Amino Acids
DOI 10.1007/s00726-014-1676-4

ORIGINAL ARTICLE

The regulatory peptide pidotimod facilitates M2 macrophage polarization and its function

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Received: 23 October 2013 / Accepted: 16 January 2014

Abstract Pidotimod is a synthetic dipeptide with biological and immunological activity in innate immune responses. It has been reported that pidotimod could promote functional maturation of dendritic cells, but little is known about the regulation of macrophages. Recent studies have demonstrated that M1 or M2 polarized macrophages are of great importance for responses to microorganism infection or host mediators. The aim of this study was to determine the effectiveness of pidotimod on mouse bone marrow-derived macrophage polarization and its function. The results showed that pidotimod had no influence on M1-polarized macrophage. While interestingly, a significant increase of M2 marker gene expression (Arg1, Fizz1, Ym1, MR) was observed ($p < 0.01$) in IL-4-induced M2 macrophage treated with pidotimod. In addition, cell surface expression of mannose receptor was dramatically enhanced using fluorescence activated cell sorter (FACS) analysis. Furthermore, the function of M2 macrophage was also determinated. The results showed that the supernatant of pidotimod-treated M2 macrophage could increase the migration ($p < 0.05$) and enhance the wound closure rate ($p < 0.05$) of MLE-12 cells. Collectively, it could be concluded that pidotimod significantly facilitated IL-4-induced M2 macrophage polarization and improves its function.

Keywords Pidotimod · M2 macrophage · Polarization · Cell migration · Wound healing

Introduction

Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4-carboxylic acid) is a synthetic dipeptide which was prepared starting from L-cysteine and L-pyroglutamic acid (Auteri et al. 1992). It has been reported that pidotimod shows biological and immunological activity on both the innate and the adaptive immune responses (Riboldi et al. 2009). In clinical trials, pidotimod is able to stimulate a protective effect against bacterial and viral infections without any interaction with most common therapeutics, such as antibiotics, pentobarbital and chlorothiazide (Coppi and Barchielli 1991; Migliorati et al. 1992; Manzardo et al. 1994), and make the activity of antiviral drugs more effective for recurrent respiratory infection in children (di Marco et al. 1992; Careddu et al. 1994). Moreover, previous studies have demonstrated that pidotimod could regulate the immune cells. The activity of natural killer (NK) cell was dramatically increased when the mouse was administrated with pidotimod Migliorati et al. 1992, 1994). It also stimulated dendritic cells (DC) to release high levels of MCP-1 and induced DC maturation to drive T cell proliferation and differentiation (Giagulli et al. 2009; Hu et al. 2012). However, little information is known about the effect of pidotimod on macrophages.

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Published online: 31 January 2014

Macrophages have been considered to be important immune cells (Martinez et al. 2009). They sense series of stimuli from both endogenous and exogenous environment, and respond with the corresponding phenotypic plasticity (Mantovani et al. 2004; Gordon and Taylor 2005; Mosser and Edwards 2008; Pollard 2009). Since the discovery of their activation and heterogeneity (Gordon 2003), polarized macrophages have been broadly classified into two different subpopulations (Mosser 2003; Edwards et al. 2006; Rolls et al. 2008). One of the subpopulations is known as classically activated macrophages (M1 macrophages) and is stimulated by lipopolysaccharides (LPS), in conjunction with endogenous cytokine such as interferon (IFN)-γ. M1 macrophages are potent cells with increased antigen presenting capacity, augmented secretion of proinflammatory cytokines such as (TNF)-α and nitric oxide (NO), and enhanced macrophage phagocytosis, which are of great importance for clearing bacteria, inhibiting viral and fungal infections (Benoit et al. 2008; Mege et al. 2011). The other subpopulation is termed alternatively activated macrophages (M2 macrophages), which can be driven in response to stimuli such as IL-4, IL-13, and glucocorticoids. M2 macrophages are characterized by the high expression of arginase-1 (Arg1), found in inflammatory zone-1 (Fizz1), chitinase-like Ym1 and mannose receptor (MR, also known as CD206) (Stein et al. 1992; Raes et al. 2002; Kreider et al. 2007). The functions of these macrophages are important for the clearance of helminthes and nematodes, the promotion of phagocytic activity and resolution of wound healing and tissue repair (Mookherjee et al. 2006; Mosser and Edwards 2008; Soehnlein and Lindbom 2010).

Despite this knowledge, there is limited information about the effect of pidotimod on M1 or M2 macrophage polarization and its function. In this study, we investigated the effectiveness of pidotimod on the polarization of mouse bone marrow-derived macrophages. The expression of M1 and M2 marker genes was evaluated. In addition, the influence of pidotimod on the macrophage function was demonstrated.

