cancer model. Thus, administration of a TLR7 agonist...
MDSC are a heterogeneous population of immature cells from the myeloid lineage. They are characterized by an absence of maturation and differentiation markers due to a block in differentiation exerted by tumor-derived factors. As these cells are known to impair tumor immunity, different strategies have been applied to target MDSC in cancer. Several approaches are under investigation to inhibit these cells pharmacologically, including the induction of MDSC apoptosis (by gemcitabine, sorafenib, or 5-fluorouracil)\textsuperscript{28-27}, the inhibition of MDSC function (with sildenafil and cyclooxygenase 2 inhibitors)\textsuperscript{28, 29} and the promotion of maturation of MDSC into non-suppressor cells (all-trans retinoic acid, vitamin D)\textsuperscript{30}. Here, we have shown that TLR7 stimulation leads to the upregulation of maturation markers on MDSC in \textit{vitro}, a finding that is in accordance with previous reports.\textsuperscript{11, 31} In contrast to what we have shown with the TLR9 agonist CpG that matures MDSC indirectly through the action of cytokines produced by dendritic cell\textsuperscript{14}, we show here that the TLR7 agonist R848 directly targeted MDSC. Importantly, we show that following treatment of mice with R848, splenic MDSC rapidly upregulate differentiation and maturation markers, demonstrating that TLR7 activation also promotes MDSC maturation \textit{in vivo}.

One of the key characteristics of MDSC is their suppressive activity on T-cell function, which is closely linked to their immature phenotype.\textsuperscript{2} Depending on their environment, MDSC are able to differentiate into fully mature APC such as dendritic cells or macrophages, or into immunosuppressive phenotypes such as tumor-associated macrophages (TAM). Indeed, exposure of m-MDSC to hypoxic stress drives the maturation of m-MDSC into TAM via the action of the transcription factor HIF1-\alpha.\textsuperscript{22} In the absence of HIF1-\alpha expression, m-MDSC differentiate into DC after hypoxic stress.\textsuperscript{22} In addition, hypoxia selectively reduces STAT3 activity in MDSC, thus, favoring differentiation to TAM.\textsuperscript{25} In the present study, we observed that TLR7 activation directs the differentiation of MDSC toward a mature APC phenotype and decreases MDSC-mediated T-cell suppression, indicating that differentiation into TAM is prevented. Furthermore, we show that TLR7 stimulation of MDSC abolishes their tumor-promoting effect. Altogether, we demonstrate that TLR7-activated MDSC lose their capacity for immunosuppression and acquire a phenotype that is closer to differentiated APC such as macrophages or dendritic cells than to TAM. The detailed molecular mechanisms leading to the altered MDSC phenotype after TLR7 stimulation remain to be elucidated. Since STAT3 activation is crucial for the immunosuppressive function of MDSC, activation of the TLR7/NF-\kappaB pathway may interact with signaling via the JAK-STAT pathway and may thus explain the effect of TLR7 stimulation on MDSC differentiation.\textsuperscript{33, 34}

Our findings show that both peripheral and intratumoral MDSC can be efficiently targeted by TLR7 agonists. We propose that TLR7 ligands could be successfully combined with other anticancer therapies to inhibit unwanted MDSC activity. For example, it was recently shown that neutralization of the chemoattractant CCL2 prevented metastasis by retaining tumor-promoting myeloid cells in the bone marrow.\textsuperscript{35} However, interruption of the anti-CCL2 treatment led to a generalized efflux of these myeloid cells and consequently increased metastasis. A combination therapy with an anti-CCL2 agent and a TLR7 agonist may be a beneficial strategy to rapidly

impacts on numbers of MDSC in the periphery as well as those located within the tumor.

The two subpopulations of MDSC, g-MDSC and m-MDSC, suppress adaptive immunity in tumor-bearing hosts.\textsuperscript{12} Reports however show that on a per cell basis ratio, m-MDSC are more suppressive than g-MDSC.\textsuperscript{13} Moreover, m-MDSC have the ability to induce expression of FOXP3 in T cells and consequently to increase the proportion of T regulatory cells in the tumor microenvironment and in secondary lymphoid organs, making them an important therapeutic target to prevent tumor-associated immunosuppression.\textsuperscript{23} We show that R848 treatment of tumor-bearing mice mainly affected m-MDSC, leading to a strong reduction of this subpopulation in the tumor but also in the spleen and within circulating MDSC. Taken together, our findings show that TLR7 activation decreases the global number of MDSC and shifts the ratio of remaining MDSC toward the less suppressive g-MDSC subpopulation.

![Figure 4](image-url)  
**Figure 4.** R848-stimulated MDSC acquire the ability to present antigen and to prime T-cell responses. BM-MDSC were stimulated for 48 h with R848 (2 \( \mu \text{g/mL} \)) or PBS. Cells were then incubated with OVA for 90 min. After washing, MDSC were co-incubated with OVA-specific, CFSE-labeled CD4\(^+\) T-cells for three days. T-cell proliferation was determined by flow cytometry. Data are representative of three independent experiments. Mean ± SEM are shown. \( *p < 0.01 \), \( **p < 0.001 \). Student’s t-test.

