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OCILRP2 signaling synergizes with LPS to induce the maturation and differentiation of murine dendritic cells

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ABSTRACT

Osteoclast Inhibitory Lectin-related Protein 2 (OCILRP2) is a typical type II transmembrane protein and belongs to C-type lectin-related protein family. It is preferentially expressed in dendritic cells (DC), B lymphocytes, and activated T lymphocytes. Upon binding to its ligand, OCILRP2 can promote CD28-mediated co-stimulation and enhance T cell activation. However, the role of OCILRP2 in DC development and activation is unclear. In this report, we present evidence that recombinant protein OCILRP2-Fc inhibits the generation and LPS-induced maturation of murine bone marrow-derived dendritic cells (BMDCs) by downregulating the expression of CD11c, MHC-II, and co-stimulators CD80 and CD86. OCILRP2-Fc also reduces the capacity of BMDCs to take up antigens, activates T cells, and secret inflammatory cytokines such as IL-6, IL-12, and TNF-α. Additionally, we show that OCILRP2-Fc may cause the aforementioned effects through inhibiting NF-κB activation. Therefore, OCILRP2 is a new regulator of DC maturation and differentiation following TLR4 activation.

1. Introduction

Dendritic cells (DC) as the main antigen-presenting cells (APC) are unique cells that can fully activate naïve T lymphocytes by providing three types of signals: (1) antigen presentation (signal 1); (2) co-stimulatory signal (signal 2); (3) polarizing (signal 3) [1–3]. Signal 1 is mediated by major histocompatibility complex (MHC) coupled with antigenic peptides that are presented to CD8 (through MHC I) or CD4 (through MHC II) T cells. Signal 2 is dependent on co-stimulatory molecules such as B7 family. Signal 3 is provided by cytokines or growth factors secreted by DC. However, the capacity of DC to activate T cells is solely dependent on the status of DC maturation. The immature DC is primarily responsive of antigen uptake and process. The matured DC are responsive of antigen presentation and provoking the expression of co-stimulatory molecules [4,5]. DC maturation is a complicated process, to which many factors contribute in different ways. For instance, DC maturation can be triggered by pathogen associated molecular patterns (PAMP) (e.g., lipopolysaccharide, LPS) through engaging the pattern recognition receptors (PRR) such as toll-like receptors (TLR) [6,7]. Other factors such as CD4-NK cells can also mediate DC maturation via contact-dependent and independent pathways [8]. The accumulating evidence demonstrated that the transcription factor NF-κB participates in driving DC maturation processes [9–12].

Osteoclast Inhibitory Lectin-related Protein 2 (OCILRP2) belongs to C-type lectin-related (Clr) protein family. It is a typical type II transmembrane protein with a short cytoplasmic domain, for which no known signaling motifs are found [13–15]. This protein has been documented to be selectively expressed in immune tissues, with the highest expression in DC, B lymphocytes, and activated T lymphocytes. Consistently, its ligand, NK-cell receptor protein 1 member f (NKRP1f) is also expressed in DC and activated lymphocytes. OCILRP2 interacting with its ligand provides an additional positive signal to enhance B7.1/CD28-mediated T cell proliferation as well as IL-2 production [16]. Silencing OCILRP2 leads to intrinsic impairment in T cell response to anti-CD3 and anti-CD28 stimulation as well as APC presenting antigens. OCILRP2-silenced T cells are incapable of proliferation, and decrease the production of IL-2 upon antigen priming. Moreover, NF-κB activation is also impaired as the result of OCILRP2 silencing [17]. Although OCILRP2 is

Abbreviations: APC, antigen-presenting cells; BMDCs, bone marrow-derived dendritic cells; Clr, C-type lectin-related; GM-CSF, granulocyte-macrophage colony stimulating factor; NKRP1f, NK-cell receptor protein 1 member f; OCILRP2, Osteoclast Inhibitory Lectin Related Protein 2; PAMP, pathogen associated molecular patterns; PRR, pattern recognition receptors; TLR, toll like-receptors.

