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Effects of Mizoribine on MHC-Restricted Exogenous Antigen Presentation in Dendritic Cells

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Mizoribine (MZR) has been shown to possess immunosuppressive activity that selectively inhibits the proliferation of lymphocytes by interfering with inosine monophosphate dehydrogenase. The efficacy of MZR is not only in patients who have had renal transplantation, but also in patients with rheumatoid arthritis (RA), lupus nephritis, and primary nephritic syndrome. Because the exact mechanism of its immunosuppressive action is not clear, the object of this study was to examine the ability of MZR to regulate the antigen presenting cells (APCs), dendritic cells (DCs). In this work, we tested whether MZR (1~10 μg/mL) could inhibit the cross-presentation of DCs. DC2.4 cells (H-2K^b) or bone marrow-derived DCs (BM-DCs) generated from BM cells of C57BL/6 mouse (H-2K^b) were cultured in the presence of MZR with OVA-microspheres, and the amount of OVA peptide-class I MHC complexes was measured by a T cell hybridoma, B3Z, that recognizes OVA (257-264 : SIINFEKL)-H-2K^b complex and expresses-galactosidase. MZR profoundly inhibited the expression of SIINFEKL-H-2K^b complexes. This inhibitory activity of MZR appeared to affect the phagocytic activity of DCs. MZR also decreased IL-2 production when we examined the effects of MZR on CD4$^+$ T cells. These results provide an understanding of the mechanism of immunosuppressive activity of MZR on the inhibition of MHC-restricted antigen presentation and phagocytic activity in relation to their actions on APCs.

Key words: Dendritic cells, Cross-presentation, H-2K^b, SIINFEKL-H-2K^b, Mizoribine

INTRODUCTION

Mizoribine (MZR) is an imidazole nucleoside isolated from *Eupenicillium brefeldianum*. MZR has been shown to possess an immunosuppressive activity that selectively inhibits the proliferation of lymphocytes by interfering with inosine monophosphate dehydrogenase (Ishikawa *et al.*, 1999). The efficacy of this agent is not only in patients who have had renal transplantation, but also in patients with rheumatoid arthritis (RA), lupus nephritis, and primary nephritic syndrome (Abe *et al.*, 2004). Moreover, the incidence of adverse effects with this drug, which include myelosuppression, hepatotoxicity and nephrotoxicity, are comparable to other immunosuppressive agents (Osakabe *et al.*, 1989). Recent studies have demonstrated that MZR suppresses the proliferation of human T cells and B cells (Shunsei and Tamiko, 1995) and clinical trials have demonstrated its efficacy in RA and lupus nephritis, in which abnormalities of B cell functions are involved (Shunsei and Tamiko, 1995). B cells are one kind of antigen presenting cells (APCs). Since the exact mechanism of its anti-arthritic action of MZR is not clear, we tested whether MZR affected the capacity of APCs, dendritic cells (DCs), to present Ags to CD8$^+$ T cells, showing that DCs cross-present a model exogenous Ags. DCs play a critical role in the initiation of immune responses and the induction of immune tolerance. We wished to determine whether presentation of exogenous Ags could be inhibited by MZR, which has been used as a suppressor of immune response. Protein must be processed to small peptides in order to be presented either on MHC class II molecules for activation of CD4$^+$ T cells or MHC class I molecules for activation of CD8$^+$ T cells.

Therefore, the aim of this study was to examine the

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effects of MZR on the *in vitro* function of DCs for the characterization of mechanism of MZR as immunosuppressive agents. We used OVA as an exogenous Ag in conjunction with MZR, and then compared the change in cross-presentation of MZR-treated DCs to that of the control group along with the level of MHC I and MHC II molecules.

MATERIALS AND METHODS

Animals

Five- to 8-week-old C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were used. The mice were maintained in pathogen-free conditions in animal facilities at the University of Sahmyook, Seoul, Korea.

Mizoribine (MZR)

Mizoribine was obtained from Chong Kun Dang Pharmaceutical Co. (Seoul, Korea).