![Figure 5](image-url)  
**Figure 5.** R848-treated MDSC lose their supporting function for promoting tumor growth. Balb/c mice were subcutaneously injected with CT26 alone or co-injected with CT26 and MDSC treated or not with R848 as in Fig. 3. Tumor inoculations were done with 2.5 \( \times 10^5 \) CT26 cells (empty circle), or 2.5 \( \times 10^5 \) CT26 and 2.5 \( \times 10^5 \) BM-MDSC without stimulation (empty square) or 2.5\( \times 10^5 \) CT26 and 2.5 \( \times 10^5 \) BM-MDSC stimulated with R848 (full square). Mean ± SEM of tumor area measured for individual mice (\( n = 4 \)) are shown. \( *p < 0.05 \), \( **p < 0.01 \). ANOVA with Bonferroni’s post-test.
mature myeloid progenitor cells and prevent MDSC-associated immunosuppression upon cessation of the anti-CCL2 treatment. MDSC targeting by TLR7 agonists could thus open new strategies for combination immunotherapy.

Materials and methods

Mice

Female BALB/c and C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France). TCR transgenic OT-II mice were obtained from Charles River (Sulzfeld, Germany). Mice were housed under specific pathogen-free conditions at the animal facility of the University of Fribourg and used at 6–12 weeks of age for in vivo experiments. All animal procedures were conducted in strict compliance with the Swiss federal legislation for animal experimentation.

Cell culture and tumor inoculation

The CT26 tumor cell line was cultured in complete medium: RPMI 1640, 1% (v/v) sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol (all from PAA Laboratories) and 10% (v/v) fetal calf serum (Life Technologies, Grand Island, NY). CT26 tumor inoculation was performed with 2.5 × 10⁵ cells injected in the flank of 6–8-week-old mice. Tumor area was calculated by the product of the perpendicular diameters of individual tumors and expressed in square millimeter. 4T1 primary tumors were initiated by orthotopic injection of 4T1 (ATCC; CRL-2539) mammary carcinoma cells into the fourth mammary fat pad of female BALB/c mice as described.³⁶

Primary cell isolation

Bone marrow cells were flushed from tibia and femur and passed through a 40 μm cell strainer. Splenocytes were obtained by cutting the spleen into small pieces and passing through a 40 μm cell strainer. Blood was taken by cardiac puncture directly after mice were euthanized. Single cell suspensions from tumor tissue were obtained by cutting tumors in small pieces followed by incubation with "Tumor Dissociation Kit" enzymes (Miltenyi Biotec) for 45 min at 37°C under agitation. Tumor lysates were then washed once with PBS and passed through 100 μm and 40 μm cell strainers. MDSC isolation was performed from single cell suspensions of tumors or splenocytes using the Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s protocol.

Preparation of bone marrow-derived MDSC (BM-MDSC)

BM-MDSC were obtained from primary bone marrow cells as previously described.¹⁵ After red blood cell lysis (BD Pharm Lyse, BD Biosciences), cells were cultured in complete medium supplemented with 40 ng/mL GM-CSF and 40 ng/mL IL-6 (both from PeproTech, Rocky Hill, NJ) and harvested at day 4. The percentage of Gr1⁺CD11b⁺ cells was over 90%.

In vitro stimulation experiments

Stimulation of the cells was carried out in complete medium with agonists at the concentrations described in the figure legends. For proliferation assays, T cells were purified from the spleen of naive mice by negative selection with the Pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were stained with CFSE (Biolegend, San Diego, CA) according to the manufacturer’s protocol and incubated in 96-well plates at 8 × 10⁵ T cells/well with different ratios of MDSC. Then, cells were stimulated for 3 d with Dynabead mouse T-cell activator anti-CD3/anti-CD28 (Thermo Scientific, Waltham, MA). To measure T-cell proliferation, CFSE dilution was assessed by flow cytometry.³⁷ For antigen presentation assays, MDSC were stimulated with R848 (2 μg/mL) for 48 h and incubated for 1.5 h with OVA protein (1 μg/mL) (Invivogen, Toulouse, France). 8 × 10⁵ OVA-specific T cells from OT-II mice labeled with CFSE (5 μM) were added to the culture medium. After 3 d, cell proliferation was monitored by flow cytometry.

Flow cytometry

Cells were incubated with Fc-blocking antibody (BioLegend, San Diego, CA) for 15 min and stained with fluorescently labeled antibodies. Anti-mouse CD11b (M1/70), CD11c (N418), CD3 (17A2), CD4⁺ (RM4-5), Gr1 (RB6-8C5), Ly6c (HK1.4), Ly6g (1A8), F4/80 (BM8), MHC-I H-2Kd (SF1-1.1.1), MHC-II (M5/114.15.2), and CD80 (16-10A1) were purchased from BioLegend (San Diego, CA). All cell acquisitions were recorded using the MACSQuant system from Miltenyi Biotec. Data were analyzed using FlowJo v10.0.8 Software (Tree Stat, Inc., Ashland, USA).

Statistical analysis

Data were analyzed by either unpaired Student’s t-test or by ANOVA as appropriate followed by Bonferroni post-tests. Results are shown in column graphs as the mean ± standard error of the mean (SEM). The number of asterisks in the figures indicates the level of statistical significance as follows: *p < 0.05; **p < 0.01; ***p < 0.001. All data were analyzed using GraphPad Prism 5.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Anne Oberson for excellent technical assistance and Jérôme Widmer for help with animal experimentation.

Funding

This work was supported by the Swiss Cancer League Grant KLS-2910-02-2012 (to C.B. and C.H.), Swiss National Science Foundation Grants 31003A_138284, 310030-156372, ProDoc PDFMP3_137079 and National Center of Competence in Research Bio-Inspired Materials (to C.B.), Swiss National Science Foundation Grants 31003A_135738 and Medic
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