Dectin-2-mediated initiation of immune responses caused by influenza virus hemagglutinin

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ABSTRACT

Antigen-presenting cells express pattern recognition receptors (PRRs), which sense pathogen-associated molecular patterns from microorganisms and lead to the induction of inflammatory responses. C-type lectin receptors (CLRs), the representative PRRs, bind to microbial polysaccharides, among which Dectin-2 and Mincle recognize mannose-containing polysaccharides. Because influenza virus (IFV) hemagglutinin (HA) is rich in mannose polysaccharides, Dectin-2 or Mincle may contribute to the recognition of HA. In this study, we addressed the possible involvement of Dectin-2 and Mincle in the viral recognition and the initiation of cytokine production. Interleukin (IL)-12p40 and IL-6 production by bone marrow–derived dendritic cells (BM-DCs) upon stimulation with HA was significantly reduced in Dectin-2 knockout (KO) mice compared to wild-type (WT) mice whereas there was no difference between WT mice and Mincle KO mice. BM-DCs that were treated with Syk inhibitor resulted in a significant reduction of cytokine production upon stimulation with HA. The treatment of BM-DCs with methyl-α-D-mannopyranoside (ManP) also led to a significant reduction in cytokine production by BM-DCs that were stimulated with HA, except for the A/H1N1pdm09 subtype. IL-12p40 and IL-6 synthesis by BM-DCs was completely diminished upon stimulation with HA treated with concanavalin A (ConA)-bound sepharose beads. Finally, GFP expression was detected in reporter cells that were transfected with the Dectin-2 gene, but not with the Mincle gene, when stimulated with HA derived from the A/H3N2 subtype. These data suggested that Dectin-2 may be a key molecule as the sensor for IFV to initiate the immune response and regulate the pathogenesis of IFV infection.

INTRODUCTION

Influenza infection is the most prevalent viral infection throughout the world. Worldwide, approximately 300,000 to 650,000 people die per year from
severe respiratory disease (World Health Organization 2018). Although an inactivated quadrivalent vaccine has become popular for preventing infection with influenza, this vaccine is insufficient for complete prevention (Osterholm et al. 2012). Elderly people over 65 years or individuals with underlying diseases are in the high risk groups and tend to develop severe disease such as secondary bacterial pneumonia or acute respiratory distress syndrome (ARDS). Although neuraminidase inhibitors are frequently used as the antiviral drugs for the treatment of influenza, these agents are not effective in suppressing severe influenza unless they are administered within 48 h after symptoms begin (Moscona et al. 2005). The effectiveness of anti-inflammmatory agents such as adrenocortical steroids has not been substantiated for treating patients with ARDS (Yu et al. 2008; Han et al. 2011). Thus, there are several concerns related to the protection against and treatment of influenza infection. Therefore, it is important to address the mechanism of host immune response against influenza infection for establishing effective measures to protect against severe pneumonia that is caused by this infection.

Antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, recognize pathogen-associated molecular patterns (PAMPs) for their activation, which include nucleic acids, peptides, polysaccharides, and lipids contained in microorganisms (Takeuchi et al. 2010). Intracellular self-molecules released from dead cells or damaged tissues, called damage-associated molecular pattern (DAMPs), are also important factors for inducing inflammatory responses. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs), sense these PAMPs or DAMPs, leading to the production of inflammatory cytokines and expression of co-stimulatory molecules by DCs and macrophages (Takeuchi et al. 2010; Amarant-Mendes et al. 2018). DCs rapidly produce pro-inflammatory cytokine IL-12p40 in response to viral PAMPs, and induce the activation of natural killer (NK) cells, leading to the lysis of virus-infected cells in the innate phase of influenza infection (Monteiro et al. 1998). The difference in serum IL-6 concentration is also known to be related to the severity in A/H1N1pdm09-infected patients in the early period of infection (Bradley-Stewart et al. 2013). Thus, these cytokines are of great importance in accelerating the host protective immune response against influenza infection.