Cell culture

DC2.4 cells were obtained from bone marrow cells infected with a retrovirus encoding *myc* and *raf* using supernatant from NIH J2 Leuk cells as previously described (Shen *et al.*, 1997). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and 1% (v/v) penicillin (10000 U/mL)/streptomycin (10000 U/mL) (P/S); the supplements were purchased from Sigma Chemical Co. (St. Louis, MO. U.S.A.). B3Z, a T cell hybridoma that recognizes K^b with peptide 257-264 (SIINFEKL) of OVA, was previously described (Yeh *et al.*, 1998). B3Z contains a DNA construct coding for the *lacZ* gene under the control of the IL-2 regulatory elements. Upon activation of B3Z through the TCR, the *lacZ* gene is expressed, allowing determination of the activation of the T cell hybrid through colorimetric assays.

Generation of bone marrow derived dendritic cells

The protocol of Lee *et al.* (Lee *et al.*, 2005) was used to generate DCs from bone marrow. Briefly, bone marrow cells obtained from femurs of Balb/c or C57BL/6 mouse were cultured in a 6 well plate (5×10^6/well) in a culture medium supplemented with 400 U/mL rmGranulocyte-macrophage colony-stimulating factor (rmGM-CSF) and 100 U/mL rmIL-4. On culture days 4 and 5, nonadherent cells were removed and fresh medium containing the cytokines after gentle shaking. Cells were used from day 7.

B3Z T hybridoma activation assay

Activation of B3Z cells was measured by *lacZ* activity. Briefly, 4×10^4 DC2.4 cells/well of 96-well plate were treated with different concentration of MZR (0.1, 1, 10, 100 μg/mL) in 5.5% CO_2 for 2 h at 37°C, and then particulate OVA (50 μg/mL) was added for another 2 h. The cells were washed with phosphate-buffered saline and then incubated with 1×10^5 B3Z cells/well in a 96-well plate to evaluate antigen presentation. After 4 h, the supernatant was removed, and the cells were lysed in 50 μL of lysis buffer (0.1% Triton X-100, 250 mM Tris, pH 8.0) and kept at -70°C for 30 min. The plates were thawed at room temperature for 30 min, and 50 μL of PBS containing 0.5% BSA was added to each well. They were then overlaid with 100 μL of substrate [1 mg/mL of chlorophenol red-β-D-galactopyranoside in β-galactosidase buffer (60 mM sodium dibasic phosphate buffer (pH 8.0), 1 mM magnesium sulfate, 10 mM KCl, 50 mM β-mercaptoethanol)]. The plates were incubated in 5.5% CO_2 for 12 h at 37°C and were measured by an ELISA reader at 580 nm.

MHC class II-restricted presentation assay

DCs treated with mitomycin C were added to 96-well microtiter plates (4×10^4 cells/well), incubated for 2 h at 37°C, and then 50 μg/mL of OVA microspheres and various amounts of MZR (0.1, 1, 10, 100 μg/mL) were added. After a 2-h incubation at 37°C, unphagocytized OVA-microspheres and MZR were removed by suction. The plate was then washed twice with 300 μL/well of pre-warmed DMEM, and OVA-specific $CD4^+$ T (1×10^5 cells/well) DOBW cells were added (Lee *et al.*, 2005). After a 24-h incubation at 37°C, the plate was centrifuged at 1,800 rpm, and the culture supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit (BD Biosciences).

Antibodies and flow cytometry

DC2.4 (1×10^6 cells/mL) cells were cultured in DMEM for 2 h. The media were changed, and the cells were incubated in the presence of either MZR (100 μg/mL) or medium for an additional 2 h. Treated cells were then scraped into PBS-0.1% sodium azide with 1% FBS (PBS-washing buffer, pH 7.2) and washed twice in washing buffer at 4°C. Before cells were stained with FITC (Fluorescein isothiocyanate)-monoclonal antibody, the cell surface Fc receptors were blocked by incubating cells with 20 μg/10^6 cells of purified anti-CD-16 for 30 min at 4°C. The residual antibody was removed by washing. The monoclonal antibody, anti-H2-K^b (clone AF6-88.5), and an isotype-matched control antibody were purchased from BD Biosciences. Anti-H2-K^b and Anti-I-A^b-FITC were then added, and the cells were kept at 4°C for 30 min. The other monoclonal antibody used for staining, 25-D1.16, was purified from ascitic fluid collected from mice injected with 25-D1.16 clone 21 cells, and then digested with pepsin to obtain $F(ab')_2$ fragments. FITC-labeled anti-mouse IgG_1 antibody was purchased from BD Biosciences.

