Arch. Pharm. Res. (2014) 37:1234–1240
DOI 10.1007/s12272-014-0379-4

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Resiquimod, a TLR7/8 agonist, promotes differentiation of myeloid-derived suppressor cells into macrophages and dendritic cells

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Received: 10 February 2014 / Accepted: 24 March 2014 / Published online: 19 April 2014

Abstract Myeloid-derived suppressor cells (MDSCs) accumulate in cancer patients and tumor-bearing mice, subsequently suppressing the host immune system. MDSCs represent a group of immature myeloid cells expressing CD11b and Gr-1. Here, we show that a Toll-like receptor (TLR) agonist, resiquimod, which binds to TLR7 and TLR8, induces the differentiation of MDSCs into mature myeloid cells. MDSCs were isolated from mice bearing mammary carcinoma 4T1 cells, and the purified MDSCs were cultured in the presence of resiquimod for 5 days. Phenotypic analysis showed that the resiquimod-treated MDSCs differentiated into $F4/80^+$ macrophages and $CD11c^+/I\text{-}A^{d+}$ dendritic cells. Functional analysis showed that the MDSCs also lost their suppressive activity on T cells. Resiquimod-treated MDSCs significantly enhanced the proliferation of T cells that were treated with anti-CD3 and anti-CD28 monoclonal antibodies. These results show that resiquimod induces the differentiation of MDSCs into macrophages and dendritic cells, and also suggest that resiquimod may improve cancer immunotherapy by reducing immunosuppressive MDSCs.

Keywords R848 · TLR7/8 · Myeloid-derived suppressor cell · Mammary carcinoma

Introduction

Many tumors induce the accumulation of myeloid lineage cells in the body. These myeloid-derived suppressor cells (MDSCs) have potent immunosuppressive activity on the adaptive and innate immune responses (Sica and Bronte 2007; Rabinovich et al. 2007; Gabrilovich and Nagaraj 2009). MDSCs inhibit the activation and proliferation of CD4 T cells and CD8 T cells. Moreover, MDSCs suppress the functions of natural killer (NK) cells, macrophages, and dendritic cells. MDSCs are usually defined as $CD11b^+Gr\text{-}1^+$ cells in the mouse (Gabrilovich et al. 2007). These $CD11b^+Gr\text{-}1^+$ cells are immature myeloid cells, and under normal differentiation conditions, they will differentiate into mature dendritic cells (DCs), macrophages, and/or granulocytes expressing CD11c, CD11b, and/or Gr-1 markers, respectively. In tumor-bearing individuals, the presence of tumor-derived factors blocks the differentiation of the immature $CD11b^+Gr\text{-}1^+$ cells, thereby resulting in their accumulation (Serafini et al. 2006; Gabrilovich et al., 2007). Tumor-derived factors inducing the accumulation of MDSCs are interleukin (IL)-6, IL-10, vascular endothelial growth factor, and granulocyte macrophage-colony stimulating factor (GM-CSF) (Gabrilovich et al. 2012; Ostrand-Rosenberg et al. 2012).

Several agents have been used to induce the differentiation of MDSCs into mature antigen presenting cells (APCs). These include GM-CSF plus IL-4, GM-CSF plus tumor necrosis factor-α, macrophage-colony stimulating factor, all-*trans*-retinoic acid, and 1-α 25-dihydroxyvitamin D3 (Wiers et al. 2000; Almand et al. 2001; Kusmartsev et al. 2003). MDSC levels have also been reduced in tumor-bearing mice by using chemotherapeutic drugs such as gemcitabine (Suzuki et al. 2005; Le et al. 2009).

Toll-like receptors (TLRs) are expressed in many types of immune cells and function as the key sensors of microbial products (Takeda et al. 2003). While most TLRs are expressed on the cell surface, TLR3, TLR7, TLR8 and TLR9 are almost exclusively expressed in intracellular

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compartments such as the endosomes (Kanzler et al. 2007). The agonists for TLR3 and TLR7-9 are nucleic acids generated from viral and bacterial infections, such as double-stranded RNA (TLR3), unmethylated DNA (TLR9), and single-stranded RNA (TLR7 and TLR8) (Kanzler et al. 2007; Park et al. 2012; Vasilakos and Tomai 2013). TLR7 is expressed in B cells and plasmacytoid DCs, and TLR8 is expressed in monocytes and myeloid DCs (Chuang and Ulevitch 2000; Iwasaki and Medzhitov 2004). Resiquimod (R-848) is a synthetic imidazoquinoline-like molecule that binds to TLR7 and TLR8 (Jurk et al. 2002). Similar to other TLR agonists, resiquimod is an immune-response modifier possessing antiviral and antitumor activity (Thomsen et al. 2004; Lee et al. 2013; Vasilakos and Tomai 2013).