Materials and methods

Mice and reagents

C57BL/6 mice were used at 6–12 weeks of age, which were purchased from Shanghai Slac Animal Inc. and maintained in Laboratory Animal Center of Zhejiang University. All animal experiments were conducted in accordance with experimental protocols approved by the Institutional Animal Care and Use Committee of Zhejiang University. LPS derived from *Escherichia coli* 0111: B4 was obtained from Sigma, America. Mouse rIL-4 and mouse rIFN-γ were obtained from PeproTech, America.

Bone marrow isolation, differentiation, and polarization in vitro

Mouse bone marrow cells were flushed from the femur with sterilized pH 7.4 PBS buffer as described previously (Ji et al. 2013). The cells were then differentiated into bone marrow-derived macrophages (BMDMs) in DMEM media (Hyclone, America) containing 10 % FCS (Gibco, America), 50 U/ml penicillin/streptomycin (Biotime, China), and 10 ng/ml M-CSF (PeproTech, America), or with BMDM media including 100 ng/ml LPS and 10 ng/ml rIFN-γ to generate M1-BMDM, or in BMDM media with 10 ng/ml rIL-4 to generate alternatively activated M2-BMDM. The BMDM was cultured in 37 °C, 5 % CO_2, for 3–24 h in the presence or absence of 1 μg/ml pidotimod (Santa Cruz, America).

Cell viability assay

Cell viability was determined by MTT assay. BMDMs, 1×10^4/well, were seeded in a 96-well plate (Corning, America) overnight before administration. The cells were then treated with different concentrations of pidotimod for 12 or 24 h, followed by culturing with 5 mg/ml of MTT working solution for 4 h at 37 °C. After being incubated with 100 μl of DMSO for 10 min, the cells were detected using SpectraMax M5 (MD, America) to measure the absorbance at 490 nm.

Quantitative real-time PCR

RNA was obtained using the RNAiso plus method (TAKARA), and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (TAKARA). Transcriptional changes were then identified by quantitative real-time PCR (QRT-PCR), which was performed using the Premix Ex Taq™ with SYBR Green (TAKARA) as per the manufacturer's instructions, and the ABI 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Fold changes were calculated after normalizing the change in expression of the gene of interest to the housekeeping gene β-actin using the threshold cycle values. The primer sequences (Invitrogen) used for QRT-PCR are as follows: IL-6 forward, 5′-AGTTGCCTTCTTGGGACTGA-3′, IL-6 reverse, 5′-TCC ACGATTTCCCAGAGAAC-3′, TNF-α forward, 5′-CTGG GACAGTGACCTGGACT-3′, TNF-α reverse, 5′-GCACCT CAGGGA AGAGTCTG-3′, IL-1β forward, 5′-GCAACTG TTCCTGAACTCAACT-3′, IL-1β reverse, 5′-ATCTTTTG

GGGTCCGTCAACT-3′, iNOS forward, 5′-CAGCTGGGC TGTACAAACCTT-3′, iNOS reverse, 5′-CATTGGAAGT GAAGCGTTTCG-3′, Arg1 forward, 5′-CTGCAGCACTT GGATCAGGAACCTG-3′, Arg1 reverse, 5′-GGAGTAGC CTGTGTGCACCTGGAA-3′, Ym1 forward, 5′-GGATGG CTACACTGGAGAAA-3′, Ym1 reverse, 5′-AGAAGGGT CACTCAGGATAA-3′, MR forward, 5′-GCAGACTGCAC CTCTGCCGG-3′, MR reverse, 5′-TGCTGCTTGCAGCTT GCCCT-3′, Fizz1 forward, 5′-CCCTCCACTGTAACGAA G-3′, Fizz1 reverse, 5′-GTGGTCCAGTCAACGAGTAA-3′, β-actin forward, 5′-AACAGTCCGCCTAGAAGCAC-3′, β-actin reverse, 5′-CGTTGACATCCGTAAAGACC-3′.

Flow cytometric analysis

BMDMs were trypsinized and incubated with an antibody against mouse F4/80 and MR for 30 min on ice and washed with PBS. A total of 100,000 events per sample were collected using a FACSCalibur flow cytometer (BD Biosciences), and the percentage of $F4/80^{+}MR^{+}$ events was determined using FlowJo7.6.1 software (BD Biosciences).

Chamber cell-migration assay

Migration of MLE-12 cells was conducted in Transwell™ cell culture chambers with 8.0 μm pore size polycarbonate membrane (Corning, USA) as described previously (Jiang et al. 2008). Briefly, MLE-12 cells were trypsinized and washed three times with 1–2 ml PBS. 2×10^4 cells were resuspended in 300 μl DMEM without FCS and seeded to the upper chamber. 300 μl supernatant of M2 macrophage cultured in the presence or absence of pidotimod was added to the lower chamber. After 24 h of incubation, cells on the upper surface of the filter were removed with a cotton swab, and the cells trapped in the membrane pores or adherent to the undersurface were fixed with 4 % paraformaldehyde, stained with 1 μg/ml DAPI (Roche) and counted. Photographs of three random fields were taken and the cells were counted to calculate the average number of cells that had migrated using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

Wound-healing assay

The in vitro wound-healing assay was performed as previously described (Liang et al. 2007). MLE-12 cells were grown to confluence in 24-well plate. Confluent cell monolayers were mechanically wounded by scraping a conventional pipette tip across the monolayer. To remove loose cells and debris, wounded MLE-12 cells were washed three times with PBS (pH 7.4), and then were incubated for 12 h in a 37 °C incubator containing 5 % CO_2 with the supernatants collected at 24 h from M2 macrophages in the presence or absence of pidotimod. A magnification of 10× was used to allow a major surface area to be covered. Photographs were taken and analyzed using ImageJ software to measure the re-epithelialization area.