Supernatant of 25-D1.16 were then added to the DC2.4 cells, and the cells were kept at 4°C for 30 min. Cells stained using mouse IgG-FITC served as a control for non-specific binding. Following staining, the cells were washed and fixed in cold PBS containing 1% paraformaldehyde (pH 7.2). Flow cytometry analysis was performed on an EPICS V analyzer (Coulter, Hialeah, FL). Fluorescence intensity was determined on 50,000 cells from each sample using logarithmic amplification.

RESULTS

MZR inhibits cross-presentation of exogenous antigen in both DC2.4 cells and BM-derived DCs

Using the above-described assay systems and B3Z T hybridoma activation assays, we examined the effects of MZR on the MHC-restricted presentation of exogenous antigen. Treatment of the DC2.4 cell line (Fig. 1A) or BM-DCs with MZR (Fig. 1B) resulted in a population of cells with an inhibited ability to present particulate OVA. The cross-presentation inhibitory activity was also examined in normal DCs generated from BM cells of C57BL/6 mouse. Since cross-presentation is required for the effective generation of CTL responses, it is important to know whether MZR modulation of cross-presentation capability will be a feasible method for suppressing the cross-presentation capability of DCs.

MZR inhibits phagocytic activity of DCs

To test whether the antigen presentation-inhibitory activity of MZR (100 μg/mL) was due to inhibition of phagocytic activity, DC2.4 cells were incubated with microspheres containing both OVA and FITC, washed, and then harvested by gentle pipetting after cooling on ice. Flow cytometric analysis of the harvested cells showed that MZR inhibited phagocytic activity in the DC2.4 cell line (Fig. 2A) and in BM-DCs (Fig. 2B).

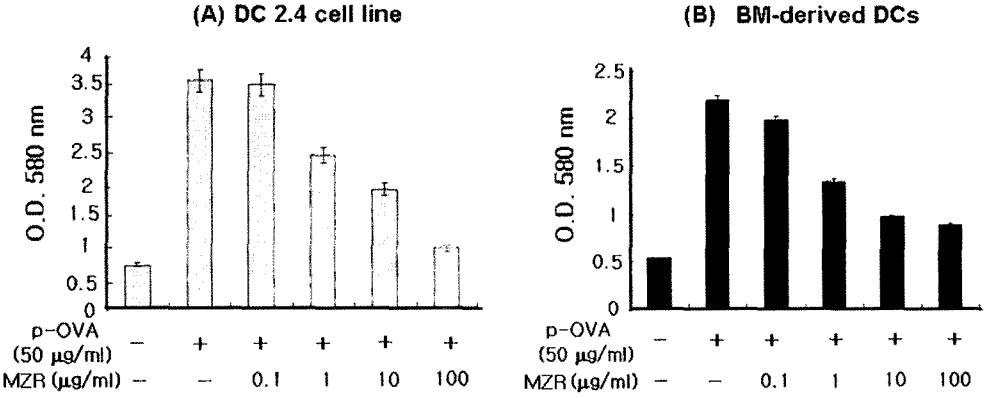

Fig. 1. DCs were incubated with the indicated amount of microencapsulated OVA and co-cultured with a T cell hybridoma, B3Z cells. The amount of b-galactosidase expressed in the B3Z cells was determined with an ELISA reader. These results are representative of more than ten experiments. An indicated amount of Mizoribine (MZR: 0.1, 1, 10, 100 μg/mL) was added to cultures of DC2.4 cells (A) or BM-derived DCs (B) together with OVA-microspheres.

