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# Anti-inflammatory modulation of human myeloid-derived dendritic cell subsets by lenalidomide

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ABSTRACT

Although immunomodulatory drugs (IMiDs) were originally developed as anti-inflammatory drugs, they are effective for multiple myeloma. In order to gain further insights into the immunomodulatory mechanisms of IMiDs for the treatment of inflammatory disorders and myeloma, we investigated the influence of a representative IMiD, lenalidomide, on human primary dendritic cell (DC) subsets: myeloid-derived $CD1c^+$ DCs, $CD141^+$ DCs, and plasmacytoid DCs. Lenalidomide did not affect the viability or expression of costimulatory molecules, but it potently suppressed the production of the key inflammatory cytokines IL-12 and IL-23, and enhanced the production of the anti-inflammatory cytokine IL-10 by $CD1c^+$ DCs. Lenalidomide also suppressed the production of IFN-α by $CD141^+$ DCs but not that by plasmacytoid DCs. Lenalidomide likely targets pathways downstream of the nuclear translocation of the transcription factors nuclear factor κB (NF-κB) and IFN regulatory 5 (IRF5) in $CD1c^+$ DCs. Consistent with the direct immunomodulatory effects on DCs, lenalidomide decreased the capacity of $CD1c^+$ DCs to induce differentiation of naïve $CD4^+$ T cells into effector cells producing immune activating and myeloma-promoting cytokines. This study demonstrated that lenalidomide has anti-inflammatory effects via the modulation of cytokine production by human myeloid-derived DCs. Such effects on DCs may allow for beneficial immunomodulation aiding in the treatment of inflammatory disorders and multiple myeloma.

## 1. Introduction

Thalidomide and its analogs, called Immunomodulatory drugs (IMiDs), were originally found to have anti-inflammatory effects and have been explored for treatment of inflammatory disorders, such as erythema nodosum leprosum and autoimmune diseases [1]. Thereafter, IMiDs were also found to be effective for multiple myeloma [1], and now represent an integral component of myeloma therapies [2]. One IMiD, lenalidomide, is included in several current regimens to treat myeloma due to its high efficacy and safety profile. Lenalidomide enhances the activity of T cells [3] and NK cells [4], and is thus likely to enhance anti-myeloma immunity, although it is apparently inconsistent with the originally discovered anti-inflammatory effects. Mechanistically, it has been revealed that IMiDs bind to cereblon, a component of E3 ubiquitin ligase complex [5], and alter its activity, which results in the pleiotropic action of IMiDs [6,7].

Dendritic cells (DCs) are central to the initiation and regulation of immune responses as the most potent antigen-presenting cells [8]. Human DCs in blood and lymphoid tissues are composed of distinctive subsets: myeloid-derived DCs, termed conventional DCs (cDCs), and plasmacytoid DCs (pDCs) [9]. cDCs are further subdivided into CD1c (BDCA-1)$^+$ DCs and CD141 (BDCA-3)$^+$ DCs [9]. In addition, monocytes

*Abbreviations:* 4E-BP, 4E-binding protein; cDC, conventional DC; c-MAF, c-masculoaponeurotic fibrosarcoma; DC, dendritic cell; ERK, extracellular-signal-regulated kinase; GSK3, glycogen synthase kinase 3; IKK, inhibitory κB kinase; IKZF, ikaros family zinc finger protein; IMiD, immunomodulatory drug; IRF, interferon regulatory factor; JNK, JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; MoDC, monocyte-derived DC; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NF-κB, nuclear factor κB; p70S6K, p70 S6 kinase; pDC, plasmacytoid DC; PI3K, phosphoinositide 3-kinase; poly I:C, polyinosinic-polycytidylic acid; TLR, Toll-like receptor

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can differentiate into DCs in the presence of specific cytokine mixtures, mostly GM-CSF and IL-4, in vitro [10]. It has recently been reported that lenalidomide enhances the immunostimulatory activity of human monocyte-derived DCs (MoDCs) [11,12]. However, MoDCs considerably differ from blood cDCs and pDCs in their gene expression profiles [13]. In addition, the physiological relevance of MoDCs is still unclear, although it has been proposed that they are induced after monocytes are recruited to inflammatory tissues [14]. Therefore, it is important to examine the effects of lenalidomide on DCs isolated from blood or tissues, rather than on MoDCs, to fully understand the mechanisms by which lenalidomide modulates immune environments through DCs.

Cytokines from DCs stimulated with pathogen-derived components significantly affect the induction of different $CD4^+$ T cell responses [15]. IL-12p70 (bioactive IL-12) produced by myeloid-derived DCs is vital for the induction of a Th1 response [16], and also induces IL-21-producing T follicular helper-like cells [17]. Another member of the IL-12 family, IL-23, augments Th17 responses, which leads to numerous inflammatory disorders, such as psoriasis and inflammatory bowel disease [18]. IFN-α secreted by pDCs and $CD141^+$ DCs upon viral stimulation [19–21] induces antiviral immunity and modulates a broad array of immune responses [22]. In contrast, IL-10 inhibits proinflammatory responses, thereby preventing tissue damage caused by excessive immune reaction [23]. Thus, it is important to elucidate the influence of lenalidomide (i) on the production of the key cytokines (IL-12, IL-23, IFN-α, and IL-10) by DCs and (ii) on the resulting functional differentiation of $CD4^+$ T cells, in order to clarify the immunological effects of lenalidomide.