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dominantly expressed in DC, its role in DC maturation and activation is still unclear.

In this paper, we report that recombinant OCILRP2-Fc protein impaired the mouse bone marrow-derived dendritic cells (BMDCs) development and activation in vitro. This suppression was attributed to restrained transcription factor NF-κB activation as a result of blockade of OCILRP2 signaling. These findings suggest that OCILRP2 signaling plays an important role in DC maturation and functional differentiation.

2. Materials and methods

2.1. Reagents and mice

Recombinant OCILRP2-Fc which fused with OCILRP2 extracellular domain and human IgG1 Fc fragment was produced by cultured CHO cells, and purified by using protein-A column. Cytokine detecting ELISA kits, recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF) and interleukin-4 (rmIL-4) were purchased from R&D system. FITC-conjugated anti-mouse CD11c, CD80, MHC-II, and PE-conjugated anti-mouse CD86 antibodies were bought from eBioscience. Polyclonal anti-IκB antibody was bought from Cell Signaling Technology. LPS and FITC-labeled dextran (M.W. = 40,000) were purchased from Sigma–Aldrich. 6–8 weeks old BALB/c mice were purchased from Beijing Laboratory Animal Research Centre. All experiments were approved by the Animal Ethics Board of Henan University and performed in accordance with EU Directive 2010/63/EU.

2.2. Dendritic cells generation and culture

BMDCs were prepared as described previously [18] with a little modifications. Briefly, bone marrow cells were collected from tibias and femurs of 6–8 weeks old BALB/c mice, and passed through a nylon mesh to remove small pieces of bone and debris, then red blood cells were lysed within ACK lysis buffer (0.15 M NH_4Cl, 1.0 M $KHCO_3$, 0.1 mM Na_2EDTA). After washing, cells were resuspended in RPMI 1640 complete medium supplemented with 10% FBS, rmGM-CSF (20 ng/ml), rmIL-4 (20 ng/ml), 2-mercaptoethanol (50 mM) and with recombinant OCILRP2-Fc protein (10 μg/ml) or control human IgG. Cells were cultured in 24-well plates at 1×10^6 cells/ml, 1 ml in each well. Non-adherent cells were discarded after having been cultured for 24 h, and medium was replaced every other day. On day 6, cells were cultured for additional 24 h in the presence of LPS (500 ng/ml) to induce BMDCs maturation. Non-adherent and loose adherent cells consisting of mostly BMDCs were harvested and purified by MACS using anti-CD11c antibody–coupled magnetic beads (Miltenyi Biotec).

2.3. Cell surface markers expression analysis

Bone marrow cells were cultured with OCILRP2-Fc or control human IgG for 5 days, then cells were harvested or stimulated by LPS for another day. 1×10^6 cells were incubated respectively with fluorescence-conjugated anti-mouse CD11c, CD80, MHC-II or CD86 monoclonal antibody for 30 min on ice, the appropriate conjugated isotype-matched IgGs were used as control. After washing 3 times, samples were acquired on FACS Calibur with CellQuest software and analyzed with FlowJo software.

2.4. Fluorescein isothiocyanate (FITC)-dextran uptake

2×10^6 Immature BMDCs or LPS-induced mature BMDCs with different treatment were incubated with 1 mg/ml of FITC-dextran for 2 h at 37 °C. Parallel cultured BMDCs were pre-cooled and incubated with FITC-dextran at 4 °C as negative control. Subsequently, cells were harvested and washed 3 times with cold PBS, then acquired on FACS Calibur.