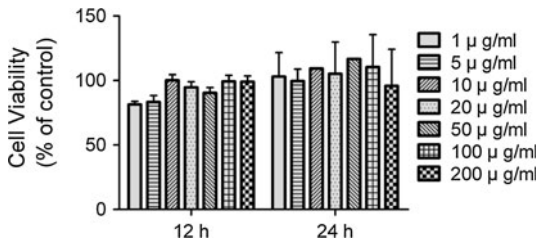

Fig. 1 Effects of pidotimod on BMDMs' viability. BMDMs were exposed in series concentrations of pidotimod for 12 or 24 h, respectively. BMDMs' viability was determined by MTT assay. BMDMs' viability in absence of pidotimod administration was taken as 100 %. The experiment was repeated for three times and the results were expressed as mean ± SD

Statistical analysis

All data were expressed as mean ± SD of at least three independent experiments. The data were evaluated whether they followed the normal distribution by the Kolmogorov–Smirnov test. The result showed that the data fitted the normal distribution. The Levene test of homogeneity of variance was further performed. When the data fitted the homogeneity of variance, one-way ANOVA was used, and for the data that did not fit the homogeneity of variance, Brown–Forsythe analysis was performed. All statistical analyses were performed by IBM SPSS Statistics 20. Values of $p < 0.05$ were considered a statistically significant difference.

Results

Pidotimod had no cytotoxicity on BMDMs

The cytotoxic effects of pidotimod on BMDMs were evaluated by using the MTT assay. BMDMs were cultured with 1–200 μg/ml pidotimod for an indicated time. The results of MTT assay showed that there were no changes of BMDMs' viability after 12 or 24 h exposure to various doses of pidotimod compared to the dimethyl sulfoxide (DMSO)-equivalent controls (Fig. 1), suggesting that no cytotoxicity of pidotimod was detected on BMDMs.

Pidotimod did not influence the M1-BMDMs

To clarify the effect of pidotimod on macrophages polarization, we first examined the hallmarks of M1 and M2 macrophages expression in BMDMs treated with

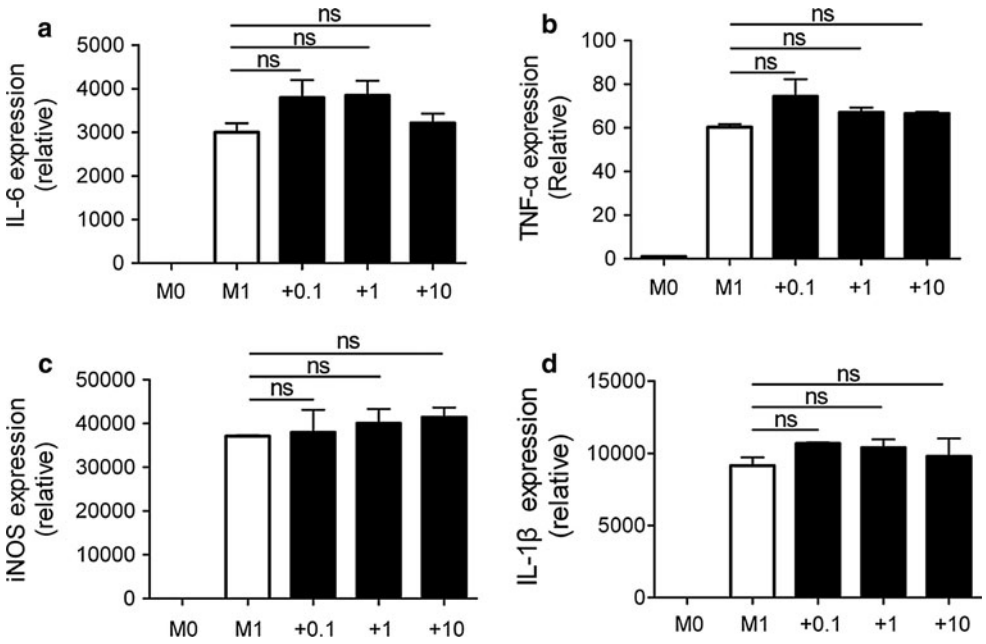

Fig. 2 IL-6, TNF-α, iNOS, and IL-1β expression by M1-BMDMs. IL-6 (**a**), TNF-α (**b**), iNOS (**c**) and IL-1β (**d**) mRNA expressed by nonpolarized (M0) or M1-BMDM polarized for 3 h. BMDMs were polarized to the M1 polarization in the absence (*white bars*) and presence (*black bars*) of 0.1, 1, 10 μg/ml pidotimod compared with the housekeeping gene β-actin. Data are representative of three experiments

pidotimod. After an indicated time administration, no changes were found in the expression of M1 or M2 marker genes, compared with the BMDMs without treatment (data not shown). It showed that pidotimod had no effect on macrophage polarization.

