Allergology International. 2010;59:★★★-★★★
DOI: 10.2332/allergolint.09-OA-0103

ORIGINAL ARTICLE

Curdlan Induces DC-Mediated Th17 Polarization via Jagged1 Activation in Human Dendritic Cells

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ABSTRACT

Background: Th17-inducing activity is carried by certain polysaccharides such as β-glucan derived from *Candia albicans*. Our previous studies have shown that Th1- and Th2-inducing activities can be qualitatively evaluated by the expression patterns of Notch ligand isoforms, using human monocyte-derived dendritic cells (Mo-DCs) and some leukemic cell lines such as THP-1. The association of Th17-inducing activities with Notch ligand expression patterns has been unclear.
Methods: Mo-DCs from healthy volunteers were co-cultured with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce a mixed lymphocyte reaction, in the presence of adjuvants, such as curdlan. Culture supernatants were assayed for IFNγ, IL-5 and IL-17 by an enzyme-linked immunosorbent assay (ELISA). Notch ligand expression on Mo-DCs and THP-1 cells was evaluated by using RT-PCR.
Results: The present study shows that curdlan, one of the β-glucans, has the ability to induce DC-mediated Th17 differentiation. It is also interesting to note that Jagged1 mRNA in Mo-DCs and THP-1 cells is up-regulated by curdlan. Furthermore, polyclonal anti-Jagged1 antibody inhibited such DC-mediated Th17 differentiation.
Conclusions: This study suggests that curdlan induces human DC-mediated Th17 polarization via Jagged1 activation in DCs.

KEY WORDS

adjuvant, dendritic cells, Notch ligand, Th17, β-gulcan

INTRODUCTION

The different classes of specific immune responses are driven by the biased development of antigen-specific effector $CD4^+$ T-cell subsets such as Th1, Th2 and Th17 cells that activate different components of cellular and humoral immunity. The Th17 lineage characteristically produces high levels of IL-17, and it represents a significant revision of the Th1-Th2 paradigm.[1,2] Moreover, Th17 cells have been reported to play not only critical roles in the immune responses to extracellular microorganisms, such as fungi, but also a pathogenetic role in autoimmunity.[1,3-6] Dendritic cells (DCs) play a pivotal role in the differentiation of naïve CD4 T helper cells towards Th1 or Th2 cells. Environmental molecules, such as LPS, certain nucleic acids and fungus-derived glycoprotein molecules alter the DC function and thereby induce Th1 differentiation.[7] Because such DCs induce Th1 responses, they are designated DC1, and DC1-inducing molecules are called Th1 adjuvants. DC2 and Th2 adjuvants have also been reported. DCs matured in the presence of forskolin or prostaglandin E2 (PGE2) induce the differentiation of naïve CD4 T cells towards Th2.[8]

Notch signaling pathways are highly conserved in organisms ranging from invertebrates to mammals and they are involved in cell fate choice during development.[9] A correlation has also been observed between the Notch ligand mRNA levels in monocyte-

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Received 9 March 2009. Accepted for publication 3 September 2009.
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derived DCs (Mo-DCs) and leukemic APCs with Th1/Th2 adjuvant activities.[10] Increased expression of Delta1 and Delta4 mRNA on DCs can predict Th2 and Th1 adjuvant activities, respectively. Indeed, in other studies using mouse models, Notch directly regulates Gata-3 expression during Th2 differentiation.[11,12]

The present study used a mixed lymphocyte reaction (MLR) between human monocyte-derived DCs and allogeneic naïve $CD4^+$ T cells to show that curdlan, one of the β-glucans, has the ability to induce DC-mediated Th17 differentiation. Jagged1 mRNA is up-regulated by curdlan. Moreover, polyclonal anti-Jagged1 antibody (Ab) inhibited such DC-mediated Th17 differentiation.

METHODS

PREPARATION OF HUMAN Mo-DCs, T LYMPHOCYTES AND PMA-DERIVED THP-1

This study using peripheral blood of healthy volunteers was approved by the Saitama Medical University Ethics Committee. Human $CD14^+$ cells were isolated from adult blood buffy coat specimens from healthy volunteer blood donors after the separation of peripheral blood mononuclear cells (PBMC) by Ficoll-Paque (GE Healthcare, Sweden) centrifugation and positive selection using CD14 MicroBeads (Miltenyi Biotec, Germany). These cells were further depleted using a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) and separated into $CD45RO^+$ memory T cells and $CD45RA^+$ naïve T cells using CD45RO MicroBeads (Miltenyi Biotec). A FACScan flow cytometer (Becton Dickinson, USA) showed the purity of these cells to be more than 99%. The $CD14^+$ cells were suspended in an RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 ng/ml of IL-4 (Primmune, Japan) and GM-CSF (Peprotech, USA) and then were cultured at 37℃ in a humidified atmosphere with 5% CO_2. After 5 days, the cells were harvested as immature Mo-DCs. To prepare THP-1-devided macrophage-like cells, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) as previously reported.[13]

