Monophosphoryl lipid A induces bone marrow precursor cells to differentiate into myeloid-derived suppressor cells

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Abstract. Myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs) are important in the immune response. *In vitro*, DCs are derived from myeloid precursors by stimulation with granulocyte macrophage colony-stimulating factor and interleukin-4. Previous studies demonstrated that lipopolysaccharide (LPS) in combination with interferon-γ inhibited DC development but enhanced MDSC functions. Monophosphoryl lipid A (MPL), derived from LPS, is a unique immunomodulatory Toll-like receptor 4 agonist. In the present study, MPL was used to disturb DC differentiation from myeloid precursors and it was observed that prolonged stimulation with MPL led to the accumulation of MDSCs *in vitro* and *in vivo*. In conclusion, it was demonstrated that stimulation by MPL from the beginning of cell differentiation disturbed the development of DCs and led to the accumulation of MDSCs.

Introduction

Monophosphoryl lipid A (MPL) is a lipopolysaccharide (LPS)-derived Toll-like receptor 4 (TLR4) agonist that exhibits unique immunomodulatory properties at doses that are nonpyrogenic. In addition, it is a chemically detoxified lipid A moiety derived from *Salmonella minnesota* R595 LPS (1). Clinically, MPL is a component of several vaccine formulations. It has been demonstrated that MPL induces a strong phagocytic and a low inflammatory response via TLR4 (2).

Myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs) are derived from the same myeloid precursors; however, they exhibit different roles in the immune response. DCs demonstrated the capacity to initiate an innate and adaptive response (3,4), while MDSCs have been observed to suppress immune responses via arginase, inducible nitric oxide synthase (5,6), reactive oxygen species (7-14) and $Foxp3^+$ regulatory cells (15,16). The mechanisms that are involved in the differentiation of myeloid precursor cells into MDSCs instead of DCs have not been fully elucidated. It has been demonstrated that the development of DCs from bone marrow (BM) precursor cells *in vitro* is impaired in the presence of LPS (17). Another study showed that a combination of LPS and IFN-γ inhibited DC development; however, enhanced the suppressive functions of MDSCs, including NO release and T cell suppression (18). In addition, other conserved structural patterns of microbial components, such as double-stranded RNA showed the capacity to regulate MDSC versus DC development from the same myeloid precursor (19).

As MPL is an active immunomodulator, in the present study, the involvement of MPL was investigated in the differentiation of myeloid precursor cells into MDSCs versus DCs. It was demonstrated *in vivo* and *in vitro*, that sustained stimulation with MPL inhibited the expansion of DCs and induced the development and expansion of MDSCs.

Materials and methods

Mice and treatments. Approximately 50 male and female wild-type C57BL/6 mice (age, 5-6 weeks) were purchased from the Chinese Academy of Sciences (Shanghai, China). DO11.10 $OVA_{323-339}$-specific TCR-transgenic mice with a

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C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in a specific pathogen-free facility for all experiments. All animal procedures were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, National Academy Press, Washington, DC, revised 1996), with the approval of the Laboratory Animal Center and Ethics Committee of the Second Military Medical University (Shanghai, China).

Reagents. Recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, and an enzyme-linked immunosorbent assay (ELISA) kit for murine IL-12, IL-6, tumor necrosis factor (TNF)-α, IL-10 and transforming growth factor (TGF)-β were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescein-conjugated mAbs to CD4, CD11b, CD80, CD86, Ia, CD40, CD11c, CCR7 and isotype control were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fluorescein-conjugated mAbs to Gr1 were obtained from eBioscience (San Diego, CA, USA). LPS, 7-Aminoactinomycin D (7-AAD) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Carlsbad, CA, USA).

Preparation of MPL from LPS of the gram-negative bacteria Salmonella minnesota R595. The MPL was prepared by eliminating the core oligosaccharide, hydrolyzing 1-phosphate from the reducing end glucosamine and removing the acyl chain from the 3' position of the disaccharide (2,20,21).

Preparation of DCs from mouse BM and DC pre-treatment. BM mononuclear cells were prepared from mouse femur BM suspensions by depletion of red blood cells. The cells were then cultured at a density of $2x10^6$ cells/ml in 6-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 ng/ml recombinant mouse GM-CSF and 1 ng/ml recombinant mouse IL-4. Nonadherent cells were gently washed out on day 4 of culture. On day 5, the dendritic proliferating clusters were collected and purified by anti-CD11c microbeads as immature dendritic cells (imDCs). ImDCs were stimulated with LPS (100 ng/ml) for another 2 days and then collected as mature DCs (mDCs). These cells were cultured in 24-well plates using $1x10^6$ cell/well. All groups were cultured with granulocyte macrophage colony-stimulating factor and IL-4 throughout. MPL (25 μg/ml) was administered to the long stimulation group on day 0 and to the short stimulation group on day 5. On day 6, all cells were collected separately for further analysis (22).