Next we investigated whether pidotimod contributed to LPS and IFN-γ-induced M1 macrophage polarization. RNA was extracted from BMDMs 3 h after LPS and IFN-γ administration in the presence or absence of pidotimod. M1 markers such as IL-6, TNF-α, iNOS, and IL-1β did not differ between M1 macrophages and M1 macrophages treated with pidotimod (Fig. 2). These data confirmed that pidotimod did not affect the polarization of M1 macrophage.

Pidotimod facilitated M2-BMDM polarization

Meanwhile, we evaluated the effect of pidotimod on M2 macrophage differentiation. BMDMs were induced IL-4 with or without different concentrations of pidotimod. The expression of genes encoding Arg1, Fizz1, Ym1, and MR was considerably higher in pidotimod-treated M2-BMDMs compared with M2-BMDMs (Fig. 3). The addition of 0.1 μg/ml pidotimod resulted a significant increase of Arg1 mRNA expression after 24 h administration relative to M2-BMDMs (Fig. 3a), whereas the expression of other M2 markers was not altered. And the hallmarks of M2 macrophages except Fizz1 (Fig. 3b) were dramatically elevated, when the concentration of pidotimod was 10 μg/ml. However, a significant improvement was observed in the expression of all these marker genes at the concentrations of 1 μg/ml pidotimod. The results indicated that the pidotimod could increase the expression of M2 marker genes in M2-BMDMs and 1 μg/ml concentration of pidotimod was used in the following experiments.

In addition to determine the influence of pidotimod on the progress of M2 polarization, we performed the experiment involving a different pidotimod stimulation time. Compared with M2-BMDMs, pidotimod-treated M2-BMDMs expressed higher levels of mRNA encoding Arg1, Fizz1, Ym1, and MR, the hallmarks of M2 macrophages (Fig. 4). Arg1 gene expression was 2.23- or 2.21-fold higher with the addition of pidotimod relative to IL-4 administration for 12 or 24 h (Fig. 4a). After 6 h culturing, the expression of Fizz1 gene was dramatically enhanced compared with M2 macrophages (Fig. 4b). We found that the production of Ym1 (Fig. 4c) and MR (Fig. 4d) upon pidotimod and IL-4 stimulation was significantly increased at the mRNA level after 24 and 12 h incubation. Furthermore, the surface expression of MR on M2-BMDMs was dramatically enhanced from 0.433 to 1.80 % in the presence of 1 μg/ml pidotimod (Fig. 5). Collectively, our data demonstrated that pidotimod could facilitate M2 macrophage polarization.

Effect of M2-BMDMs on migration properties was increased while treated with pidotimod

To analyze whether pidotimod could facilitate on the function of M2 macrophage, we next explored the effects of activated macrophages on the migratory behavior of MLE-12 cells. As shown in Fig. 6, after 24 h of culture in conditioned medium, MLE-12 cells in M2-BMDMs group had much more migration cells than that in M0-BMDMs