In this study, we examined the effects of lenalidomide on human primary DCs in blood: $CD1c^+$ DCs, $CD141^+$ DCs, and pDCs in vitro. We found that lenalidomide strongly suppressed the production of immunostimulatory cytokines (IL-12, IL-23, IFN-α) and enhanced that of an anti-inflammatory cytokine (IL-10) by cDCs ($CD1c^+$ DCs and $CD141^+$ DCs). The effects of lenalidomide on $CD1c^+$ DCs were reflected in the attenuated differentiation of naïve $CD4^+$ T cells toward proinflammation. In contrast, lenalidomide did not suppress IFN-α production by pDCs, having differential effects on human cDCs and pDCs. These data shed new light on the immunomodulatory effects of IMiDs.

## 2. Materials and methods

### 2.1. Culture media

RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated FCS (Gibco BRL, San Francisco, CA, USA), 2 mM L-glutamine, penicillin G, streptomycin (Gibco BRL), and 10 mM HEPES (Nacalai tesque) was used for cell culture.

### 2.2. Isolation and culture of DCs

This study was approved by the Institutional Review Board of the Graduate School of Medicine, Kyoto University (Approval No. C123), and abides by the tenets of the Declaration of Helsinki. All specimens from humans were obtained from healthy donors with written informed consent. Human blood DCs were isolated as previously described [24]. Briefly, total peripheral blood mononuclear cells (PBMCs) obtained from 400 mL of blood were depleted of $CD3^+$, $CD14^+$, and $CD16^+$ cells using Dynabeads® goat anti-mouse IgG (Invitrogen Dynal, Carlsbad, CA, USA). Among forward scatter$^{intermediate}$lineage(CD3, 14, 16, 20)$^-CD4^+$ cells, $CD11c^+CD141^{-/low}$ cells, $CD141^{high}$ cells, and $CD11c^-CD141^{-/low}$ cells were sorted as $CD1c^+$ DCs, $CD141^+$ DCs, and pDCs, respectively, by the FACSAria™ II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). The purity of each subset was more than 98%. Monocytes were purified from PBMCs using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

The isolated blood DC subsets were cultured in round-bottom 96-well plates. $CD1c^+$ DCs were plated at $1 \times 10^5$ cells /200 µL, and cultured with 800 U/mL recombinant human GM-CSF (Genzyme, Cambridge, MA, USA), 10 ng/mL IFN-γ (R & D Systems, Minneapolis, MN, USA), and the indicated combinations of Toll-like receptor (TLR) ligands: 100 ng/mL lipopolysaccharide (LPS) (from *Escherichia coli* O111:B4, Sigma-Aldrich, St. Louis, MO, USA), 10 µg/mL R848 (InvivoGen, San Diego, CA, USA), and 20 µg/mL polyinosinic-polycytidylic acid (poly I:C) (GE Healthcare, Chicago, IL, USA). Indicated concentrations of lenalidomide (provided by Celgene Corporation, Summit, NJ, USA) and 10 µg/mL anti-IL-10 monoclonal antibody (mAb) (BD Biosciences) were added 1 h before adding the TLR ligands. $CD141^+$ DCs were plated at $3 \times 10^4$ cells /200 µL, and cultured with 800 U/mL GM-CSF, 10 ng/mL IFN-γ, 10 µg/mL R848, and 20 µg/mL poly I:C. pDCs were plated at $4 \times 10^4$ cells /200 µL, and cultured with 1 µM ODN2216 (Operon Biotechnologies, Alameda, CA, USA). Monocytes were plated at $1 \times 10^5$ cells /200 µL, and cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 (Peprotech, Rocky Hill, CT, USA) for 6 d to induce immature MoDCs. The cells were then stimulated with 10 ng/mL IFN-γ, 100 ng/mL LPS, 10 µg/mL R848, and 20 µg/mL poly I:C. Cells and supernatants were collected 24 h after TLR stimulation for flow cytometry and cytokine measurements, respectively. Cell viability was measured by trypan blue exclusion.

### 2.3. DC-T cell co-culture

Naïve $CD4^+$ T cells were isolated from PBMCs by negative selection using the Naïve $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec). $CD1c^+$ DCs were stimulated with 10 µg/mL R848 in the absence or presence of 1 µM lenalidomide. After 24-h stimulation, DCs were harvested and washed, and then cultured at $1 \times 10^4$ cells with allogenic naïve $CD4^+$ T cells ($1 \times 10^5$ cells) in the absence or presence of 1 µM lenalidomide for 7 days in round-bottom 96-well plates. The $CD4^+$ T cells were re-stimulated with plate-bound CD3 mAb (OKT3) and 1 µg/mL soluble anti-CD28 mAb (BD Biosciences) in flat-bottom 96-well plates for 24 h. The supernatants were analyzed for cytokines by ELISA.