DC-MEDIATED T CELL DIFFERENTIATION ASSAY

After washing, immature Mo-DCs were stimulated with curdlan purchased from Wako, Japan. In some experiments, immature Mo-DCs were pretreated with goat anti-human Jagged-1 Ab or goat IgG as a control for 1 hour before curdlan stimulation. Two days after the incubation with curdlan, cellular components were further co-cultured with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce an MLR in RPMI 1640 medium supplemented with 10% human serum for 6-8 days. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs (BD Pharmingen, USA). Culture supernatants were harvested after 48 hours to be assayed for IFNγ, IL-5 and IL-17 by an enzyme-linked immunosorbent assay (ELISA) using IFNγ, IL-5 and IL-17 ELISA kits (R&D systems, USA).

STIMULATION OF APCs WITH ADJUVANTS

Mo-DCs were stimulated with curdlan, and PMA-treated THP-1 cells were stimulated with curdlan, laminarin (SIGMA, Japan) or zymosan (SIGMA) for 4 hours. Thereafter, the total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, USA).

RT-PCR ANALYSIS OF NOTCH LIGAND GENE EXPRESSION

RNA was extracted using an RNeasy Mini kit (Qiagen, Germany), first-strand cDNA synthesis was performed using Omniscript reverse transcription for polymerase chain reaction (RT-PCR; Qiagen), and cDNA was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, USA). Primers of Notch ligand genes and β-actin[10] were synthesized as follows: Jagged1 sense 5'-AGTCACTGGCACGGTTG TAG-3', Jagged1 antisense 5'-TCGCTGTATCTGTCC ACCTG-3', Jagged2 sense 5'-GATTGGCGGCTATTA CTGTG-3', Jagged2 antisense 5'-AGGCAGTCGTCAA TGTTCTC-3', Delta1 sense 5'-AGACGGAGACCATG AACAAC-3' Delta1 antisense 5'-AGATGCTTCTCCCA CCCCTGA-3', Delta3 sense 5'-GTGAATGCCGATGC CTAGAG-3' Delta3 antisense 5'-GGTCCATCTGCAC ATGTCAC-3', Delta4 sense 5'-TGACCACTTCGGCCA CTATG-3', Delta4 antisense 5'-AGTTGGAGCCGGTG AAGTTG-3', β-actin sense 5'-CATCACCATTGGCAAT GAGC-3', β-actin sense 5'-CGATCCACACGGAGTAC TTG-3'.

Each PCR product was separated by electrophoresis on a 1.5% agarose gel, analyzed using the densitometer (BIO-RAD Laboratories, Japan) and the Jagged1/β-actin ratio was calculated. In some experiments, quantitative real time PCR was performed using Taqman gene expression systems (Applied Biosystems), a Jagged1 probe (ID: Hs01070036_m1) and a β-actin probe purchased from Applied Biosystems.

STATISTICAL ANALYSIS

Comparisons between sets of two groups were performed using Student's two-tailed *t*-test, while sets of more than two groups were compared by ANOVA.

RESULTS

DC-MEDIATED Th17 POLARIZATION BY CURDLAN

The cytokine profiles in the MLR induced by co-culture were determined in allogeneic $CD45RA^+$ naïve T cells and Mo-DCs stimulated with curdlan to test the induction of Th17 differentiation by human Mo-DCs stimulated with curdlan. As expected, 0.1-100 ng/ml curdlan led to IL-17 production. Stimulation at 1 ng/ml curdlan most markedly induced IL-17