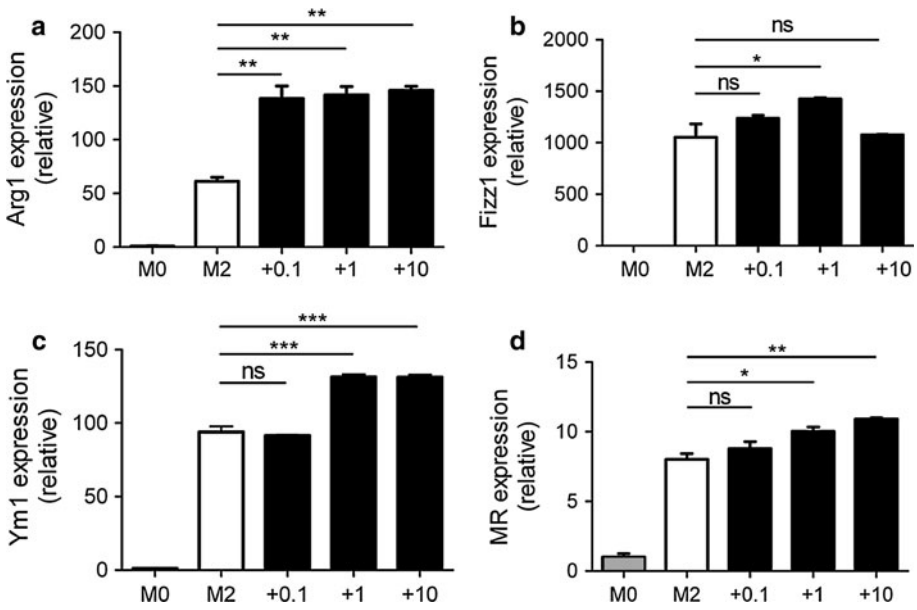

Fig. 3 Arg1, Fizz1, Ym1, and MR expression by M2-BMDMs with different concentration pidotimod treatment. Levels of Arg1 (**a**), Fizz1 (**b**), Ym1 (**c**) and MR (**d**) produced by nonpolarized (M0, *gray bars*) or M2-BMDM polarized for 24 h with rIL-4 in the absence (*white bars*) or presence (*black bars*) of 0.1, 1, 10 μg/ml pidotimod compared with the housekeeping gene β-actin. Data are representative of three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

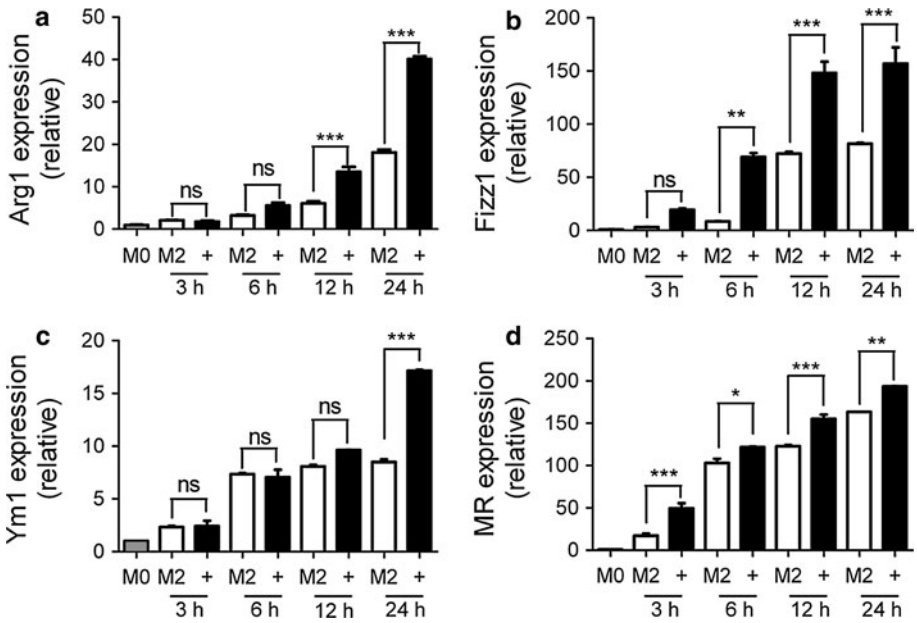

Fig. 4 Arg1, Fizz1, Ym1, and MR expression by M2-BMDMs for different culture time. The relative quantity of Arg1 (**a**), Fizz1 (**b**), Ym1 (**c**) and MR (**d**) mRNA transcribed by nonpolarized (M0, *gray bars*) or M2-BMDM polarized after 3, 6, 12 and 24 h incubation with rIL-4 in the absence (*white bars*) or presence (*black bars*) of 1 μg/ml pidotimod. Data are representative of three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

group ($p < 0.01$). While, the number of MLE-12 cells in the permeating septum in M2-BMDMs stimulated with pidotimod group was the highest among the three groups (Fig. 6a) and increased 1.47-fold ($p < 0.05$) of cell migration relative to M2-BMDM group (Fig. 6b). It suggested that M2 macrophage function in cell migration could be further increased in the presence of pidotimod.

Pidotimod enhanced wound-healing properties of MLE-12 cells

Furthermore, a functional wound-healing assay was performed using bronchial cells in an in vitro scrape model. A monolayer of MLE-12 cells was wounded by mechanical removal of cells and supernatants from M2-BMDMs treated with IL-4 alone, or IL-4 and pidotimod, and M0-BMDMs for 24 h poststimulation (as per Fig. 7a) were applied to wounded epithelial monolayers. The resulting migration of MLE-12 cells into the empty site was examined from images taken over 0 and 12 h after wound formation. As shown in Fig. 7, supernatants from M2-BMDMs were able to increase wound closure compared with that from the M0-BMDMs ($p < 0.001$). In addition, supernatants from M2-BMDMs treated with pidotimod improved re-epithelialization relative to the M2-BMDMs ($p < 0.05$). The data obtained suggested that pidotimod