### 2.4. ELISA

Cytokines in culture supernatants released from DCs or T cells were analyzed by ELISA, using ELISA MAX™ Standard SET human IL-12p70, ELISA MAX™ Standard SET human IL-10, ELISA MAX™ Standard SET human IL-2, ELISA MAX™ Standard SET human IFN-γ, ELISA MAX™ Deluxe SET human IL-17A (BioLegend, San Diego, CA, USA), the Human IL-23 ELISA Ready-SET-Go® Set (eBioscience, Waltham, MA, USA), the Human IFN-α (pan specific) ELISA development kit, and the Human IL-21 ELISA development kit (MABTECH, Nacka Strand, Sweden).

### 2.5. Flow cytometry

For the analysis of cell surface proteins, $CD1c^+$ DCs were stained with FITC-conjugated mouse anti-human CD80 or FITC-conjugated mouse anti-human CD86 mAb (BD Biosciences). Dead cells were excluded by staining with propidium iodide.

### 2.6. Immunofluorescence microscopy

$CD1c^+$ DCs were stimulated with TLR ligands for 1 h in the absence or presence of 1 µM lenalidomide. After fixation by 2% paraformaldehyde for 15 min, cells were permeabilized with 0.5% Triton X-100 and blocked with 5% goat serum for 1 h. Subsequently, cells were incubated with rabbit anti-IFN regulatory factor 5 (IRF5; Cell Signaling Technologies, Danvers, CO, USA) or nuclear factor κB p65 (NF-κB p65; Santa Cruz Biotechnology, Dallas, TX, USA) mAb at 4 °C overnight. Alexa Fluor® 488-conjugated goat anti-rabbit IgG antibody (Invitrogen,

Carlsbad, CA, USA) was added for 90 min at room temperature. The stained cells were attached to slides using a Cytospin centrifuge, mounted in VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and examined using the confocal microscope Leica TCS SP8 (Leica, Wetzlar, Germany). Data were analyzed with LAS X software (Leica).

### 2.7. Reverse transcription and real-time PCR

CD1c$^+$ DCs were cultured with GM-CSF, IFN-γ, and TLR ligands for 8 h. One μM lenalidomide was added 1 h before adding the TLR ligands. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). First-strand cDNA synthesis was carried out with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed on the Thermal Cycler Dice® Real Time System (TaKaRa, Kusatsu, Japan). c-masculoaponeurotic fibrosarcoma (c-MAF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Osaka, Japan) and gene-specific oligonucleotide primers as follows: *c-MAF*, 5′-AGCAGTTGGTG ACCATGTCG-3′ (forward); 5′-TGGAGATCTCCTGCTTGAGG-3′ (reverse) [25] and *GAPDH*, 5′-GAGTCAACGGATTTGGTCGT-3′ (forward); 5′-GACAAGCTTCCCGTTCTCAG-3′ (reverse). The mRNA expression levels of each gene were normalized to that of *GAPDH*.

### 2.8. Statistical analysis

Data are presented as the mean ± SE. Statistical comparisons were performed using the paired two-tailed t-test. Differences with $P < .05$ were considered significant.

## 3. Results

### 3.1. A combination of TLR ligands potently induces CD1c$^+$ DCs to produce IL-12p70

Although LPS is sufficient to induce DCs to produce IL-12p40, a combination of TLR ligands together with IFN-γ was reported as necessary to induce DCs to produce high levels of IL-12p70, the bioactive form of IL-12 [26–28]. Thus, we first evaluated which combination of TLR ligands induced CD1c$^+$ DCs to produce IL-12p70 most potently. As CD1c$^+$ DCs express multiple TLRs, including TLR3, TLR4, and TLR8 [29], we used 3 TLR ligands: LPS (TLR4), R848 (TLR7/8), and poly I:C (TLR3). Stimulation with a single TLR ligand, LPS plus poly I:C, or R848 plus poly I:C, induced CD1c$^+$ DCs to produce only low levels of IL-12p70. In contrast, LPS plus R848 induced production of a high level of IL-12p70 (Supplementary Fig. 1). The addition of poly I:C to LPS plus R848 further enhanced production. Thus, we used the 3 TLR ligands (LPS, R848, poly I:C) in combination with GM-CSF and IFN-γ to stimulate CD1c$^+$ DCs in the following experiments to examine the effects of lenalidomide.

### 3.2. Lenalidomide does not reduce the viability or expression of co-stimulatory molecules

Next, we investigated whether the representative IMiD lenalidomide affects the viability of CD1c$^+$ DCs. We added lenalidomide at different concentrations together with the 3 TLR ligands. Lenalidomide did not reduce the viability of CD1c$^+$ DCs, CD141$^+$ DCs, or pDCs (Supplementary Fig. 2).

We also examined whether lenalidomide affects the expression of co-stimulatory molecules CD80 and CD86 on CD1c$^+$ DCs. The TLR ligands upregulated the expression of CD80 and CD86 (Supplementary Fig. 3), whereas lenalidomide did not affect this upregulation of CD80 and CD86 induced by the TLR ligands.

### 3.3. Lenalidomide potently suppresses production of IL-12p70 and IL-23, but enhances that of IL-10 by CD1c$^+$ DCs

We next explored whether lenalidomide affects the production of 3 key cytokines, IL-12p70, IL-23, and IL-10 by CD1c$^+$ DCs stimulated with the TLR ligands. Lenalidomide potently suppressed the production of IL-12p70 and IL-23, whereas it reciprocally enhanced the production of IL-10 at as low as 100 nM, which is only 6% of the maximal concentration in plasma after administration of 25 mg of lenalidomide [30] (Fig. 1).