2.5. Allogeneic mixed leukocyte reaction

Graded numbers of BMDCs were cultured with 2×10^5 $CD4^+$ T cells in triplicate in round-bottom 96-well plates, $CD4^+$ T cells were purified from allogeneic mouse spleen by MACS (Miltenyi Biotec). After 3 days of culturing, each well was pulsed with 1 μCi [^{3}H]thymidine and incubated for additional 18 h. And then the incorporation of radionuclide into DNA was measured by liquid scintillation counter.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [17]. In brief, BMDCs with different treatment were harvested, cells were lysed within 100 μl of Buffer C (20 mM Hepes, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSA, 1 mM DTT, leupeptin and aprotinin) plus 0.1% NP40 followed by sonication. Soluble fractions were separated from sonicated cell lysate by centrifugation (14,000 rpm). 5 μg of cell lysate were incubated with ^{32}P-labeled IgκB probe at room temperature for 20 min and then resolved on 6% native polyacrylamide gel.

2.7. Western blotting

Whole cell lysate was prepared by using lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM PMSF and 1 × protease inhibitor cocktail (Roche). Protein concentrations were determined by BCA assay. Equal quantities of samples were run on 10% SDS–PAGE gels and transferred onto nitrocellulose membrane. The membrane was probed with polyclonal anti-IκB antibody (1:500 dilution) and followed by horseradish peroxidase-conjugated secondary antibody, specific bands were visualized via the ECL kit according to the manufacturer's instruction.

2.8. Cytokine ELISA

The same number of immature BMDCs with different treatment were seeded in each well of 24-well plate followed by LPS (500 ng/ml) stimulating. Cell culture supernatants were collected at different time points (0, 6, 12, 24, and 48 h post-stimulation with LPS). Cytokine concentrations were determined by ELISA kits according to the manufacturer's instructions.

2.9. Statistical analyses

SPSS13.0 software was used for the statistic analysis. Data were presented as mean ± standard deviations (SE), and analyzed using two-way ANOVA and paired student's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. OCILRP2-Fc suppresses BMDC generation and maturation

In order to investigate whether OCILRP2 signaling affects DC generation and maturation, we surveyed cell surface marker expression in both immature BMDCs and LPS-induced mature BMDCs with OCILRP2-Fc treatment. 74% of immature BMDCs and 85% of mature BMDCs were CD11c positive, before purification. The expression level of CD11c in OCILRP2-Fc treated cells was