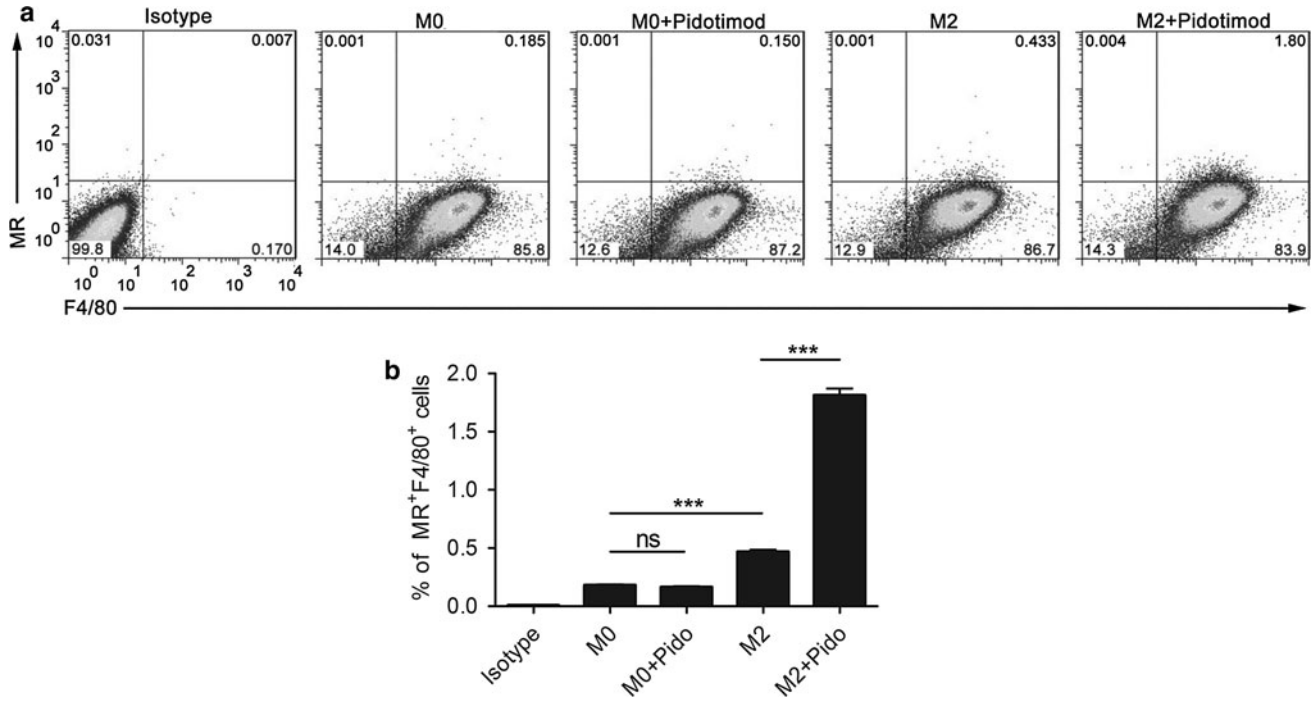

Fig. 5 Pidotimod increased MR expression in M2-BMDMs. Representative flow cytometry dot plots showing the percent of surface MR (*y*-axis) produced in the absence or presence of 1 μg/ml pidotimod in $F4/80^+$ macrophages (*x*-axis) after 24 h treatment (**a**) and the percentage of $MR^+F4/80^+$ cells in pidotimod-treated M2-BMDMs was significantly higher than M2-BMDMs (**b**). Data are representative of three experiments. $^{***}p < 0.001$