Pharmacological agents that inhibit the accumulation or the immunosuppressive function of MDSCs would improve the efficacy of immune-based cancer therapies. During our search for MDSC differentiation-inducing agents, we found that resiquimod efficiently induces MDSC differentiation into macrophages and dendritic cells. Resiquimod, therefore, could be a useful agent to potentially enhance the effects of cancer immunotherapies.

Materials and methods

Tumor cell line and cell culture

The 4T1 mammary tumor cell line was kindly provided by Dr. Wang Jae Lee, College of Medicine, Seoul National University, Seoul, South Korea. The 4T1 cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone). Purified MDSCs were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone).

Mice

Male BALB/c mice aged 8–10 weeks were purchased from OrientBio (Gyeonggi, South Korea). The mice were maintained in specific pathogen-free facility of Laboratory Animal Research Center of Chungbuk National University, and were handled according to the institutional protocols approved by the Animal Care Committee of Chungbuk National University.

Isolation of MDSCs

Balb/c mice were inoculated subcutaneously in the flank with 1×10^6 4T1 mammary carcinoma cells. Tumor-bearing mice were sacrificed when the average tumor volume of mice in the untreated control group reached 1,000 mm^3. The spleens were collected and MDSCs were isolated from the spleens using magnetic-activated cell sorting, as previously described (Nagaraj et al. 2010). Briefly, erythrocyte-depleted splenocytes were depleted of $CD19^+$ cells and $CD11c^+$ cells via magnetic selection using anti-CD19 and anti-CD11c microbeads and LD columns (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. The residual $CD11b^+$ cells were positively selected using anti-CD11b microbeads in LS columns (Miltenyi Biotec) to obtain the purified MDSCs. The purity of $CD11b^+Gr\text{-}1^+$ cells ranged from 88 to 92 %.

Phenotypic analysis

Cells were stained with monoclonal antibodies recognizing murine cell surface molecules after blocking of FcR-binding using an anti-CD16/CD32 monoclonal antibody (mAb) (clone 2.4G2), as previously described (Lee et al. 2001). All of the mAbs including anti-mouse CD11b and anti-mouse Ly-6G/6C (Gr-1) mAbs were purchased from BD Biosciences (San Jose, CA, USA). Flow cytometric analysis was performed using the FACSCanto system (BD Bioscience).

Functional assay

The T cell-stimulatory activity of MDSCs cultured in the presence (5 μg/ml) or absence of resiquimod for 5 days was evaluated using T cells that were treated with anti-CD3 mAb and anti-CD28 mAb (BD Bioscience). Briefly, CD4 T cell or CD8 T cell populations were isolated from the spleens of normal BALB/c mice using mouse CD4 T cell or CD8 T cell enrichment kits (StemSep, Vancouver, Canada) according to the manufacturer's instructions. Isolated T cells (1×10^5 cells/well) were treated with 100 ng/ml anti-CD3 mAb (BD Bioscience) and 200 ng/ml anti-CD28 mAb (BD Bioscience) on ice for 10 min, and then were mixed with the indicated numbers of MDSCs. The cells were cultured for 48 h at 37 °C and pulsed with 1 μCi/ml [^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA, USA) for an additional 18 h. Thymidine uptake was measured using a scintillation counter.

Statistical analysis

The statistical significance of the difference between values of the control and treatment groups in the various assays was assessed using Student *t* test.