It has been reported that IL-10 suppresses the production of IL-12p70 by DCs [31]. Thus, we added a neutralizing anti-IL-10 mAb to evaluate whether the increased production of endogenous IL-10 is responsible for the decreased production of IL-12p70 by lenalidomide-treated CD1c$^+$ DCs. Anti-IL-10 mAb partially abrogated the suppressive effects of lenalidomide on IL-12p70 production (Fig. 2). This indicates that the increased production of endogenous IL-10 reduces the production of IL-12p70.

We also examined whether lenalidomide affects the production of IL-12p70, IL-23, and IL-10 by MoDCs. Lenalidomide suppressed the production of IL-12p70 and IL-23 whereas enhanced that of IL-10 by MoDCs, although to a lesser extent for CD1c$^+$ DCs (Supplementary Fig. 4).

### 3.4. Lenalidomide suppresses production of IFN-α by CD141$^+$ DCs but not by pDCs

Type I IFNs are key cytokines that modulate a broad array of immune responses [22]. CD141$^+$ DCs and pDCs potently produce type I IFNs in response to nucleic acids [19–21]. Thus, we tested whether lenalidomide affects the production of IFN-α by CD141$^+$ DCs and pDCs stimulated with specific TLR ligands. Lenalidomide suppressed IFN-α production by CD141$^+$ DCs but not by pDCs (Fig. 3). Taken together, lenalidomide suppressed the production of immunostimulatory cytokines by myeloid-derived DCs (CD1c$^+$ DCs and CD141$^+$ DCs). In contrast, lenalidomide did not suppress the production of IFN-α by pDCs.

### 3.5. Lenalidomide does not suppress the signaling pathways leading to nuclear translocation of NF-κBp65 and IRF5 or expression of c-MAF

Activation of the transcription factors NF-κB [32] and IRF5 [33] is crucial for production of proinflammatory cytokines. c-MAF is an important transcription factor for IL-10 production by myeloid cells [34]. Thus, we investigated whether the activity or expression of these transcription factors in CD1c$^+$ DCs is affected by lenalidomide. The TLR ligands induced nuclear translocation of NF-κBp65 and IRF5, but it was not abrogated by lenalidomide (Fig. 4). Furthermore, lenalidomide did not alter the expression level of c-MAF mRNA (Supplementary Fig. 5). These data suggest that lenalidomide modulates the production of IL-12, IL-23, and IL-10 independently of the nuclear translocation of NF-κB and IRF5 and expression of c-MAF.

### 3.6. Treatment of CD1c$^+$ DCs with lenalidomide reduces the differentiation of naïve CD4$^+$ T cells to helper T cells producing proinflammatory cytokines

Inducing naïve CD4$^+$ T cells to acquire the ability to secrete cytokines is the major task of DCs [35]. Thus, we explored the cytokine-producing properties of naïve CD4$^+$ T cells stimulated with allogeneic CD1c$^+$ DCs treated with lenalidomide. We treated DCs and T cells with lenalidomide during one or both of the 2 culture periods: during stimulation of DCs with a TLR ligand (R848) and/or during co-culture of DCs and T cells (Fig. 5A). We then measured the secretion of cytokines (IL-2, IFN-γ, IL-17, IL-21, and IL-10) by T cells. When lenalidomide was added during the DC-T cell co-culture, T cells acquired the ability to secrete higher levels of all cytokines compared with culturing without lenalidomide (Fig. 5B). This is consistent with the previous reports that