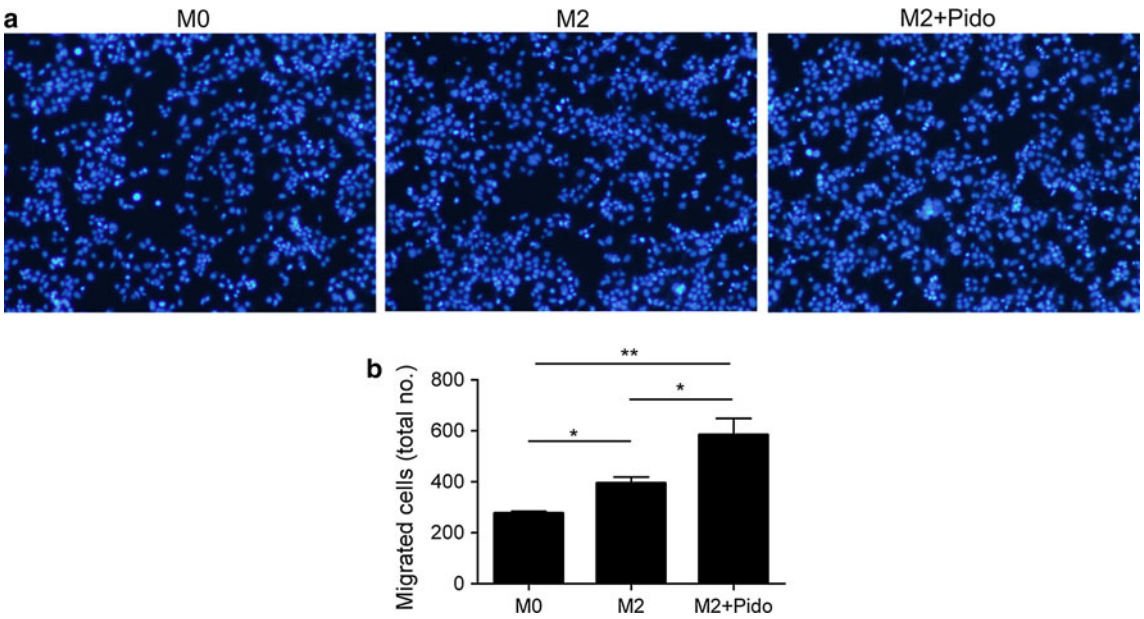

Fig. 6 Transwell migration assay of MLE-12 cell lines (×10). (**a**) Transwell filter was used to measure the migration ability of MLE-12 culture with the supernatant of nonpolarized (M0) or M2-BMDMs in the absence (M2) or presence (M2 + Pido) of 1 μg/ml pidotimod for 24 h. Migrated cells at the lower surface of the transwell filter were stained and counted. (**b**) The numbers of the migrated cells in the supernatant of M2-BMDMs in the presence of pidotimod were significantly higher than that of the M2-BMDMs supernatant. $^{*}p < 0.05$, $^{**}p < 0.01$

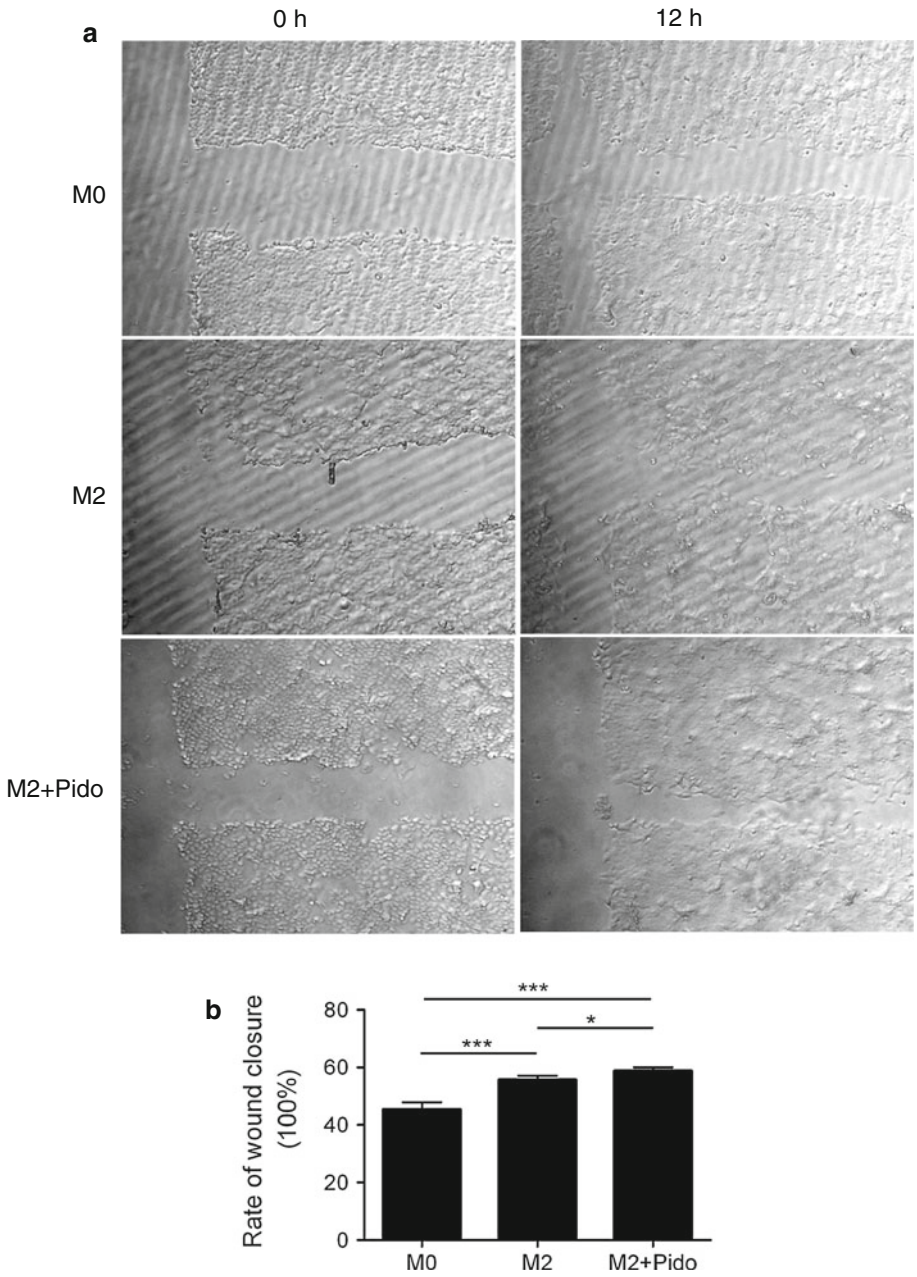

Fig. 7 Cell migration of MLE-12 cell lines in vitro scratch wound-healing assay (×10). (**a**) MLE-12 cells were treated with the supernatant of nonpolarized (M0) or M2-BMDMs in the absence (M2) or presence (M2 + Pido) of 1 μg/ml pidotimod. Photographs were taken at 0 and 12 h, respectively, after the wound was made. (**b**) The rate of wound closure of MLE-12 was measured. $*p < 0.05$, $***p < 0.001$