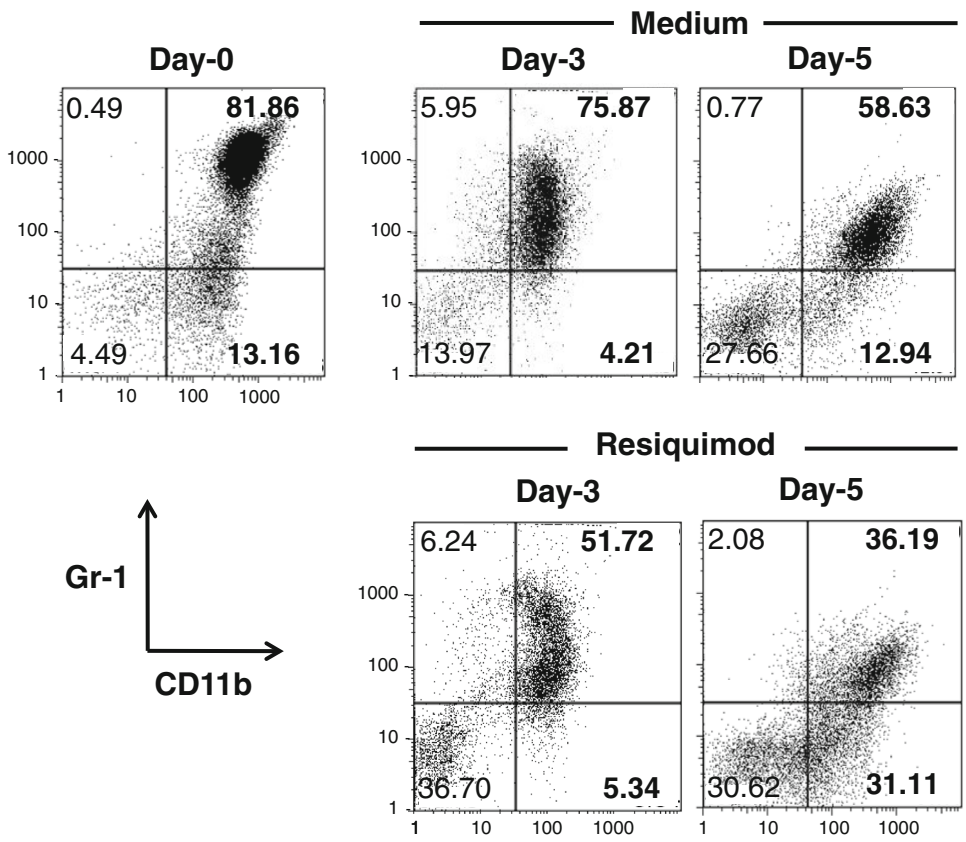

Fig. 1 Resiquimod reduces the number of $CD11b^{+}Gr\text{-}1^{+}$ cells when added to purified MDSC culture. MDSCs were purified from the spleens of 4T1 tumor-bearing mice when the average tumor volume reached approximately 1,000 mm^3. The MDSCs (1×10^5/well) were cultured in the presence (5 μg/ml) or absence of resiquimod for indicated durations. Cells were then harvested and stained with anti-mouse CD11b and anti-mouse Ly-6G/6C (Gr-1) mAbs. Representative dot blots of one experiment of the three independent experiments are shown. The Day-0 cells were purified MDSCs, which were used for culturing with resiquimod

Results

Resiquimod reduces the number of $CD11b^{+}Gr\text{-}1^{+}$ cells when added to purified MDSC cultures

The 4T1 mouse mammary tumors are well-known for inducing the accumulation of large numbers of highly immunosuppressive MDSCs in the spleen, tumor mass, and bone marrow (Gabrilovich and Nagaraj 2009). To examine the effects of resiquimod on MDSC differentiation, MDSCs that were isolated from the spleens of 4T1 tumor-bearing mice were cultured in the presence or absence of resiquimod for 5 days. The concentration of resiquimod was 5 μg/ml. The changes in the percentage of $CD11b^{+}Gr\text{-}1^{+}$ cells were analyzed on days 3 and 5 after the initiation of the culture. Resiquimod substantially reduced the number of $CD11b^{+}Gr\text{-}1^{+}$ cells in the culture (Fig. 1). When examined on day 5, resiquimod-treated group contained 36.2 % $CD11b^{+}Gr\text{-}1^{+}$ cells, while the control untreated group contained 58.6 %. By contrast, resiquimod considerably increased the number of $CD11b^{+}Gr\text{-}1^{-}$ cells in the culture. On day 5, resiquimod-treated group contained 31.1 % $CD11b^{+}Gr\text{-}1^{-}$ cells, while the control untreated group contained 12.9 %. There was essentially no difference in the cell viability between the MDSCs cultured in the absence of resiquimod and those cultured in the presence of resiquimod. These results suggest that resiquimod induced the differentiation of MDSCs into myeloid cells.