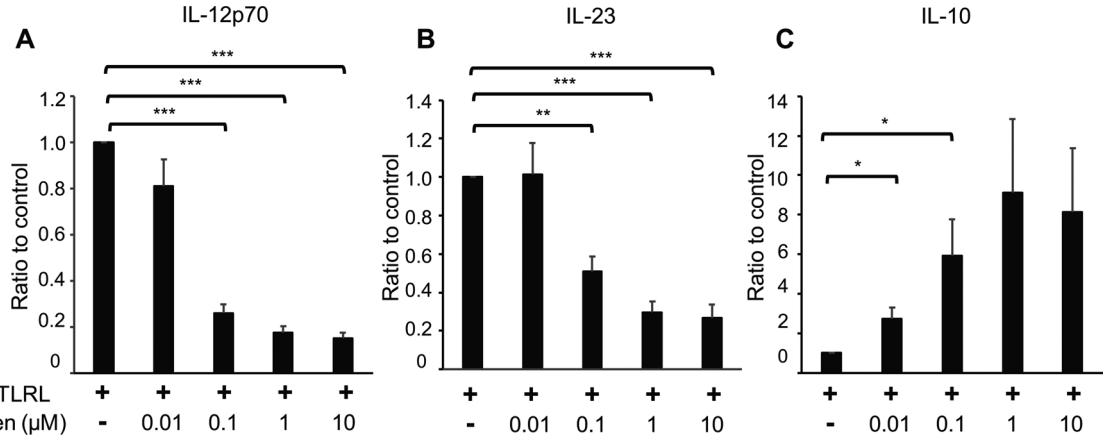


**Fig. 1.** Lenalidomide affects cytokine production by CD1c$^+$ DCs stimulated with TLR ligands.
CD1c$^+$ DCs were stimulated with GM-CSF, IFN-γ, LPS, R848, and poly I:C (TLR ligands [TLRL]) in the absence or presence of the indicated concentrations of lenalidomide (Len) for 24 h. The concentrations of (A) IL-12p70, (B) IL-23, and (C) IL-10 in the supernatants were measured in duplicate by ELISA. As the absolute concentrations varied depending on the donors, the values were normalized to those obtained without lenalidomide. The data are shown as means ± SE of 6 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$. The means and ranges of absolute cytokine concentrations from DCs cultured without lenalidomide were as follows: IL-12p70, 12.3 ng/mL (4.6–19.7 ng/mL); IL-23, 7.4 ng/mL (2.4–16.8 ng/mL); IL-10, 2061 pg/mL (117–5093 pg/mL).

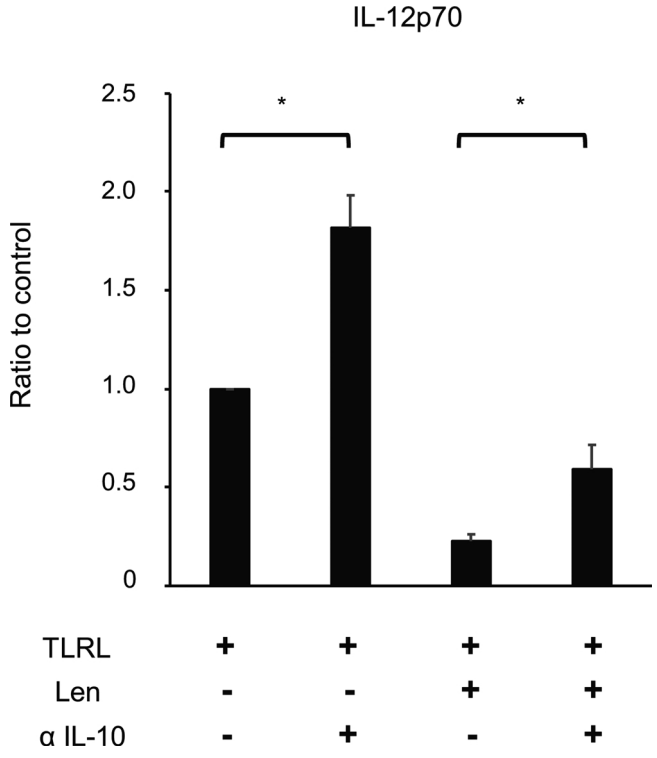


**Fig. 2.** Neutralizing anti-IL-10 mAb increases production of IL-12p70 by CD1c$^+$ DCs.
The cells were stimulated with GM-CSF, IFN-γ, LPS, R848, and poly I:C in the absence or presence of lenalidomide and anti-IL-10 mAb for 24 h. The concentration of IL-12p70 in the supernatants was measured in duplicate by ELISA. The values were normalized to those obtained without lenalidomide and anti-IL-10 mAb. The data are shown as means ± SE of 4 independent experiments. *$P < .05$. The mean and range of absolute concentrations of IL-12p70 from DCs cultured with TLR ligands alone were 41.6 ng/mL (4.8–20.0 ng/mL).

lenalidomide enhances the production of immunostimulatory cytokines by T cells [3]. However, treatment of DCs with lenalidomide during TLR stimulation reduced the capacity of DCs to induce cytokine-producing T cells irrespective of the presence of lenalidomide during the DC-T cell co-culture. These data demonstrate that lenalidomide reduces the ability of CD1c$^+$ DCs to induce proinflammatory CD4$^+$ T cell responses.

## 4. Discussion

In this study, we examined the influences of lenalidomide on the production of key immunomodulatory cytokines IL-12p70, IL-23, IL-10, and IFN-α by different human DC subsets. We found that lenalidomide suppressed the production of immunostimulatory cytokines by human cDCs but not by pDCs. This is the first report of the immunosuppressive effects of lenalidomide on human primary DC subsets isolated from blood.

Human DC subsets have distinctive and overlapping functions. Although CD141$^+$ DCs were reported to have superior antigen cross-presenting capacity [36], more recent studies have reported that CD1c$^+$ DCs and CD141$^+$ DCs have similar cross-presenting capacity upon stimulation with specific combinations of TLR ligands [28,37]. CD1c$^+$ DCs produce more IL-12p70 than CD141$^+$ DCs upon appropriate stimulation [28,38]. In contrast, CD141$^+$ DCs but not CD1c$^+$ DCs produce IFN-α in response to the TLR3 ligand poly I:C [20,21]. pDCs produce high levels of IFN-α upon ligation of TLR7 or TLR9 [19]. In vitro-derived MoDCs, widely used as a human DC model, are distinct from blood cDCs and pDCs in the steady state of their gene expression profiles [13]. Although MoDCs were proposed to correspond to DCs observed in inflammatory tissues [14], an *in vivo* counterpart of MoDCs is still unclear. Given this heterogeneity of human DCs and physiological relevance of blood cDCs and pDCs, we focused on cDCs and pDCs isolated from blood to clarify the influences of lenalidomide on human DCs.