could facilitate further improvement in re-epithelialization of IL-4-induced M2 macrophages.

Discussion

Macrophages play an essential role in homeostasis and defense (Goerdt and Orfanos 1999; Mantovani et al. 2002; Gordon and Taylor 2005) and can be polarized by different stimuli from the microenvironment to mount M1 or M2 macrophages (Mantovani et al. 2005; Gordon and Taylor 2005; Mosser and Edwards 2008). M1 macrophages are considered potent effector cells that produce kinds of proinflammatory cytokines and clear intracellular microorganisms (Saccani et al. 2006; Murray and Wynn 2011). While, M2 macrophages are able to tune inflammatory responses, enhance phagocytosis, scavenge debris, and promote tissue remodeling and repair (Benoit et al. 2008; Kang et al. 2008). Pidotimod is a biological immunoregulator with essential immunoregulatory function. As demonstrated in many clinical trials, pidotimod is able to play a protective role in bacterial and viral infection processes. Treatment with pidotimod can activate NK cells and promote DC cell maturation (Coppi and Manzardo 1994; Migliorati et al. 1994; Giagulli et al. 2009). Furthermore, it is able to improve macrophage activity and humoral immune functions (Coppi and Manzardo 1994).

However, it is still unclear whether pidotimod plays a role in macrophage polarization. Here, we focused on the effect of pidotimod on macrophage polarization. Our data showed that pidotimod did not facilitate M1 macrophage polarization, while it could facilitate M2 macrophage polarization, as significant up-regulation of M2 macrophage marker expression was obtained. These findings are supported by previous observations revealing that the phenomenon of macrophages undergoing M2 polarization tends to show enhanced marker gene expression (Stein et al. 1992; Bronte and Zanovello 2005; Nair et al. 2005). Moreover, it has been observed that when treating M2 macrophage with pidotimod, MR, a major IL-4-induced alternative activated macrophage marker was significantly enhanced.

It is known that one of the characteristics of alternative macrophage is the facilitation of wound-healing processes (Mosser and Edwards 2008). Previous reports showed that this function has been connected with the effect of IL-4, causing the expression of arginase-1 and consequently expressed polyamines, which are essential for wound repair (Hesse et al. 2001). In consistent with these results, pidotimod-treated M2-BMDM group was identified to enhance the rate of wound closure compared with M2-BMDM group ($p < 0.05$). And the dramatic up-regulation of Arg1 mRNA expression in pidotimod-treated M2-BMDM group ($p < 0.05$) also supported this wound repair function. Moreover, the major event in the wound repair process is the cell spreading and migration (Gordon and Martinez 2010). We established a transplantation model by incubating MLE-12 cells with the supernatant of M0-BMDMs, M2-BMDMs or pidotimod-treated M2-BMDMs. We found that M2-BMDM increased the MLE-12 migration relative to M0-BMDM group ($p < 0.05$), while compared to M2-BMDM group, stimulated with pidotimod which enhanced the number of migrated cells ($p < 0.05$).

Our data investigated a connection between pidotimod and M2 macrophage polarization, which is known to be of great importance in wound healing and tissue repair. Therefore, it would be very interesting to explore the mechanism that how pidotimod facilitated the polarization of M2 macrophage. Previous studies have shown that IL-4-induced M2 macrophages polarization is depended on the activation of signal transducer and activator of transcription 6 (STAT6) (Mikita et al. 1996), transcription factor Irf4 is a vital for the induction of M2 macrophage responses (Satoh et al. 2010) and the nuclear receptor peroxisome-proliferator-activated receptor-γ (PPARγ) has been reported to be an important regulator of this macrophage phenotype (Heilbronn and Campbell 2008). Thus, the mechanisms of pidotimod facilitating M2 macrophage polarization by which signal transduction occurs might be deeply speculated.

In conclusion, this study demonstrated a novel role for dipeptide pidotimod in the facilitation of M2 macrophage polarization and function. These findings might give a new insight into the function of pidotimod as an immunoregulator through regulating immune responses.

Acknowledgments This work was supported by the grants from the planning subject of 'the twelfth five-year-plan' in national science and technology for the rural development in China (No.2013BAD10B03).

Conflict of interest The authors declare that they have no conflict of interest.

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