Analysis of DC phagocytic ability. The cells of all groups were incubated at 37˚C for 4 h with fluorescein isothiocyanate (FITC)-conjugated OVA at a final concentration of 100 μg/ml in RPMI-1640 medium containing 10% FCS, were washed twice with ice-cold phosphate-buffered saline (PBS; pH 7.2), containing 0.1% NaN_3 and 0.5% BSA, and were resuspended in chilled PBS for immediate flow cytometry. Cells were incubated with OVA-FITC at 4˚C.

Assay for cytokines and NO. Cytokines in the supernatant of the DC system were assayed with ELISA kits. NO production was assayed by the measurement of the nitrite concentration with the Griess assay.

Assays for Ag-specific $CD4^+$ T-cell response. For the assay of the Ag-specific $CD4^+$ T-cell proliferation, splenic $CD4^+$ T cells from DO11.10 $OVA_{323-339}$-specific TCR-transgenic mice were positively selected with anti-CD4-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic-activated cell sorting. The cells were cocultured with DCs treated as indicated in the presence of $OVA_{323-339}$ peptide at a ratio of 1:10 (DC:T) in round-bottom 96-well plates ($1x10^5$ T cells/200μl/well) for 5 days. The proliferation of the T cells was analyzed by double staining with anti-$CD4^+$ and 7-AAD^-, and cells were counted by a fluorescence activated cell sorter (FACS).

Assay to determine the percentage of MDSCs and DCs in vivo. Six of the wild-type C57BL/6 mice were administered with MPL via the tail vein once (short stimulation) or once daily for 5 days (long stimulation). In total, there were 12 mice with 6 in the control group. The administration dosage was dependent upon the mouse body weight (0.2 mg/kg or 2 mg/kg). Following the preparation of single-cell suspensions from the mouse spleen, cells were stained with Ab-$CD11c^+$ conjugated FITC, Ab-$CD11b^+$ conjugated R-Phycoerythrin (PE) or Ab-$Gr1^+$ conjugated FITC as indicated in the manufacturer's instructions.

Statistical analysis. Comparisons between experimental groups and relevant controls were performed by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BM precursor cells exhibit a distinct phenotype and cytokine profile following long stimulation with MPL. Mouse BM-DCs were produced by standard protocol using GM-CSF and IL-4 (23-26). *In vitro*, the process of differentiation from myeloid precursor cells to DCs required 5 or 6 days. On day 0, in the long stimulation group, myeloid precursor cells were isolated from mouse BM, washed and co-cultured with GM-CSF, IL-4 and MPL (20 μg/ml) for 5 days. In the short stimulation group, myeloid precursor cells were treated with GM-CSF and IL-4 only until day 5. On day 5, cells in the short stimulation group cells were treated once with MPL (20 μg/ml). On day 6, cells from the two groups and the control group were harvested for further analysis. It was demonstrated that cells from the long stimulation group exhibited a lower expression of CD40, CD80, CD86 and Ia compared with that in the short stimulation group. Notably, CD11c expression in the cells of the long stimulation group was significantly decreased, which suggested that the development of DCs was blocked. Chemokine (C-C motif) receptor 7 (CCR7) was demonstrated to be responsible for directing the migration of DCs, in addition to the control of the cytoarchitecture, the rate of endocytosis, DC survival, migratory speed and DC maturation (27). In the present study, the expression of CCR7 was significantly decreased (Fig. 1A and B). Compared with the cytokine profile of the short stimulation and control groups, the cells in the long stimulation group secreted lower levels of IL-6, IL-12 and TNF-α and higher levels of IL-10 and NO (Fig. 1C). The cytokine profile of