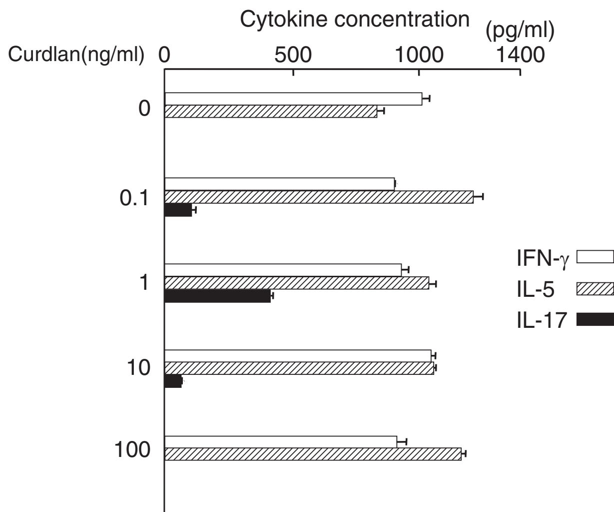


Fig. 1 DC-mediated Th17 polarization by curdlan. The $CD14^{+}$ cells were stimulated with IL-4 and GM-CSF for 5 days and the cells were harvested as immature Mo-DCs. After washing, immature Mo-DCs were incubated with 0-100 ng/ml of curdlan. Two days after the incubation with curdlan, cellular components were further co-cultured for 7 days with HLA-DR-nonshared allogeneic $CD4^{+}$ naïve T cells to induce an MLR. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs. The culture supernatants were harvested after 48 hours to be assayed for IFNγ, IL-5 and IL-17 by an ELISA.

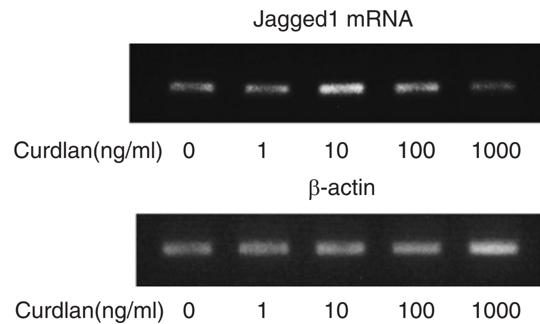


Fig. 2 The expression patterns of Notch ligand mRNA in curdlan-stimulated Mo-DCs. Mo-DCs were stimulated with curdlan for 4 hours. After stimulation, the expressions of Jagged1 mRNA and β-actin were evaluated by RT-PCR. The primers were described in "Methods". PCR products were separated by electrophoresis on a 1.5% agarose gel.

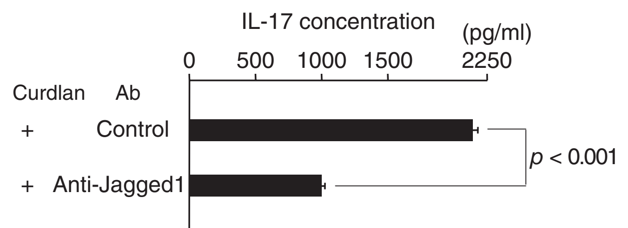


Fig. 3 Inhibition of DC-mediated Th17 polarization by polyclonal anti-Jagged1 Ab. Immature Mo-DCs were treated with 1 μg/ml of polyclonal anti-Jagged1 Ab or a control for 1 hour before curdlan stimulation (1 ng/ml). Two days after the incubation with curdlan, cellular components were further co-cultured with HLA-DR-nonshared allogeneic $CD4^{+}$ naïve T cells to induce an MLR. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs. The culture supernatants were harvested after 48 hours to be assayed for IL-17 by an ELISA.

production (Fig. 1). In contrast, curdlan had no effect on IFN-γ or IL-5 production.

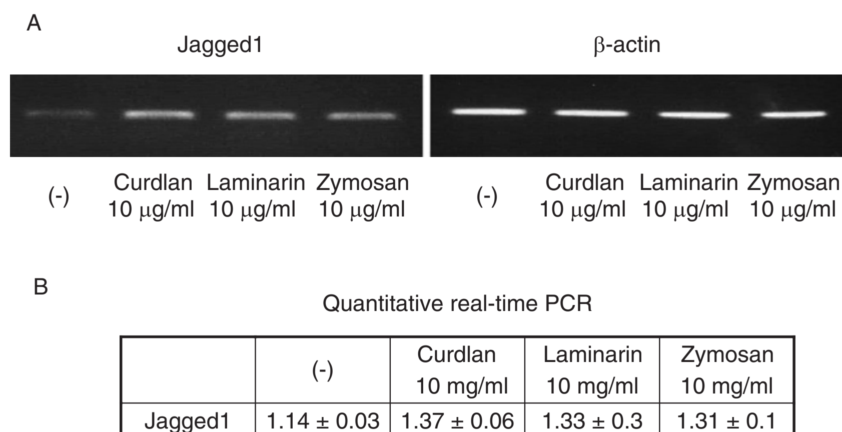
INCREASED JAGGED1 mRNA LEVEL IN Mo-DCs WAS INDUCED BY CURDLAN-STIMULATION IN THE Mo-DCs