Lenalidomide did not reduce the expression of costimulatory molecules (CD80 and CD86) by CD1c$^+$ DCs, but it markedly suppressed the production of key proinflammatory cytokines (IL-12 and IL-23), and enhanced the production of an anti-inflammatory cytokine (IL-10) by CD1c$^+$ DCs, and to a lesser extent, by MoDCs. Lenalidomide significantly affected the cytokine production by CD1c$^+$ DCs at clinically relevant concentrations (as low as approximately 6% of the maximal concentration in plasma after administration of 25 mg of lenalidomide) [30]. Lenalidomide also suppressed the production of IFN-α by CD141$^+$ DCs but not by pDCs. Thus, human DC subsets exhibit differential sensitivity to lenalidomide. The resistance of pDCs to lenalidomide may be beneficial for maintaining antiviral immunity during therapy.

The neutralizing anti-IL-10 mAb increased the IL-12 production by CD1c$^+$ DCs, consistent with the previous report that IL-10 suppresses IL-12 production by DCs in an autocrine manner [31]. This suggests

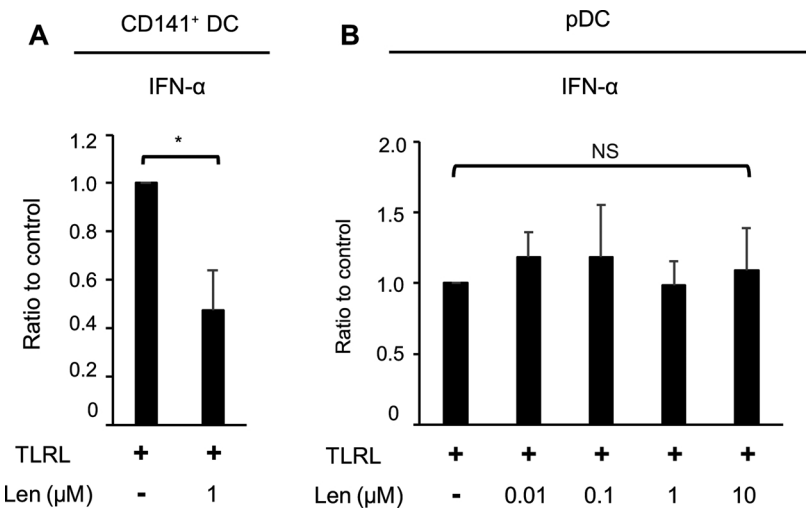


**Fig. 3.** Lenalidomide decreases production of IFN-α by CD141$^+$ DCs but not by pDCs.
(A) CD141$^+$ DCs were stimulated with GM-CSF, IFN-γ, R848, and poly I:C, and (B) pDCs were stimulated with ODN2216 for 24 h in the absence or presence of lenalidomide. The concentration of cytokines in the supernatants was measured in duplicate by ELISA. The values were normalized to those obtained without lenalidomide. The data are shown as means ± SE of 5 (A) and 6 (B) independent experiments. *$P$ < .05. NS: not significant. The means and ranges of absolute concentrations of IFN-α from DCs cultured with TLR ligands alone were (A) 805 pg/mL (52–4883 pg/mL) and (B) 32.2 ng/mL (11.4–59.7 ng/mL).

that the enhancement of IL-10 production is partly responsible for the suppression of IL-12 production by lenalidomide-treated CD1c$^+$ DCs. However, the degree of direct and IL-10-mediated indirect effects of lenalidomide on the suppression of IL-12 production remains unclear.

Transcription factors, such as NF-κB [32] and IRF5 [33], induce proinflammatory cytokines, including IL-12, whereas c-MAF plays an important role in inducing IL-10 [34]. However, we were unable to identify transcription factors targeted by lenalidomide. In addition to the inhibitory κB kinase (IKK)-NF-κB pathway, several signaling pathways, including the mitogen-activated protein kinase (MAPK) [39], phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR), and Akt-glycogen synthase kinase 3 (GSK3) pathways [40–44], regulate the IL-12 and IL-10 production by DCs stimulated with TLR ligands. Although we also analyzed the activation status of the signaling pathways by evaluating the degree of phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK1/2), p38, JUN N-terminal kinase (JNK), Akt, mTOR complex 1 (mTORC1) substrates (p70 S6 kinase [p70S6K] and 4E-binding protein 1 [4E-BP1]), and GSK3β by flow cytometry, lenalidomide did not suppress or augment phosphorylation of these molecules induced by the TLR ligands (unpublished data). Thus, lenalidomide may epigenetically regulate the transactivation of cytokine genes after nuclear translocation of the transcription factors. In this line, it has recently been reported that the histone demethylase Jmjd2d increases transactivation of IL-12p35 and p40 genes by promoting recruitment of NF-κB to their promoters, and that the expression of Jmjd2d protein is stabilized by the deubiquitinase Trabid [45]. However, we did not observe a decrease in the amount of Jmjd2d protein in lenalidomide-treated monocytes (unpublished data). It has also been reported that lenalidomide induces dissociation of a guanine nucleotide exchange factor, Rabex-5, from cereblon, resulting in suppression of LPS-induced proinflammatory cytokine production by the human monocytic leukemia cell line THP-1 [46]. Whether this pathway is involved in the cytokine modulation in DCs by lenalidomide remains to be determined. Molecular mechanisms by which lenalidomide affects cytokine production by DCs require further investigation.