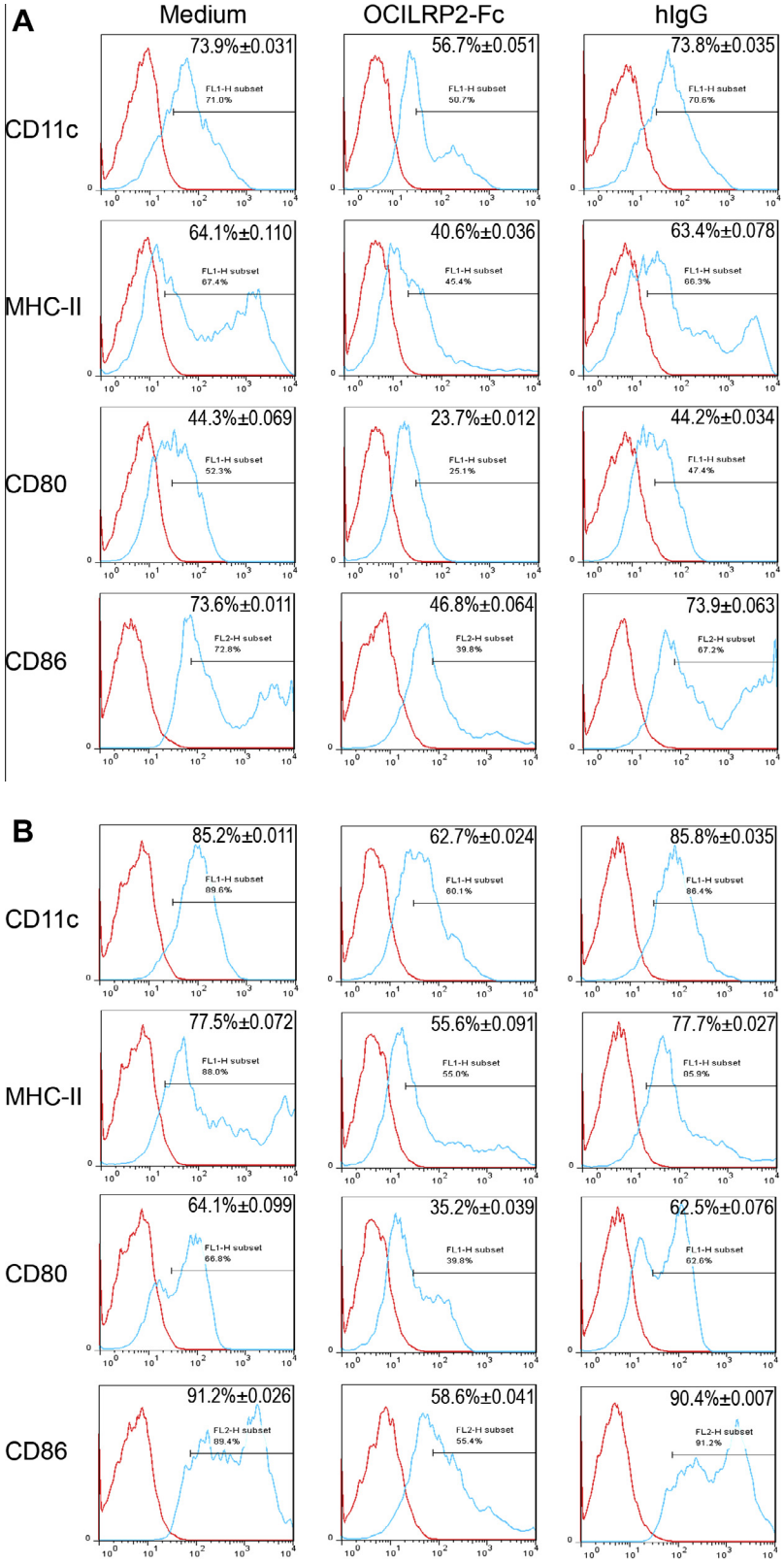

Fig. 1. OCILRP2-Fc suppressed cell surface marker expression on BMDCs. (A) Mouse bone marrow cells were cultured in RPMI 1640 complete medium supplemented with recombinant OCILRP2-Fc or control human IgG (hIgG) for 5 days. Then, cells were harvested and incubated with FITC-conjugated anti-mouse CD11c, CD80, MHC-II and PE-conjugated anti-mouse CD86 antibodies on ice for 30 min, respectively. The fluorescence intensity was measured by flow cytometry. The red curves represent fluorescence-conjugated isotype IgGs. (B) The cultured cells were stimulated by LPS for additional 24 h, and then flow cytometry was performed for cell surface marker expression by using fluorescence-conjugated antibodies. Data shown are representative of those from more than 3 experiments.