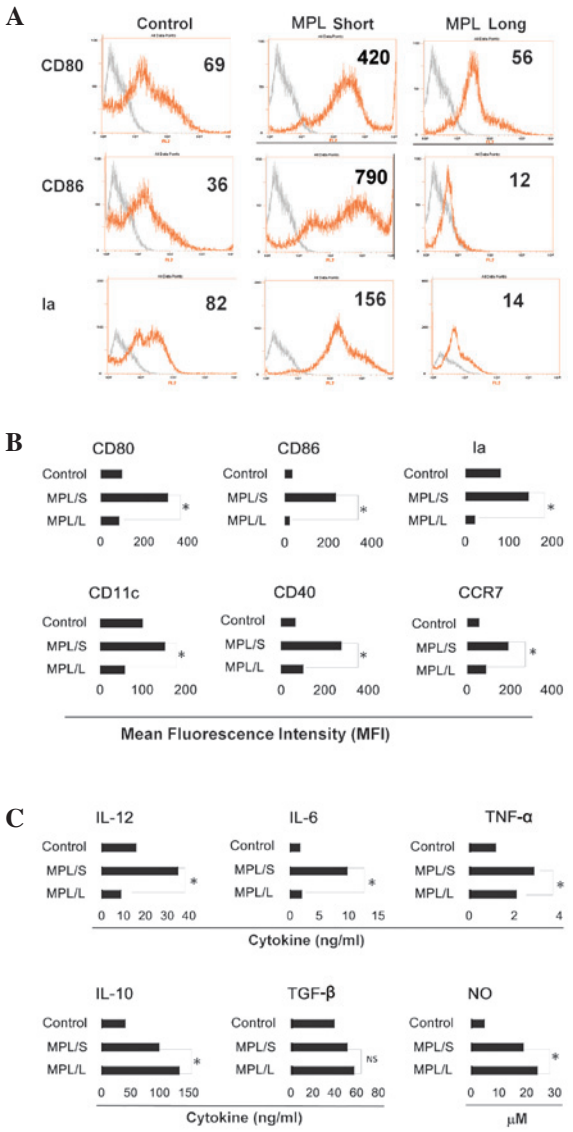

Figure 1. Phenotype and cytokine profiles of all groups. Myeloid precursor cells were generated from C57BL/6 mouse femur bone marrow suspensions by the depletion of red blood cells. These cells were cultured in 24-well plates using $1x10^6$ cell/well. All groups were cultured with granulocyte macrophage colony-stimulating factor and IL-4 throughout. MPL was administered to the long stimulation group on day 0 and to the short stimulation group on day 5. On day 6, all cells were collected separately for further analysis. (A) FACS charts of CD80, CD86 and Ia. Fold line in grey represents the blank control (cells without staining). Numbers in each chart represent the mean fluorescence intensity. (B) The expression of CD80, CD86, Ia, CD11c, CD40 and CCR7 was detected by FACS. Histograms represent the mean fluorescence intensity. Results are presented as the mean ± SD of cells from triplicate wells. (C) NO expression and the cytokine profile of cells from different groups for 24 h. On day 6, cells of different groups were collected and washed in phosphate-buffered saline 3 times and placed in the wells for another 24 h. IL-12, IL-6, tumor necrosis factor-α, IL-10 and TGF-β levels were assayed by an enzyme-linked immunosorbent assay and NO levels were assayed by a Griess assay. Results are presented as the mean ± SD of triplicate wells. *P<0.05 compared with the short stimulation group. NS, no significance; IL, interleukin; MPL, monophosphoryl lipid A; FACS, fluorescence-activated cell sorting; NO, nitric oxide; TGF, transforming growth factor.

the long stimulation group suggested that the cells may exhibit an immunosuppressive capacity.

Cells from the long stimulation group show an enhanced phagocytic ability and the ability to suppress T cell proliferation. The phenotype and cytokine profiles of cells from the MPL long stimulation group suggested that these cells may exhibit an immunosuppressive function. Thus, the cells were stained with $Gr1^+CD11b^+$ and analyzed by FACS. This demonstrated that double-positive $Gr1^+CD11b^+$ cells existed in the population (Fig. 2A and B). Therefore, it was hypothesized that these cells may be MDSCs. Subsequent to this, the phagocytic function of the different groups was analyzed, which demonstrated that cells from the long stimulation group exhibited a significantly increased phagocytic ability (Fig. 2C). In addition, the ability of DCs to stimulate antigen-specific T cell responses was investigated. It was observed that cells from the long stimulation group exhibited a reduced ability to induce the proliferation of OVA-specific $CD4^+$ T cells. Notably, cells from the long stimulation group were added to the mDCs/CD4 T cell coculture system and it was demonstrated that the T cell proliferation *in vitro* was partly suppressed (Fig. 2D).