Next, the expression patterns of Notch ligand mRNA were examined in curdlan-stimulated Mo-DCs, using RT-PCR. Figure 2 shows that 10-100 ng/ml curdlan induced increased Jagged1 mRNA in Mo-DCs. None of the other Notch ligands, i.e., Delta1, Delta3, Delta4 or Jagged2 mRNA were below the limit of detection in our PCR systems (data not shown). Experiments were repeated six times and the stimulation with 10 ng/ml, but not 100 ng/ml curdlan reproducibly upregulated Jagged1 mRNA in Mo-DCs. The Jagged1/β-actin ratio ranged from 1.3 to 1.42 ($p < 0.05$). The expression was also examined at the protein level by Western blotting and/or flow cytometry. However, antibodies capable of specifically detecting human Jagged1 without cross reactivity to other ligands are not commercially available at present.

POLYCLONAL ANTI-JAGGED1 AB INHIBITED Th17 DIFFERENTIATION

The inhibitory effect of polyclonal anti-Jagged1 antibody was examined to confirm that Jagged1 has a functional role in curdlan-mediated Th17 differentiation. Mo-DCs were incubated with anti-Jagged1 Ab for 1 hour before the addition of curdlan to the culture, followed by MLR as described in Figure 1. As shown in Figure 3, IL-17 production was significantly inhibited by anti-Jagged1 Ab in comparison to a control ($p < 0.001$).

Experiments were reproducibly repeated three times. We also observed a slight increase of IFNγ and a slight decrease of IL-5, by using anti-Jagged1 Ab (not shown), which will be discussed later in the discussion section. MLR is caused by co-culturing Mo-DCs with polyclonal $CD4^{+}$ naïve T cells. The clonal frequency of alloreactive T cells is only 10^{-4} to 10^{-3}, which resulted in very high background signals for transcriptional factors caused by non-reactive T cells. It was thereby difficult to show whether stimulating



	(-)	Curdlan 10 mg/ml	Laminarin 10 mg/ml	Zymosan 10 mg/ml
Jagged1	1.14 ± 0.03	1.37 ± 0.06	1.33 ± 0.3	1.31 ± 0.1

Fig. 4 The expression patterns of Notch ligand mRNA in the curdlan-stimulated human leukemic APC line, THP-1. THP-1 cells were incubated with PMA for 2 days to induce macrophage-like cells. PMA-derived THP-1 cells were stimulated with curdlan, laminarin or zymosan for 4 hours. **A.** After stimulation, expression of Jagged1 mRNA and β-actin were evaluated by RT-PCR. The primers were described in "Methods". PCR products were separated by electrophoresis on a 1.5% agarose gel. **B.** Expression of Jagged1 mRNA and β-actin were evaluated by quantitative real-time PCR. The probes were described in "Methods".

Notch directly regulates GATA, RORγt or Foxp3 by RT-PCR in this MLR system.

INCREASED JAGGED1 mRNA LEVEL WAS INDUCED BY *β*-GLUCAN-STIMULATION IN PMA-DERIVED THP-1

Previous studies showed that Delta1/Delta4 gene expression patterns in human leukemic APCs have a potential to evaluate Th1/Th2 adjuvant activities.[10] We therefore observed all Notch ligand isoforms in APC lines stimulated with β-glucans. As shown in Figure 4A, β-glucans, such as curdlan, zymosan and laminarin induced increased Jagged1 mRNA in PMA-derived THP-1 cells. Experiments were repeated six times and the stimulation with 100 μg/ml β-glucans reproducibly up-regulated Jagged1 mRNA in Mo-DCs. The Jagged1/β-actin ratio ranged from 1.4 to 1.5 ($p < 0.01$). Other Notch ligands exhibited no change in their expression (data not shown). Next, we observed Jagged1 mRNA in PMA-derived THP-1 cells using quantitative real-time PCR studies. As shown in Figure 4B, the stimulation with 10 μg/ml curdlan, zymosan or laminalin up-regulated Jagged1 mRNA in PMA-derived THP-1 cells ($p < 0.01$).