The most important task of DCs is to activate naïve T cells and to provide them with effector functions. Consistent with the reduced IL-12 and IL-23 production by CD1c$^+$ DCs, pretreatment of DCs with lenalidomide reduced their ability to induce differentiation of naïve CD4$^+$ T cells to Th1 (IFN-γ), Th17 (IL-17), and follicular helper T (IL-21) cytokine-producing cells. IL-10 production by T cells was also reduced in lenalidomide-pretreated DCs, likely due to the reduction of IL-12, which promotes not only IFN-γ but also IL-10 production by T cells [47]. IL-17 was previously found to promote myeloma cell growth [48]

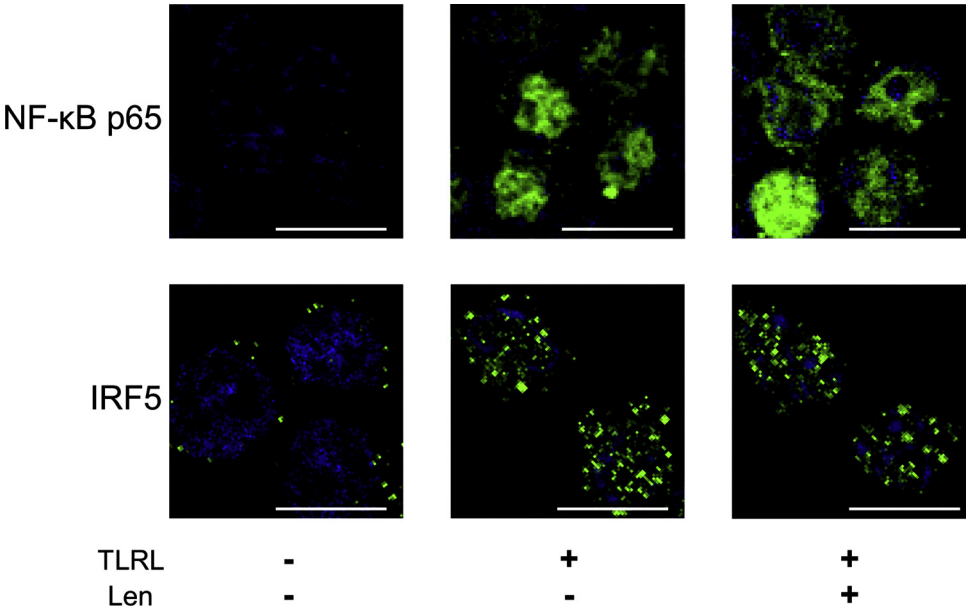


**Fig. 4.** Lenalidomide does not inhibit the nuclear translocation of NF-κBp65 and IRF5 in CD1c$^+$ DCs.
Cells were stimulated with GM-CSF, IFN-γ, LPS, R848, and poly I:C in the absence or presence of lenalidomide for 1 h, and then stained with rabbit anti-NF-κBp65 (top) or anti-IRF5 (bottom, both green) mAb. Nuclei were identified using DAPI (blue). The data shown are representative of 3 independent experiments. Scale bars, 10 μm.