Resiquimod induces differentiation of MDSCs into macrophages and dendritic cells

The phenotype of the cells derived from resiquimod-treated MDSCs was examined on day 3 and 5 after the initiation of the culture. Resiquimod efficiently induced MDSC differentiation into $F4/80^{+}$ macrophages (Fig. 2). On day 3, the resiquimod-treated MDSC cultures contained 72.5 % $F4/80^{+}$ macrophages, compared with 40.7 % in the control cultures. Treatment of MDSCs with resiquimod for an additional 2 days (total 5 days) did not change the percentage of $F4/80^{+}$ macrophages, but strongly increased the levels of F4/80 expression (Fig. 2, bottom panel). DCs, which exert the most potent antigen presenting function among professional APCs, are usually defined as cells expressing CD11c and class II MHC molecules. Therefore, we also examined the percentage of cells expressing CD11c and $I\text{-}A^{d}$. Resiquimod also induced MDSC differentiation into $CD11c^{+}/I\text{-}A^{d+}$ DCs (Fig. 3). On day 3, the resiquimod-treated MDSC cultures contained 10.5 % $CD11c^{+}/I\text{-}A^{d+}$ DCs, compared with 5.4 % in the control cultures. Treatment of MDSCs with resiquimod for an

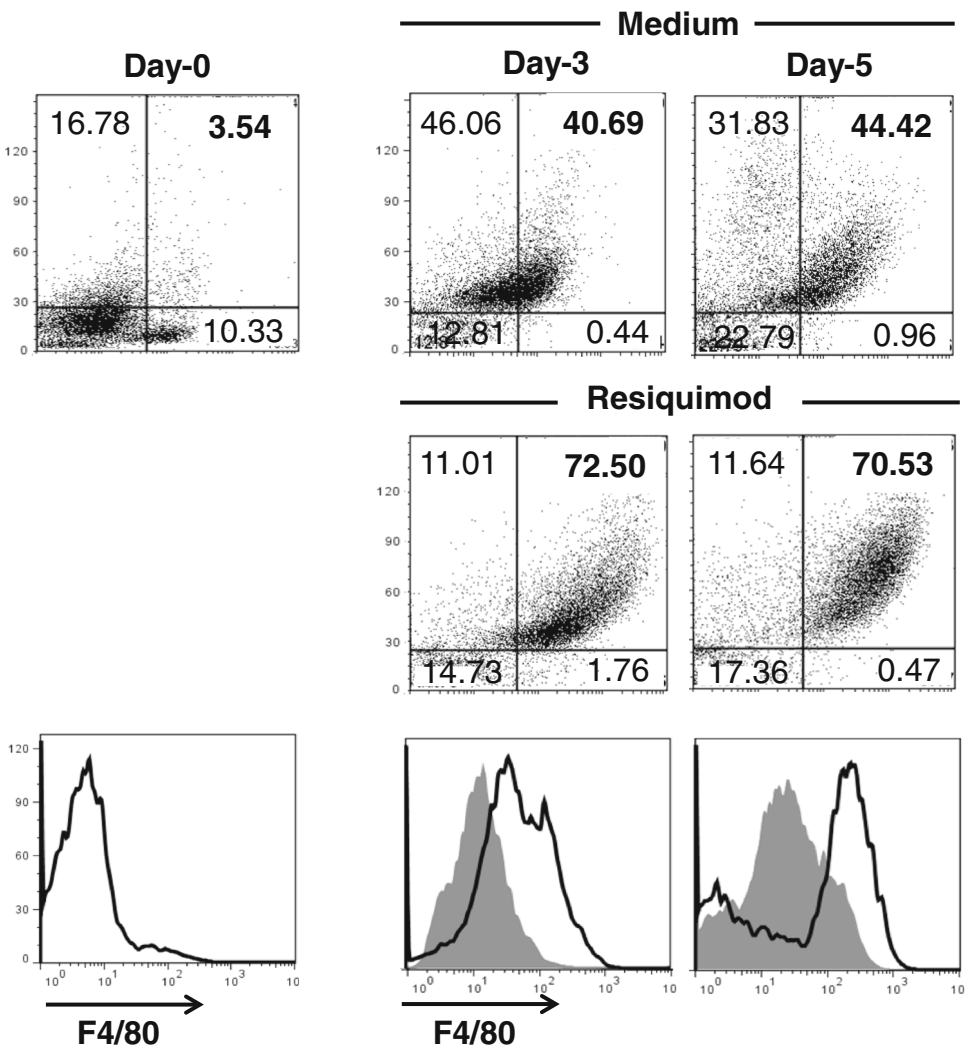

Fig. 2 Resiquimod induces differentiation of MDSCs into macrophages. Purified MDSCs (1×10^5/well) were cultured in the presence (5 μg/ml) or absence of resiquimod for the indicated durations. Cells were then harvested, and stained with anti-mouse F4/80 mAb. Representative dot blots of one experiment of the three independent experiments are shown. The Day-0 cells were purified MDSCs, which were used for culturing with resiquimod. In the overlaid histograms, *shaded histograms* represent the expression levels of F4/80 of the MDSCs cultured in the absence of resiquimod, and *thick line histograms* represent those of the MDSCS cultured in the presence of resiquimod