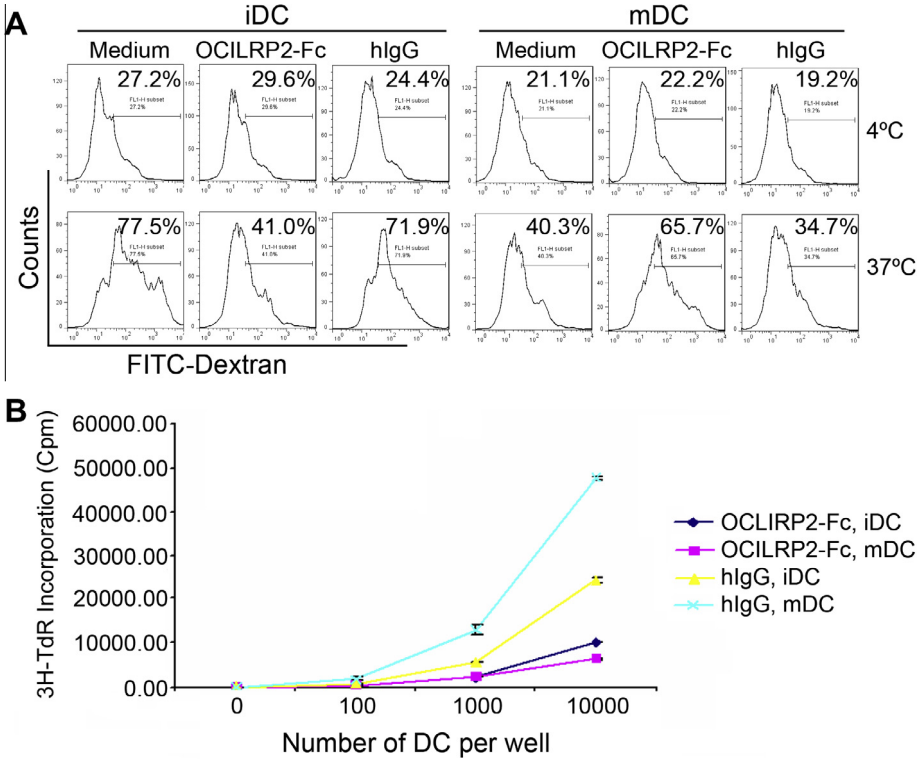

Fig. 2. OCILRP2-Fc depressed BMDC functional differentiation (A) Immature BMDCs (iDC) and mature BMDCs (mDC) treated with recombinant OCILRP2-Fc or control human IgG (hIgG) were incubated with FITC-labeled dextran for 2 h at 4 °C or 37 °C, flow cytometry was performed for endocytosis capacity of BMDCs. Cells incubated with FITC-labeled Dextran at 4 °C were considered as negative control. Data shown are representative of those from over 3 experiments. (B) Irradiated iDC or mDC with OCILRP2-Fc or hIgG treatment were incubated with $CD4^+$ T cells for 3 days, then cells were pulsed with 1 μCi [^{3}H]thymidine for additional 18 h and measured for [^{3}H]thymidine incorporation by scintillation counter. *Y* axis was expressed in radioactivity (cpm). $^*p < 0.05$, $^{**}p < 0.01$.

much lower than that of untreated cells. Similarly, the expression of MHC-II and co-stimulators CD80 and CD86 were also obviously down regulated in OCILRP2-Fc treated BMDCs compared with control cells (Fig. 1A, B). These results suggest that BMDCs generation and maturation were suppressed by recombinant OCILRP2-Fc which blocked OCILRP2 signaling.

3.2. OCILRP2-Fc depresses BMDCs functional differentiation

Since OCILRP2-Fc suppressed BMDCs maturation, we investigated whether OCILRP2-Fc affected the BMDCs' biological function. BMDCs with different treatment purified by MACS were incubated with FITC-dextran for 2 h at 37 °C, the results of Flow analysis showed that OCILRP2-Fc inhibited the immature BMDCs' endocytosis significantly, and restored the decreased endocytosis of FITC-dextran of LPS-induced mature BMDCs. As control, BMDCs incubated with FITC-dextran at 4 °C displayed poor and similar endocytosis. This data implied that OCILRP2-Fc impaired the ability of immature BMDCs for capturing antigens and maintained BMDCs in an immature stage. (Fig. 2A). For BMDCs capacity of activating naïve T cells, we assessed allostimulatory activity of OCILRP2-Fc treated BMDCs through [^{3}H]thymidine incorporation mixed lymphocytes reaction. As expected, BMDCs treated with OCILRP2-Fc were less effective on stimulating naïve allogeneic T cells activation than that without OCILRP2-Fc treatment (Fig. 2B). To further characterize the cytokine expression profile by BMDCs, cell culture supernatants that were treated with LPS for the indicated times were subjected to cytokine ELISA analysis. In contrast to OCILRP2-Fc untreated BMDCs, OCILRP2-Fc treated BMDCs decreased secretion of pro-inflammatory cytokines including IL-12, IL-6 and TNFα (Fig. 3A), and the inhibition of IL-12 release was dependent on the OCILRP2-Fc concentration (Fig. 3B). The apoptosis ratio of BMDCs with different treatments had no significant difference (data not shown). Taken together, these results indicated that OCILRP2 signaling is required for BMDCs maturation and functional differentiation.