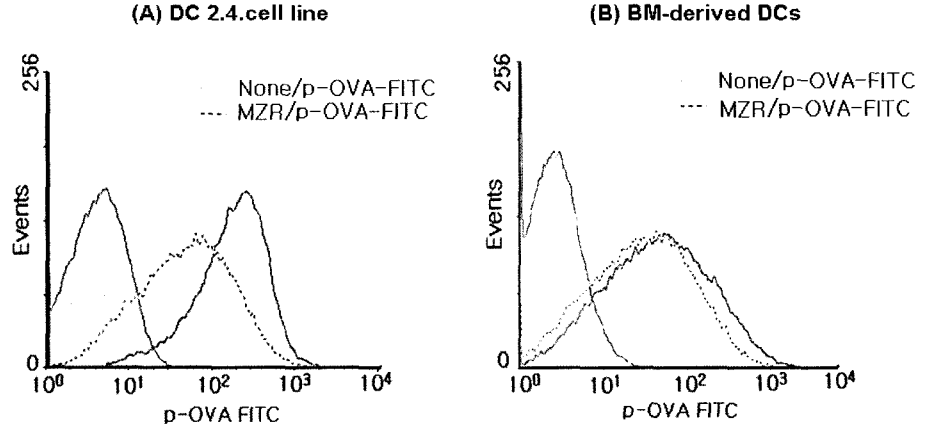

Fig. 2. DCs were incubated with microspheres containing OVA-FITC in the presence or absence of MZR (100 μg/mL) and then analyzed with a FACS. We tested DC2.4 cell line (A) and BM-derived DCs (B). Positive control represents DCs treated with p-OVA-FITC without MZR.

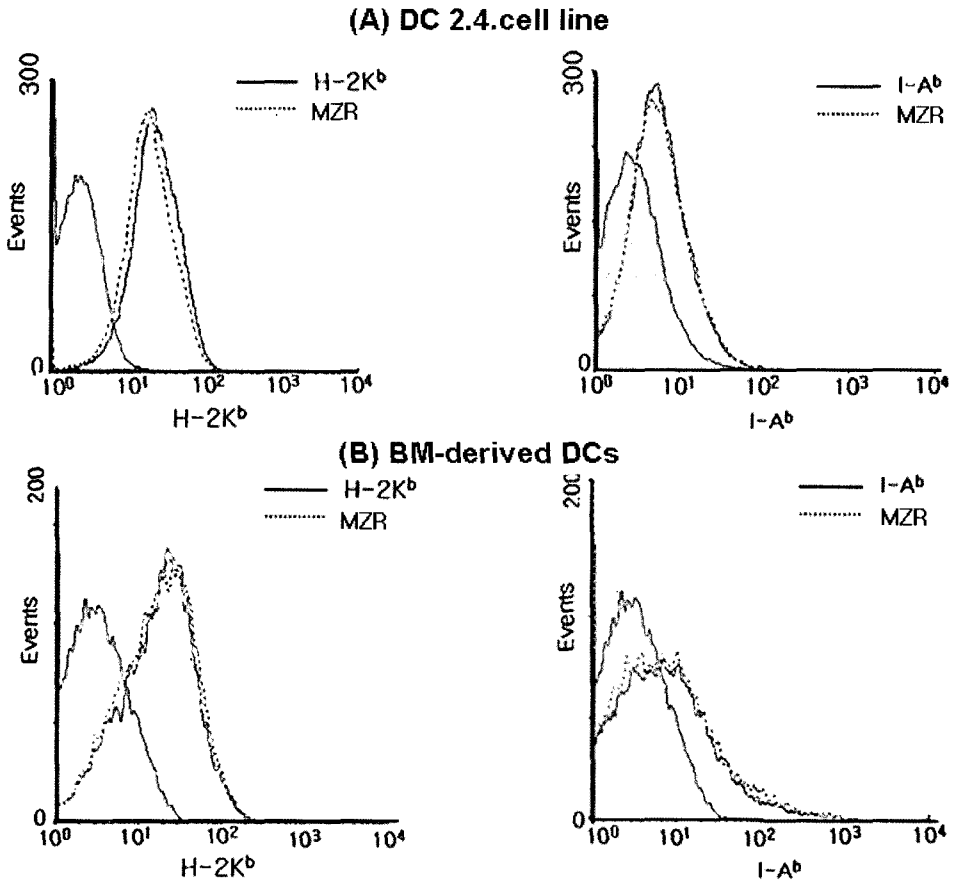

Fig. 3. DC2.4 cells (A) and BM-DCs (B) were incubated with MZR (100 μg/mL), and the expression level of H-2K^b complexes or I-A^b was determined with monoclonal antibodies. Control represents DCs treated with neither p-OVA nor MZR.

MZR does not decrease total expression of H-2K^b and I-A^b molecules

To demonstrate that MZR inhibits an extracellular event in the antigen processing pathways, DC2.4 cells were incubated with MZR (100 μg/mL) and washed. Anti-H2-K^b and Anti-I-A^b-FITC were then added, and the cells were kept on ice for 20 min. Fluorescence intensity was determined from 50,000 cells in each sample using logarithmic amplification. As shown in Fig. 3, MZR did not change the total expression of H-2 K^b and I-A^b molecules.