additional 2 days (total 5 days) further increased the percentage of $CD11c^+/I\text{-}A^{d+}$ DCs to 20.1 %, while the percentage in control cultures increased to 9.9 % (Fig. 3).

Resiquimod-treated MDSCs exert a potent T-cell stimulatory function

MDSCs have potent immunosuppressive activity on T cells (Sinha et al. 2005; Bunt et al. 2006). The suppressive activity of MDSCs isolated from the spleens of 4T1 tumor-bearing mice was initially determined using syngeneic T cells. In these experiments, purified MDSCs were co-cultured with syngeneic CD4 T cells and CD8 T cells that had been treated with 100 ng/ml anti-CD3 mAb and 200 ng/ml anti-CD28 mAb. The MDSCs isolated from the spleens of 4T1 tumor-bearing mice were equally suppressive toward both CD4 and CD8 T cells (Fig. 4).

The effects of resiquimod-treatment on the APC function of MDSCs were examined using MDSCs that were cultured in the presence or absence of resiquimod for 5 days. The cultured cells were harvested, and mixed with anti-CD3/anti-CD28 mAb-treated CD4 T cells or CD8 T cells in a ratio of 2:1. Addition of the cells harvested from resiquimod-treated MDSC cultures significantly increased the proliferation of CD4 T cells (Fig. 5a). The CD8 T cell stimulatory activities of MDSCs cultured in the presence of resiquimod for 5 days were also assessed by using the same experimental system in essence. We again found that cells harvested from resiquimod-treated MDSC cultures induced the proliferation of anti-CD3/anti-CD28 mAb-treated CD8 T cells to a significantly higher level than that of cells from untreated control MDSC cultures (Fig. 5b). These results show that the cells differentiated from MDSCs by the action of resiquimod function to induce proliferation of CD4 and CD8 T cells.

Discussion

The importance of manipulating the host-tumor interaction has become increasingly clear in the treatment of cancers,