3.3. OCILRP2-Fc inhibits NF-κB activation

In view of the inhibitory effects of OCILRP2-Fc on BMDCs maturation and functional differentiation, we took further research to uncover whether the inhibitory effects were regulated by transcription factor NF-κB. As expected, NF-κB activation in LPS-induced mature BMDCs was higher than that in immature BMDCs. However, NF-κB activation was significantly inhibited in the BMDCs treated by OCILRP2-Fc (Fig. 4A). Consistently, immunoblotting results indicated that LPS causes a significantly reduced expression of I-κB in mature BMDCs and OCILRP2-Fc could restore the expression of I-κB, both in LPS-induced mature BMDCs and bared immature BMDCs (Fig. 4B). Together, OCILRP2-Fc restrained NF-κB activation in immature BMDCs, as well as the LPS primed mature BMDCs, which suggests OCILRP2 signaling contributes to BMDCs maturation most likely through NF-κB activation pathway. Furthermore, there was no considerable difference detected on the concentration of IL-10 between the supernatant from OCILRP2-Fc treated BMDCs and that untreated (Fig. 4C), which suggests that IL-10 is not involved in the inhibitory effect of OCILRP2-Fc on the BMDCs maturation and activation.

4. Discussion

The maturation of DC is characterized by a high expression of co-stimulatory molecules such as CD80, CD86, CD83 and MHC class II molecules, the extended half-lifes of peptide–MHC