MPL expands MDSC population and suppresses DC population in vivo. To investigate the effect of MPL *in vivo*, the MPL short and long stimulation model was conducted. Mice were administered with MPL via the tail vein once daily. In the short stimulation group, mice received MPL treatment on day 0, then 24 h later, on day 1, mice were euthanized. In the long stimulation group, mice received MPL treatment every 24 h and were euthanized on day 5. The spleens were isolated from the two groups and the control to analyze the DC and MDSC population by FACS. It was demonstrated that MPL upregulated the percentage of DCs ($CD11c^+$ cells) and downregulated the percentage of MDSCs ($CD11b^+Gr1^+$ cells) in the spleen. This data is consistent with the previous results (Fig. 3A and B).

Discussion

The results of the present study suggested that the TLR4 agonist (MPL) in the long stimulation group, disturbed the development of DCs and induced myeloid precursor cells to differentiate into $CD11b^+Gr1^+$ cells (considered to be MDSCs) with an immunosuppressive function. Previous studies have demonstrated that factors that induce MDSC expansion include cyclooxygenase-2, prostaglandins (28-30), stem-cell factor (28), M-CSF, IL-6 (31), GM-CSF and vascular endothelial growth factor (32). In addition to expansion, the suppressive activity of MDSCs requires factors to induce their activation, which include IFN-γ (33,34), ligands for Toll-like receptors, IL-13 (35), IL-4 and TGF-β (35). In this study, IL-4, GM-CSF and MPL were investigated and were suggested to be involved in the differentiation of myeloid precursors into MDSCs.

MPL is a detoxified lipid A moiety derived from *Salmonella minnesota* R595 LPS. It is at least 100-fold less pyrogenic than LPS, yet maintains a number of the immunomodulatory properties of LPS (36). Previous studies have demonstrated that LPS and poly (I:C) may suppress the immune response (18,19). The results indicated that MPL derived from LPS may also impair DC development.

The cells from the long stimulation group exhibited a $CD11b^+Gr1^+$ phenotype and were able to suppress T cell proliferation. This suggests that these cells exhibit an immunosuppressive function in the immune response. However, these