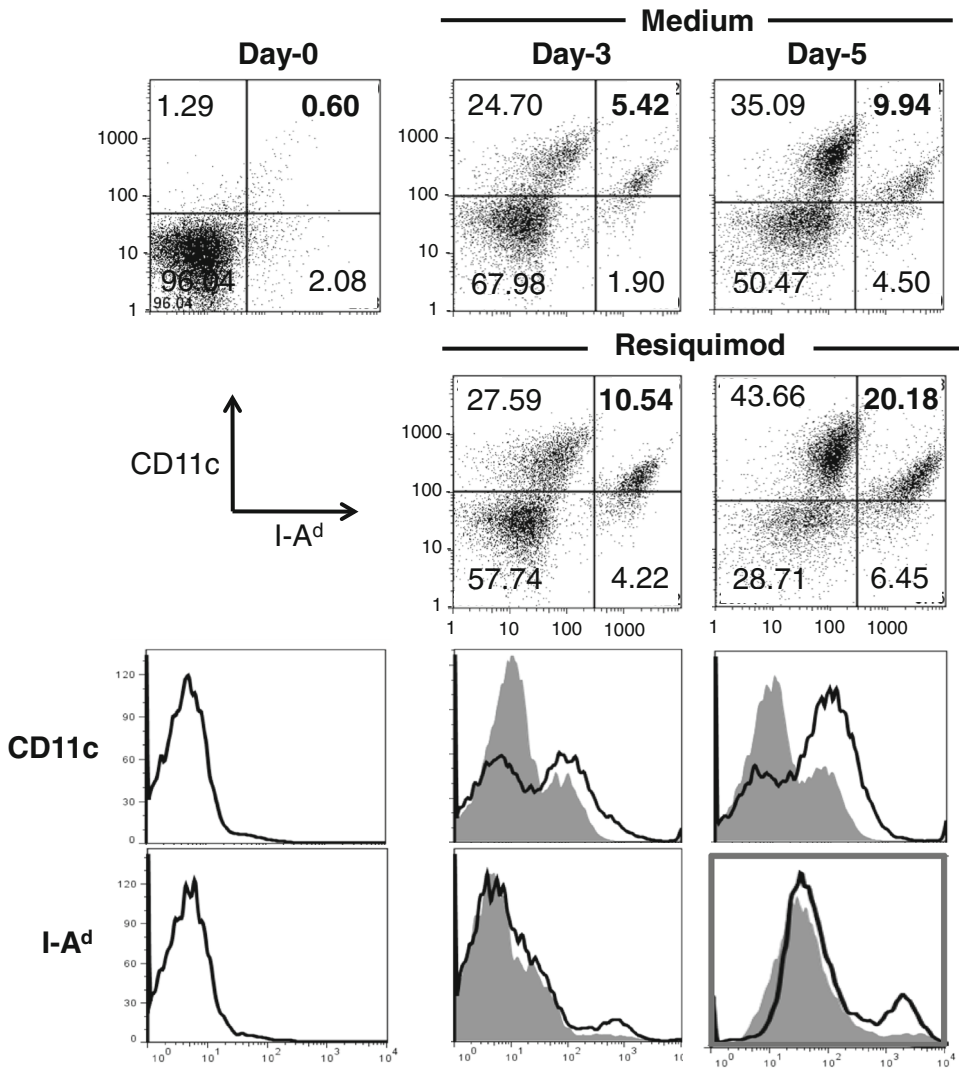

Fig. 3 Resiquimod induces differentiation of MDSCs into dendritic cells. Purified MDSCs (1×10^5/well) were cultured in the presence (5 μg/ml) or absence of resiquimod for the indicated durations. Cells were then harvested, and stained with anti-mouse CD11c and anti-mouse I-A^d mAbs. Representative dot blots of one experiment out of three independent experiments are shown. The Day-0 cells were purified MDSCs, which were used for culturing with resiquimod. In the overlaid histograms, *shaded histograms* represent the expression levels of CD11C and I-A^d of the MDSCs cultured in the absence of resiquimod, and *thick line histograms* represent those of the MDSCS cultured in the presence of resiquimod

considering that many tumors suppress the host immune system. MDSCs are one of the key cellular populations that mediate tumor-associated immune suppression (Gabrilovich and Nagaraj 2009). MDSCs inhibit the activation and proliferation of CD4 T cells and CD8 T cells. In addition, MDSCs exert immunosuppressive functions on NK cells, macrophages, and dendritic cells (Gabrilovich and Nagaraj 2009).

Resiquimod, which induces MDSC differentiation into macrophages and dendritic cells, is an attractive candidate for enhancing the effects of cancer immunotherapy, in which the MDSC population is the major factor that mediates tumor-associated immune suppression. In the present study, we showed that resiquimod induces MDSC differentiation into F4/80$^+$ macrophages and CD11c$^+$/I-A$^+$ DCs. Furthermore, we showed that the cells differentiated from MDSCs by the action of resiquimod exert higher proliferation-inducing activity on antigen-primed T cells than that by the cells obtained from untreated MDSC cultures.

Resiquimod, which is an agonist for TLR7/8, exerts antiviral and antitumor immunomodulatory activities mainly by stimulating production of various cytokines (Dockrell and Kinghorn 2001; Stanley 2002). Resiquimod has also been reported to increase the efficacy of DNA vaccination in mice (Thomsen et al. 2004). The effect of resiquimod on the induction of myeloid cell maturation is debatable. Resiquimod was reported to induce functional maturation of human epidermal Langerhan's cells, but not their phenotypic maturation (Burns et al. 2000). In a separate study, resiquimod was also shown to impair DC differentiation and maturation from human monocytes (Assier et al. 2007). However, other studies showed that resiquimod promoted the generation of functional DC population from human monocytes (Gorden et al. 2005; Hackstein et al. 2011). To our knowledge, the effect of