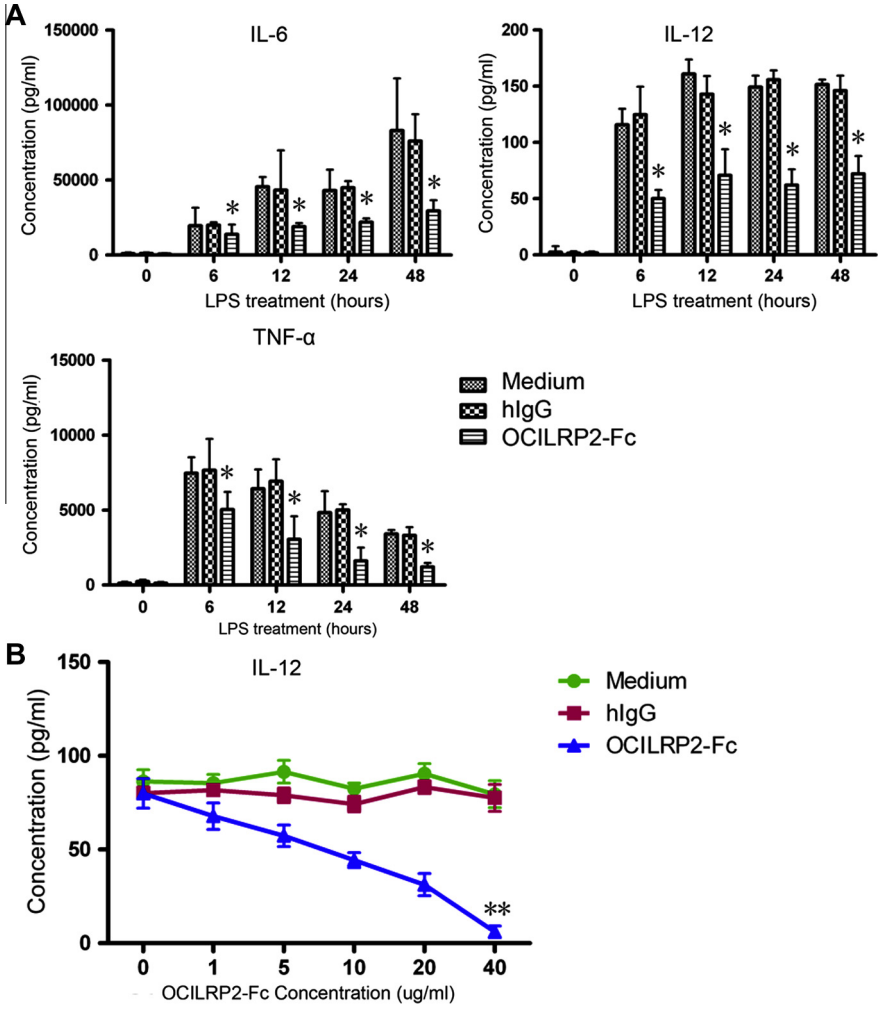

Fig. 3. OCILRP2-Fc decreased cytokine secretion of BMDCs. (A) The same number of immature BMDCs treated with OCILRP2-Fc or control IgG (hIgG) were seeded in each well of 24-well plates and stimulated with LPS. Cell culture supernatants were collected at indicated time points, cytokine concentration was measured by individual ELISA kit. (B) Immature BMDCs cultured in the presence of varying doses of OCILRP2-Fc or control IgG were stimulated by LPS for additional 24 h after having been cultured for 5 days. Cell culture supernatants were assayed by IL-12 ELISA kit for cytokine concentration. The data were accounted for 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

complexes and the increased production of chemokines and cytokines [19].The dominant expression of OCILRP2 and its ligand NKRP1f in DC implies that they probably play important roles in DC maturation. Here, we presented the evidence that OCILRP2 signaling blocked by recombinant OCILRP2-Fc downregulated expression of co-stimulatory molecules and MHC class II molecules on BMDCs, impaired the capacity of antigen uptake and allogeneic T lymphocytes activation, and decreased the secretion of pro-inflammatory cytokines including IL-6, IL-12 and TNF-α. Isotype, human IgG, had no such effect, which excluded the function of Fc cross-linking. Our results suggest that OCILRP2-mediated cell–cell interaction contributes to BMDCs maturation and activation. In this study, OCILRP2 signal is prerequisite for LPS-induced BMDC maturation, to which OCILRP2 may contribute, either directly, or in synergy with other cell surface molecules such as TLR4.

Since NKRP1f is usually considered as ITIM-less activation receptor [14,20], it probably delivers activatory signals following binding to OCILRP2. So, according to our present data, we are inclined to believe that OCILRP2-Fc blocked OCILRP2 signaling, and then impaired DC development and activation. Nevertheless, recent research suggests that the activation potential of NKRP1f is questionable, because it does not enhance NK cells' cytotoxicity or IFN-γ production upon crosslinking by using specific monoclonal antibody [21]. Because of ambiguous effects of NKRP1f, in this study, we cannot exclude the agonistic effects of OCILRP2-Fc through NKRP1f. That is to say, NKRP1f perhaps delivers inhibitory signals after activated by OCILRP2-Fc, which inhibits DC development and activation.

TLR4 is a well-characterized TLR that facilitate the activation of DC [22,23]. Although it is well accepted that LPS activates DC, there is only limited information about the role of TLR4 in DC differentiation processes. Upon ligand binding, activated TLR4 recruits MyD88 and TICAM-1 adaptor molecules and initiates signaling cascades that ultimately lead to the activation of transcription factors such as NF-κB and TRIF. Recently, many reports have demonstrated that other signaling pathways affect TLR signaling in macrophage and DC activation. Some of them are positive and synergistic [24–27], whereas others are negative effect [28–32]. We report here that Clr protein OCILRP2 may be a novel molecule that synergizes with TLR4 signaling in DC maturation.