The immune response to surface molecules of influenza virus (IFV) is not fully understood because analysis of molecular interactions between viral nucleic acid and TLRs is the main stream in the host defense against viral infection. Recently, CLRs that recognize carbohydrates have been focused upon in investigating the protective host defense mechanism against fungi. CLRs possess carbohydrate recognition domains (CRDs), which bind to polysaccharides in a calcium-dependent manner. Among CLRs, Dectin-1 recognizes fungal β-1,3-D-glucan and Dectin-2 senses high mannose polysaccharides (Shiokawa et al. 2017). Mincle (macrophage-inducible C-type lectin) binds to trehalose dimycolate (TDM), a cell wall component of Mycobacterium tuberculosis that is also called cord factor, which leads to the production of pro-inflammatory cytokines and chemokines (Ishikawa et al. 2009). Previous studies showed that C-type lectin domain family 5 member A (CLEC5A) interacts with dengue virus and that dendritic cell immunoreceptor (DCIR) recognizes human immunodeficiency virus (Chen et al. 2008; Lambert et al. 2008). Although IFV infects macrophages via DC-SIGN (CD209) in a sialic acid–independent manner (Londrigan et al. 2011), the role of CLRs in the host protective response to this virus remains to be elucidated. Recently, it has been reported that caspase recruitment domain–containing protein 9 (CARD9), a common adaptor molecule of CLRs, plays important roles in inducing an inflammatory response in the lungs of mice infected with various pathogenic microbes (Yamamoto et al. 2014; Uematsu et al. 2015). CARD9 transduces signals via immunoreceptor tyrosine-based activation motif (ITAM), followed by the phosphorylation of NF-κB and production of various cytokines (Hara et al. 2008). Because hemagglutinin (HA), a representative surface antigen of IFV, is rich in glycoproteins including high mannose polysaccharides on their surface (Deom et al. 1985), a CLR, such as Dectin-2 and Mincle, may be involved in the recognition of IFV particles via HA and the development of the pathogenic condition in this infection.

In the present study, we addressed the possible roles of CLRs, with a focus on Dectin-2 and Mincle, in the recognition of IFV via HA and the initiation of the inflammatory response that is caused by this infection. Here, we demonstrated that deficiency of Dectin-2, but not Mincle, led to the reduction of inflammatory cytokine production by bone marrow–derived DCs (BM-DCs) upon stimulation with HA from the type A and type B strains. We also found that inhibitors of spleen tyrosine kinase (Syk), an intracellular signal-transducing molecule, suppressed the synthesis of these cytokines by HA-stimulated
BM-DCs. In addition, we revealed that HA could possess a certain ligand of Dectin-2 as identified by ConA-bound molecules, which may have different abilities to stimulate BM-DCs among distinct IFV strains.

MATERIALS AND METHODS

Ethics statement. This study was performed in strict accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan, 2006. All experimental procedures involving animals followed the Regulation for Animal Experiments at Niigata University and Tohoku University and were approved by the Institutional Animal Care and Use Committee of Niigata University and the ethics committee of Tohoku University. All experiments were performed under anesthesia to minimize animal distress.

Mice. Dectin-1 gene-knockout (KO) mice, Dectin-2 KO mice, and Mince KO mice were established as described previously (Saijo et al. 2007, 2010; Yamasaki et al. 2009), and backcrossed to C57BL/6J mice for more than eight generations. Wild-type (WT) C57BL/6J mice that were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) were used as controls. Six- to eight-week-old male mice were used in the experiments. All mice were bred under specific pathogen-free conditions at the Department of Comparative and Experimental Medicine, Niigata University, and at the Animal Facility, Tohoku University Graduate School of Medicine.

Reagents. HA that was derived from IFV, including A/Singapore/GP1908/2015(IVR-180) (H1N1)pdm09, A/Singapore/INFIMH-16-0019/2016(IVR-186) (H3N2), B/Maryland/15/2016 (NYMC BX-69A) (Victoria lineage), and B/Phuket/3073/2013 (Yamagata lineage), were purchased from Denka Seiken Co., Ltd. (Niigata, Japan), and were prepared according to the manufacturer’s protocol as with the seasonal influenza HA vaccine in Japan. Briefly, the viruses were cultured with an embryonated egg and concentrated by density-gradient centrifugation. After the refinement, the HA fractions from condensed virions were extracted and stabilized. All HA was dialyzed in RPMI1640 medium three times and stored at −80°C until use. The purity of this dialyzed HA was approximately 80%. Lipopolysaccharide (LPS), α-mannan, and methyl-α-D-mannopyranoside (ManP) were purchased from Sigma Aldrich (St. Louis, MO, USA). Immunostimulatory oligodeoxynucleotide CpG1826, which is a TLR9 agonist and has a length of 20 nucleotides, was synthesized by Hokkaido System Science (Sapporo, Japan). Syk inhibitor BAY61-3606 was purchased from Selleck Chemicals (Houston, TX, USA).

Preparation and culture of dendritic cells. Bone marrow cells from C57BL/6J mice, Dectin-2 KO mice, or Mince KO mice were cultured at 2 × 10^5/mL in 10 mL of RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol containing 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF; FUJIFILM Wako Pure Chemical Corporation, Ltd., Osaka, Japan). On day 3, another 10 mL of the same complete medium was added, and on day 6, a half-volume of medium was removed and replaced by new culture medium. On day 8, CD11c+ cells were used as BM-DCs (Supporting data 1). The obtained BM-DCs were cultured with various doses of HA, Furfurman (InVivoGen, San Diego, CA, USA), α-mannan, trehalose dimycolate (TDM; InVivoGen), hot alkali-treated zymosan (dzymosan; InVivoGen), or LPS for 24 h at 37°C under 5% CO2 conditions. In TDM stimulation, indicated doses of TDM were diluted by isopropanol, and then coated on the bottom surfaces of culture wells in a 96-well culture plate for 12 h at room temperature. After evaporating the solvent, BM-DCs were inoculated in these wells.