MZR inhibits the expression of SIINFEKL-H-2K^b complexes

We next examined whether MZR inhibited the expression of OVA-specific class I MHC molecules (Fig. 4). DC2.4 cells were incubated with MZR (100 μg/mL) for 2 h in the presence of OVA-microspheres and washed. The expression level of SIINFEKL-H-2K^b complexes was then determined using the F(ab')$_2$ fragment of the SIINFEKL-H-2K^b-specific monoclonal antibody, 25-D1.16. We found that MZR profoundly inhibited the expression of SIINFEKL-H-2K^b complexes.

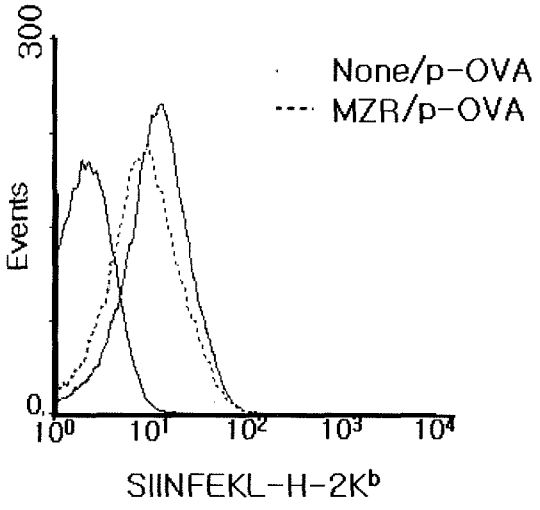

Fig. 4. DC2.4 cells were incubated with microspheres containing OVA in the presence of MZR (100 μg/mL), and levels of SIINFEKL-H-2K^b complexes were determined with monoclonal antibodies. Control represents DCs treated with p-OVA without MZR.

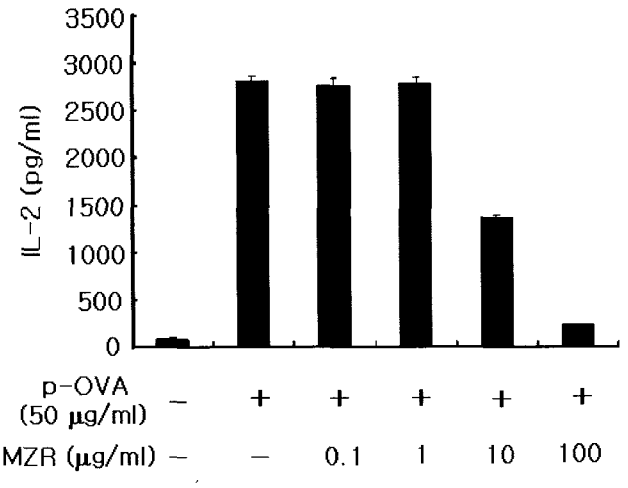

Fig. 5. Mizoribine inhibits class II MHC-restricted presentation of exogenous OVA in DCs generated from BM cells of Balb/c mouse. Indicated amounts of MZR were added to cultures of DCs generated from mouse BM cells with GM-CSF and IL-4 for 24 h, washed, and then added with OVA microspheres. After 2 h incubation, unphagocytized OVA microspheres were washed, and then co-cultured with $CD4^+$ T cells, DOBW, for 48 h. Supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit. Values represent mean ± SD of triplicates and are representative of five experiments.

Inhibition of IL-2 production with class II MHC presentation of exogenous antigen by MZR

For class II MHC-restricted presentation assays, DCs generated from BM cells of Balb/c mice ($H\text{-}2^d$) were incubated with OVA-microspheres, and the amount of OVA peptide-class II MHC complexes was measured by OVA-specific CD4 T cells isolated from spleen of DOBW mice ($H\text{-}2^d$). Exogenous antigens (Ags) are normally processed and presented on class II MHC molecules. These results showed that the classical exogenous pathway was blocked in the presence of MZR (Fig. 5).

DISCUSSION

Mizoribine (MZR) was isolated from the soil fungus *Eupenicillium brefeldianum* and has been shown to be an effective immunosuppressant (Aihara *et al.*, 2002, Abe *et al.*, 2004). There are many reports on the activity of MZR, such as antinephhritic activity (Okamoto *et al.*, 1983), blocking T cell proliferation without interfering with the initial phase of T cell activation (Turka *et al.*, 1991), suppression of the expression of cycline A in B cells and the production of humoral antibody (Hirohata *et al.*, 2000, Kobayashi *et al.*, 1983). However, little is known the exact mechanism of its immunosuppressive action of MZR. We tested whether MZR affected the capacity of APCs as dendritic cells (DCs) and macrophages to present Ags to T cells. We therefore examined the effects of MZR on the *in vitro* function of DCs for antigen presentation.