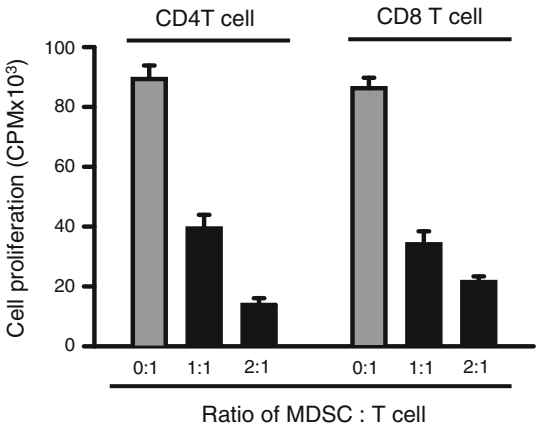

Fig. 4 Suppression of T cell proliferation by MDSCs. MDSCs were purified from the spleens of 4T1 tumor-bearing mice when the average tumor volume of the untreated group of mice reached approximately 1,000 mm^3. The MDSCs (1×10^5/well) were co-cultured with syngeneic CD4 T cells, or CD8 T cells in the presence of 100 ng/ml anti-CD3 mAb and 200 ng/ml anti-CD28 mAb. The ratio of MDSCs to T cells is indicated on the *X*-axis. The cells were cultured for 48 h and then pulsed with 1 μCi/ml [^{3}H]thymidine for an additional 18 h. The results are expressed as mean value ± SE of three indifferent experiments

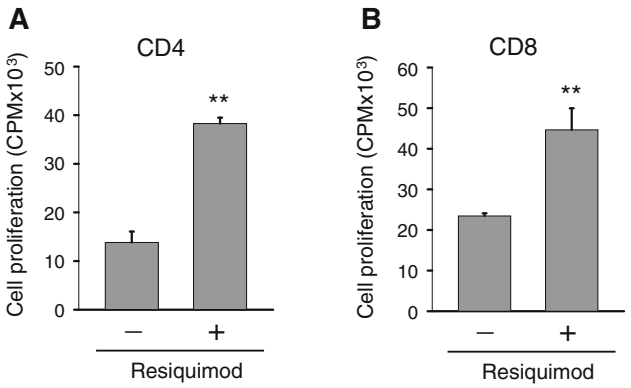

Fig. 5 Resiquimod-treated MDSCs exert potent T-cell stimulatory function. Purified MDSCs (1×10^5/well) were cultured in the presence (5 μg/ml) or absence of resiquimod for 5 days. Cells were then harvested, washed, and co-cultured with syngeneic CD4 T cells (**a**) or CD8 T cells (**b**) in the presence of 100 ng/ml anti-CD3 mAb and 200 ng/ml anti-CD28 mAb. Cells were cultured for 48 h, and then pulsed with 1 μCi/ml [^{3}H]thymidine for an additional 18 h. The results are expressed as mean value ± SE of 3 indifferent experiments. $^{**}P < 0.01$ compared with the untreated control group

resiquimod on the differentiation of MDSCs has not been studied to date. The major finding of the present study is that resiquimod induces differentiation of mouse MDSCs into mature macrophages and DCs. The present study may help in improving the current understanding of the effects of resiquimod on the differentiation of myeloid cells.

MDSCs from 4T1 tumor-bearing mice were known to be equally suppressive toward both CD4 and CD8 T cells (Sinha et al. 2005; Bunt et al. 2006). Consistent with the findings of others, we showed here that the MDSCs from 4T1 tumor-bearing mice inhibited the proliferation of CD4 and CD8 T cells that were primed with anti-CD3/anti-CD28 mAbs (Fig. 4). Using this experimental system, we showed that the cells obtained from resiquimod-treated MDSCs exerted potent stimulatory effects on the proliferation of both CD4 T cells and CD8 T cells (Fig. 5). Because phenotypic analysis showed that MDSCs cultured with resiquimod contained F4/80$^+$ macrophages and CD11c$^+$/I-A$^+$ DCs, we speculate that resiquimod differentiated MDSCs into functional macrophages and DCs. However, it is also possible that the enhanced proliferation of anti-CD3/anti-CD28 mAbs-primed T cells was due to the inhibition of the suppressive activity of MDSCs by resiquimod.

Acknowledgments This work was supported by a grant from the Next-Generation BioGreen 21 Program (PJ009619), Rural Development Administration, Republic of Korea and by the Ministry of Knowledge Economy (MKE), Korea Institute for Advancement of Technology (KIAT) through the Inter-ER Cooperation Projects (R0002019).

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