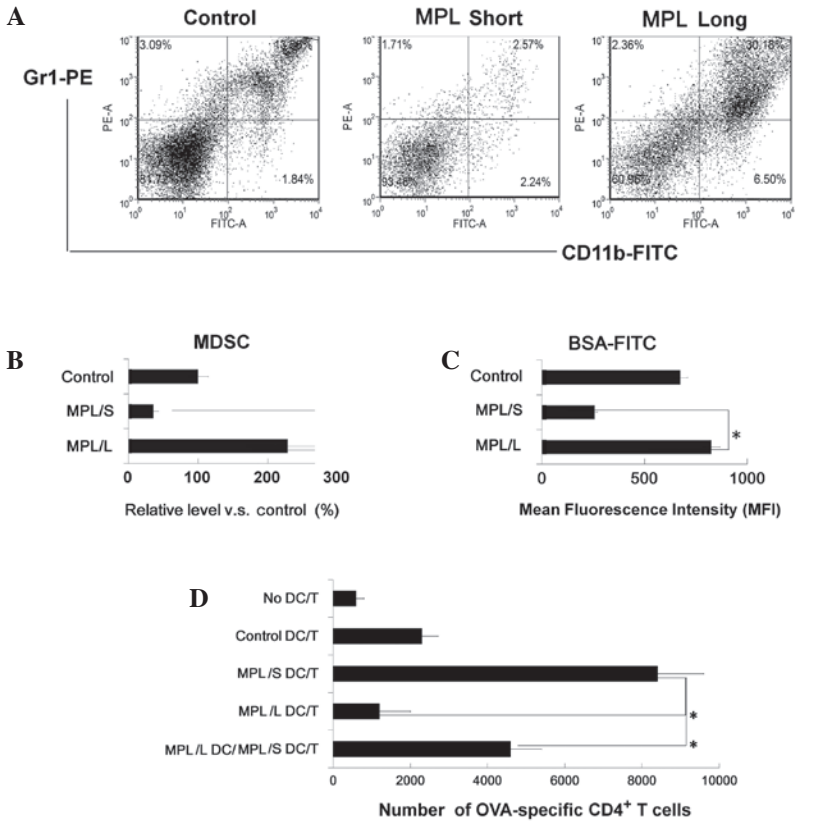

Figure 2. Grl$^+$CD11b$^+$ cells in the long stimulation group showed an enhanced phagocytic ability and inhibited CD4$^+$ T cell proliferation. (A) The phenotype of cells in the long stimulation group were analyzed by flow cytometry, the percentage of Grl$^+$CD11b$^+$ cells was higher than in the other groups. (B) The percentage of Grl$^+$CD11b$^+$ cells in all groups (the percentage in control group was referred as 100%). Phagocytic ability was assessed for OVA-FITC phagocytosis by flow cytometry. Numbers in the histograms indicate the geometric mean fluorescence of test samples, cells ($1x10^6$ cell/well) were incubated with OVA-FITC at 4˚C for 12 h, washed with phosphate-buffered saline 3 times and analyzed by a fluorescence-activated cell sorter. (D) CD4$^+$ T cells from DO11.10 $OVA_{323-339}$ specific (TCR-transgenic C57BL/6) F1 hybrid mice were cocultured with cells from all groups, 5 days later, the total number of viable CD4$^+$ T cells (CD4$^+$ 7AAD$^-$) cells in each well was measured by flow cytometry. Results are presented as the mean ± SD of three independent analyses. *P<0.05 compared with the short stimulation group. FITC, fluorescein isothiocyanate; 7-AAD, 7-aminoactinomycin D; MDSC, myeloid derived suppressor cell; MPL, monophosphoryl lipid A; DC, dendritic cell; BSA, bovine serum albumin.

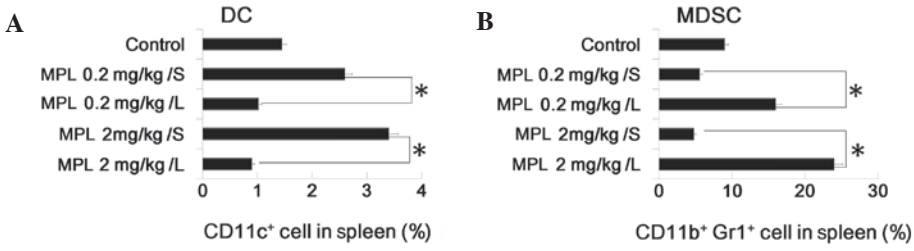

Figure 3. Long stimulation of MPL expanded Grl$^+$CD11b$^+$ cells in the spleen and short stimulation expanded CD11c$^+$ cells. Six mice were administered MPL via the tail vein once a day for 1 day (short stimulation group) or for 5 days (long stimulation group). The dosage of administration was dependent upon mouse body weight (0.2 mg/kg or 2 mg/kg). Following the preparation of single-cell suspensions of spleen, cells were stained with Ab-CD11c$^+$ conjugated FITC, Ab-CD11b$^+$ conjugated R-Phycoerythrin or Ab-Grl$^+$ conjugated FITC. These cells were then analyzed by a fluorescence-activated cell sorter. Results are presented as the mean ± SD of three independent analyses. *P<0.05 compared with the short stimulation group. (A) Percentage of CD11c$^+$ cells in the spleen of all groups. (B) Percentage of Grl$^+$CD11b$^+$ cells in the spleen of all groups. MPL, monophosphoryl lipid A; FITC, fluorescein isothiocyanate; MDSC, myeloid derived suppressor cell; DC, dendritic cell.

cells also showed enhanced phagocytic ability. A previous study demonstrated that stimulation of microglia and monocytes with MPL induced increased rates of phagocytosis of amyloid-β (Aβ) and that this mechanism may reduce the accumulation of Aβ and improve spatial memory in APPswe/PS1 mice (2). Therefore, this similar phenomenon suggests that the enhanced phagocytic ability of the CD11b$^+$Gr1$^+$ cells may have important roles in this process.

As BM precursor cells can be triggered to differentiate by conserved structural patterns of pathogens, it was questioned whether DCs only have one fate. However, Abdi *et al* (37) demonstrated that LPS-activated DCs lose their responsiveness to LPS, yet remain capable of producing inflammatory cytokines in response to signals from activated T cells, CD40-ligand and soluble T cell-derived signals. Furthermore, these DCs retained sufficient plasticity to respond differentially to the interaction

with Th0, Th1, Th2 and Th17 T cells (37). Thus, it appears that the MPL stimulated cells are not rested or exhausted, and suppress Th1 proliferation. However, the result of interaction with Th0, Th2 and Th17 cells and the understanding of whether these cells are equivalent to classical MDSCs remains to be elucidated.

In conclusion, $CD11b^+Gr1^+$ cells were generated *in vivo* and *in vitro*, and these cells showed an ability to suppress $CD4^+$ T cell proliferation and enhance phagocytic ability; however, further studies are required to fully determine this effect.

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