DISCUSSION

The present study used a mixed lymphocyte reaction (MLR) between human monocyte-derived DCs and allogeneic naïve $CD4^+$ T cells to show that curdlan, one of β-glucans, has the ability to induce DC-mediated Th17 differentiation. Jagged1 mRNA is up-regulated by curdlan. Moreover, polyclonal anti-Jagged1 antibody inhibited this DC-mediated Th17 differentiation. These findings suggest that curdlan induces human DC-mediated Th17 polarization via Jagged1 activation in DCs. Th17 responses could be observed by using human PBMC stimulated with various exogenous antigens, such as *Candida albicans*[6,14] and purified protein derivative. This observation raises the possibility that particular exogenous crude antigens stimulate the differentiation of naïve CD4 T cells. Specific adjuvant activities have been extensively described, for Th1 and Th2 responses, which are often associated with maturation and differentiation of DCs towards DC1/DC2.[15] Zymosan, one of the β-glucans, binds to dectin-1, and leads to the activation of Syk followed by the production of IL-23 from APC and IL-17 from T cells.[16] In the present study, not only polyclonal anti-Jagged1 Ab (Fig. 3) but also polyclonal anti-dectin-1 Ab inhibited Mo-DCs-mediated Th17 polarization by curdlan (data not shown). These findings collectively suggest that a consecutive signaling pathway, including β-glucan, dectin-1 and Jagged1, plays an important role in Mo-DCs maturation, thus resulting in Th17 differentiation via the Jagged1-Notch pathway. β-glucans, such as curdlan, zymosan and laminarin, can also trigger severe chronic arthritis in SKG mice, which is one of Th17-mediated disease models.[17,18] Laminarin and zymosan have been tested as well, and we successfully proved that they also carry the Th17-inducing and Jagged1-inducing activities (data not shown). We also observed a slight increase of IFNγ accompanied by a slight decrease of IL-5, by using anti-Jagged1 Ab. This might be caused by signaling mediators, such as cAMP, which is shared by Th2 and Th17 differentia-

tion pathways as shown in our previous studies.[19-21]

A correlation has been reported between the Notch ligand mRNA levels of Mo-DCs and leukemic APCs with Th1/Th2 adjuvant activities.[10] An increased expression of Delta1 and Delta4 mRNA in DCs can predict the Th2 and Th1 adjuvant activities, respectively. In this study, curdlan increased expression of Jagged1 mRNA level but not the other Notch ligands. The same results were obtained from analysis using human leukemic APCs, THP-1, as reported in previous Th1/Th2 adjuvant studies.[13] Although an increased expression of Jagged1 mRNA was most efficiently induced by 10 ng/ml curdlan in Mo-DCs, 10 μg/ml of curdlan was needed in PMA-stimulated THP-1 cells. This discrepancy may be attributable to the fact that PMA-stimulated THP-1 cells are not physiological DCs. Our results suggested that increased expression of Jagged1 mRNA in Mo-DCs or leukemic APCs can predict Th17 adjuvant activity. Furthermore, polyclonal anti-Jagged1 Ab inhibited Mo-DCs-mediated Th17 polarization by curdlan. Indeed, in other studies using mouse models, Notch directly regulates the Gata-3 expression during Th2 differentiation.[11,12] These data collectively suggest that Jagged1 is not only a marker for DC17 but also a functional molecule for the induction of Th17 differentiation.

The detection of Th17 adjuvant activities has long been dependent on an assay for the cytokine profiles induced on T cells by co-culturing with APCs stimulated with adjuvants. This assay requires a large amount of labor and more than 2 weeks due to the preparation of cells from healthy donors and a large number of culture processes. In addition, the heterogeneities of cell sources and donor-to-donor variances may also lead to difficulties in obtaining stable and reproducible results. The present study demonstrated, for the first time, that increased Jagged1 mRNA in PMA-derived THP-1 cells have the potential to scrutinize and evaluate Th17 adjuvant candidates out of a large number of environmental substances and natural products, without possible instability arising from cell sources and polymorphisms. It is also interesting to note that this assay system can be used for the evaluation of live microorganisms, because 4-hour incubation with live probiotic bacteria or *Candida albicans* did not decrease the viability of the THP-1 cells, while successfully inducing Delta1/Delta4 and Jagged1, respectively (data not shown).[10] We are currently scrutinizing bacteria and fungi that carry Th17-inducing activity, which might be associated with infection-related autoimmunity.

ACKNOWLEDGEMENTS

This work was supported in part by a Saitama Medical University Internal Grant and a Research Grant-In-Aid for Scientific Research by the Ministry of Health, Labour and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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