NF-κB activation is essential for DC development and functional differentiation. NF-κB participates in the up-regulation of the expression of MHC II and co-stimulatory molecules, the release of pro-inflammatory cytokines and chemokines, and DC migration and recruitment [9,33]. Here, the fact that impaired OCILRP2 signaling suppressed LPS-induced NF-κB activation in BMDCs suggests that OCILRP2 may potentiate TLR4 signaling through its effects on NF-κB activation. Nevertheless, OCILRP2 has no activated motif in cytoplasmic domain, which implies adaptors would be needed to contribute to the signal transduction. C-type

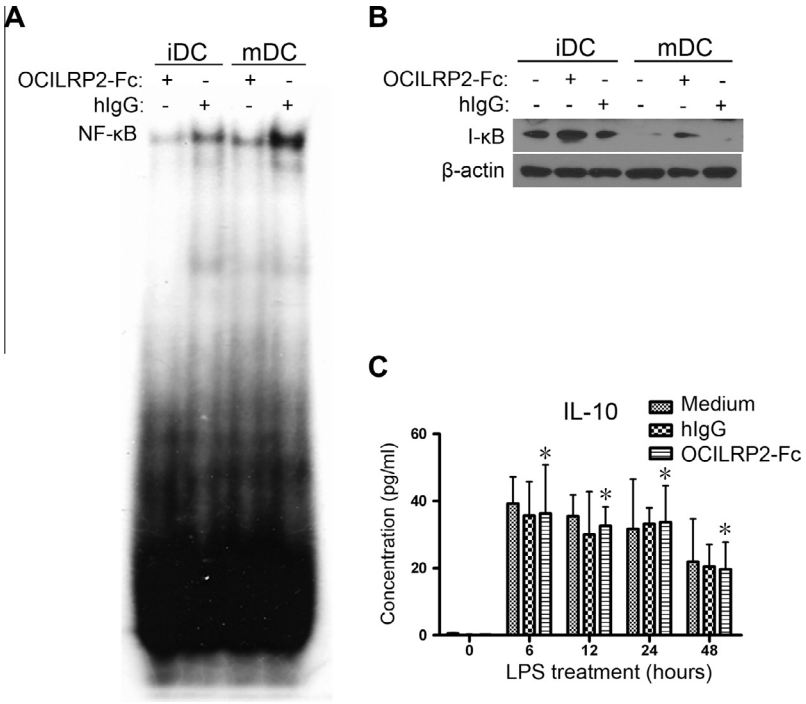

Fig. 4. OCILRP2-Fc restrained NF-κB activity. (A) Nuclear protein extracts prepared from immature or mature BMDCs with different treatment were incubated with ^{32}P-labeled IgκB probe for 20 min at room temperature. The DNA binding reactions were resolved on 6% native polyacrylamide gel. (B) Whole lysates of BMDCs treated with OCILRP2-Fc or control hIgG were subject to western blot. I-κB was probed by using anti-IκB polyclonal antibody and followed by HRP-conjugated secondary antibody, β-actin as inner control. Data shown are representative of those from 3 experiments. (C) Cell culture supernatants were collected at indicated time points after LPS stimulation. Cytokine concentration was measured by IL-10 ELISA kit. *$p > 0.05$.

lectin-like receptors NKG2D and CLEC5A transmits a signal through association with adaptor DAP12 and DAP10 [34,35]. It is not certain whether OCILRP2 associate with DAP10 or DAP12 or both, and which pathway OCILRP2 would take to facilitate DC maturation and activation. The relevant research is still under investigation in our laboratory.

Given their potent function in antigen presentation and T cells activation, DC has been explored as vaccine vehicles. The maturation stage of DC is important for their ability to function as vehicles for DC-based immunotherapy. Mature DC directs Th1-polarized immune response and are considered as potent cells that initiate tumor-specific immunity [36]. Immature DC presenting antigenic peptides to T cells without co-stimulatory signal results in T cell anergy [37], promotes the proliferation of $CD4^+CD25^+$ regulatory T cells [38], suppresses IL-17 production and downregulates DC-dependent Th17 cell differentiation [39,40]. DC activity may elicit tolerance rather than immunity if the DC maturation is not appropriately induced [41]. We report here that OCILRP2 as a new molecule plays important roles in DC maturation and could be a therapeutic target of drug development. Adjusting OCILRP2 signaling to regulate DC maturation by recombinant OCILRP2-Fc or its ligand can be explored as a potential strategy in DC vaccine development for autoimmune disease and malignant tumors.

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