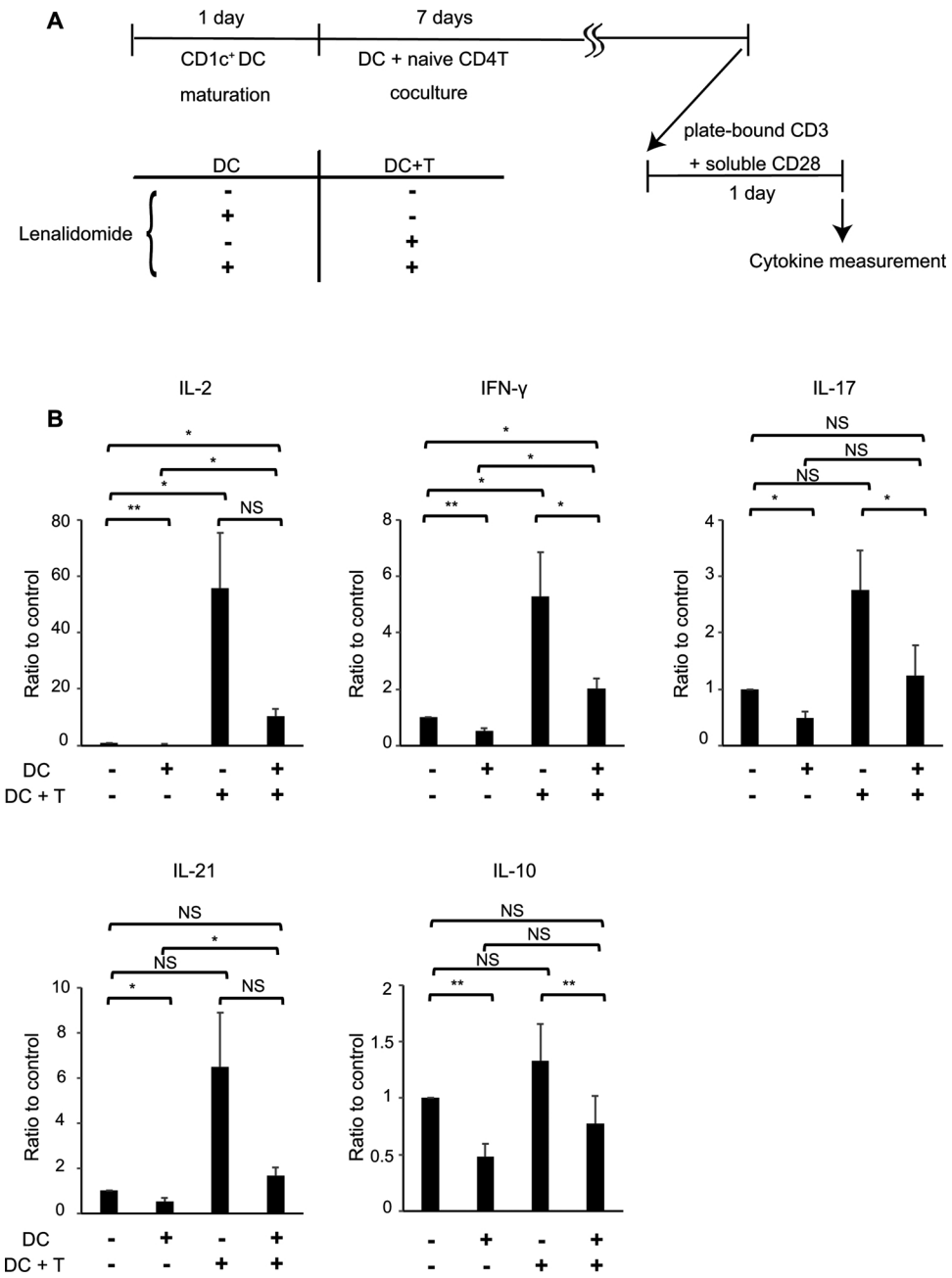


**Fig. 5.** Lenalidomide downregulates the capacity of CD1c$^+$ DCs to induce cytokine production from CD4$^+$ T cells.
(A) Schema of culture schedule. CD1c$^+$ DCs were stimulated with GM-CSF, IFN-γ, and R848 in the absence or presence of lenalidomide for 24 h. Then, allogeneic naïve CD4$^+$ T cells were co-cultured with CD1c$^+$ DCs in the absence or presence of lenalidomide for 7 d. (B) CD4$^+$ T cells co-cultured with DCs were re-stimulated for 24 h, and the supernatants were analyzed for cytokines by ELISA. There were 4 culture conditions based on the absence or presence of lenalidomide during DC activation and DC-T cell co-culture as depicted in (A). The values are normalized to those obtained from CD4$^+$ T cells co-cultured with lenalidomide-untreated DCs in the absence of lenalidomide. The data are shown as the mean ± SE of 7 (IFN-γ, IL-10), 6 (IL-2, IL-21), and 5 (IL-17) independent experiments. *$P$ < .05; **$P$ < .01. NS: not significant. The means and ranges of absolute cytokine concentrations from T cells cocultured with lenalidomide-untreated DCs in the absence of lenalidomide were as follows: IL-2, 731 pg/mL (21.5–1228 pg/mL); IFN-γ, 124 ng/mL (5.0–340 ng/mL); IL-17, 1003 pg/mL (299–2647 pg/mL); IL-21, 297 pg/mL (32.1–988 pg/mL); IL-10, 6.6 ng/mL (1.6–13.2 ng/mL).

and bone destruction by activating osteoclasts [49]. IL-21 promotes proliferation and survival of myeloma cells [50]. Therefore, lenalidomide may alleviate myeloma pathology by attenuating IL-17 and IL-21 production by T cells through acting on DCs. However, direct action of lenalidomide on T cells strongly enhanced the production of IL-2, IFN-γ, IL-17, and IL-21, as observed in culture conditions where lenalidomide was present during DC-T cell co-culture. Such direct effects of lenalidomide on T cells are in agreement with the report that lenalidomide enhances IL-2 production by T cells through degradation of ikaros family zinc finger protein 1 (IKZF1) and IKZF3 [6], likely augmenting antitumor immunity.

IMiDs were originally developed as anti-inflammatory drugs that inhibit production of TNF-α by LPS-stimulated monocytes [51,52], and may thus be a treatment option for autoimmune/inflammatory disorders such as rheumatoid arthritis, cutaneous lupus, and inflammatory bowel disease [53–55]. The suppression of proinflammatory cytokine production by DCs may be instrumental in mitigating inflammation in such diseases by lenalidomide.

## 5. Conclusions

Lenalidomide potently suppressed the production of key proinflammatory cytokines IL-12, IL-23, and IFN-α, and reciprocally enhanced the production of the anti-inflammatory cytokine IL-10 by human myeloid-derived DC subsets, CD1c$^+$ DCs and CD141$^+$ DCs. Accordingly, lenalidomide-treated CD1c$^+$ DCs had decreased production of inflammatory and myeloma-promoting cytokines by CD4$^+$ T cells. These findings may partly explain the contradictory anti-inflammatory and anti-myeloma effects of IMiDs, as well as shed new light on the roles of lenalidomide in treatment of myeloma and inflammatory disorders.

## Conflicts of interest

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.05.012.

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