There have been numerous reports of the transfer of molecules from APCs to T cells in mouse and rat systems (Sharrow *et al.*, 1981). The first such observations were made during immunoelectron microscopic analysis of mouse thymic sections, where murine thymocytes were noted to express MHC class II molecules, presumably acquired from thymic epithelium. More recently, the capture of MHC class I and class II molecules by mouse and rat T cells from APCs has been described (Huang *et al.*, 1994; Hudrisier *et al.*, 2001; Arnold *et al.*, 1997; Patel *et al.*, 1999; Arnol and Mannine, 1999). Additional reports of the transfer of co-stimulatory molecules from APCs to T cells have been made (Hwang *et al.*, 2000; Sabzevari *et al.*, 2001). Indeed, such acquired MHC:peptide complexes appeared to be functional, in that $CD8^+$ T cells became sensitive to peptide-specific lysis by neighboring T cells after acquisition of MHC I:peptide complexes from APCs (Huang *et al.*, 1999; Hudrisier *et al.*, 2001). Also, naive T cells that had acquired CD80 from APCs were capable of inducing IL-2 production by responder T cells (Sabzevari *et al.*, 2001).

In vivo and *in vitro*, professional antigen-presenting cells (APCs), particularly dendritic cells (DCs), are capable of processing cell-associated antigens for presentation on major histocompatibility complex (MHC) class I molecules (Brossart and Bevan *et al.*, 1997). *In vitro*, several forms of antigen can access the exogenous pathway for cross-presentation, apparently through phagocytic or nonphagocytic mechanisms (Larsson *et al.*, 2001). Antigens such as particulate antigens, immune complexes, and heat shock proteins (HSPs) have been shown to be loaded onto MHC class I molecules in fashions either independent of or dependent on proteasome degradation, transport into the ER *via* TAP, and MHC class I peptide loading in the ER (Yewdell *et al.*, 1999).

Our study of murine DCs revealed many unique features of cross-presentation *in vitro*. Cross-presentation was inhibited in the presence of MZR, consistent with the interpretation that internalization and possibly processing of OVA occurred. In all of the experiments described in the present study, DCs were exposed to MZR for only 2 h in the presence of OVA-microspheres. Thus, it is obvious that the inhibitory activity of MZR on MHC-restricted antigen processing is initiated immediately after exposure to these materials. The concentration of MZR that was shown to inhibit MHC-restricted antigen processing is below that of a cytotoxic dose. Expression of $H\text{-}2K^b$ and $I\text{-}A^b$ was similar in the presence of MZR. DCs can internalize exogenous antigens *via* different mechanisms such as clathrin-mediated endocytosis, fluid-phase endocytosis, macropinocytosis, or phagocytosis (Steinman *et al.*, 1999). To evaluate if uptake via macropinocytosis or phagocytosis

is involved in the cross-presentation of cell-associated OVA by DCs, We inhibited these processes using MZR.

In this work, we tested whether MZR (1~100 μg/mL) inhibited the presentation of DCs by selectively alleviating the cross-presentation of CD8 T cells. Following MZR treatment, decreased secretion of IL-2 in response to the bone marrow derived DCs was observed. We also examined the phagocytic activity of DCs when treated with MZR using FITC-conjugated OVA protein as an exogenous antigen. MZR inhibited their capacity for phagocytosis compared to OVA only. This observation showed that cross-presentation of DCs was inhibited by MZR through the downregulation of phagocytosis in a dose-dependent manner. When cells were incubated with microspheres containing OVA in the presence of MZR, the expression level of SIINFEKL-H-2K^b complex was inhibited while the total expression of H-2K^b and I-A^b molecules were not changed by MZR when MZR was used to treat cells in the absence of OVA.

In summary, the current studies have shown that MZR can regulate class I MHC-restricted presentation of exogenous antigen through downregulation of phagocytosis in both the DC2.4 cell line and bone marrow-derived DCs generated *in vitro*. Further studies will be required to characterize the role of MZR in the intracellular processing events of antigen processing and expression pathways.

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