Cytokine assay. Interleukin (IL)-12p40 and IL-6 concentrations in culture supernatants were measured using an ELISA kit (BioLegend, San Diego, CA, USA). The sensitivity was 4 pg/mL and 2 pg/mL for IL-12p40 and IL-6, respectively.

NFAT-GFP reporter assay. Nuclear factor of activated T cells (NFAT)-GFP reporter cells expressing indicated CLRs were prepared as previously described (Ohtsuka et al. 2004). Briefly, T cell hybridoma 2B4 was transfected with the NFAT-GFP construct, which was prepared by fusing three tandem NFAT-binding sites with enhanced GFP cDNA. NFAT-GFP transfected cells were stimulated with anti-TCR-β antibody and GFP-positive cells were collected. This cell line was transfected with the Dectin-1, Dectin-2, or Mince gene, together with the FcRγ gene.
Except for in the Dectin-1-transfected cell, and the same cell line that lacked the receptor gene was used as a control. These reporter cells were stimulated with 10 μg/mL of various types of HA for 20 to 24 h, and the GFP expression on CD3+ cells was analyzed using a flow cytometer.

**Effect of incubation with ConA-bound sepharose on HA-induced BM-DCs activation.** Concanavalin A (ConA)-bound sepharose 4B (GE Healthcare Life Sciences, Buckinghamshire, England) was used in accordance with the manufacturer’s procedure. Briefly, ConA-sepharose beads were washed using distilled water and binding buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) for preparation before stimulation. Each HA was incubated with ConA-sepharose beads for 15 min at room temperature. After incubation, the treated samples were centrifuged, and the supernatants (ConA-unbound fraction) were collected. In some experiments, the incubated ConA-sepharose beads were washed using binding buffer and incubated with 62.5 mM ManP for 15 min at room temperature. The ConA-bound sepharose beads were centrifuged, and the supernatants (ConA-bound fraction) were collected.

**Statistical analysis.** Data are expressed as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Welch’s t-test. A p value less than 0.05 was considered to be significant.

**RESULTS**

**Cytokine production by BM-DCs upon stimulation with HA**

To elucidate the involvement of Dectin-2 or Mincle in HA-induced BM-DC activation, BM-DCs from Dectin-2 KO mice or Mincle KO mice were cultured with various types of HA, and cytokine production in the culture supernatants was measured. As shown in Fig. 1, HA derived from type B strain induced higher levels of cytokine synthesis than that from type A strain. IL-12p40 and IL-6 production upon stimulation with HA, except for the A/H1N1pdm09 subtype, were significantly reduced in Dectin-2 KO mice compared to WT mice (Fig. 1A). These cytokines were also significantly decreased in Dectin-2 KO mice compared to WT mice after stimulation with Furfurman, a specific ligand of Dectin-2. However, there was no apparent difference in the production of both cytokines between WT mice and Mincle KO mice whereas IL-6 production upon stimulation with type B strain was slightly increased in Mincle KO mice compared to WT mice (Fig. 1B).

We also investigated whether Dectin-1, which is a β-glucan receptor, was involved in the cytokine production by HA-stimulated BM-DCs. Dectin-1 deficiency showed no or slight decrease on the initiation of cytokine production (Supporting data 2). These results indicated that Dectin-2 was involved in the production of IL-12p40 and IL-6 by BM-DCs that were stimulated with HA, and this suggested that Dectin-2 may play an important role in the induction of the inflammatory response that was caused by IFV infection.

**Involvement of intracellular signaling molecule Syk in cytokine production by HA-stimulated BM-DCs**

Spleen tyrosine kinase (Syk) is an essential molecule for signaling via CLRs (Yamasaki et al. 2008; Robinson et al. 2009). To elucidate the involvement of Syk-mediated signaling in the cytokine production via triggering of Dectin-2 with HA stimulation, BM-DCs were treated with a Syk inhibitor BAY61-3606 and cytokine production by these cells was analyzed. As shown in Fig. 2, IL-12p40 production was significantly reduced in Syk inhibitor–treated BM-DCs upon stimulation with HA from the A/H3N2 subtype and B/Victoria lineage compared to the vehicle control. IL-6 was reduced only by B/Victoria lineage stimulation whereas there was no difference between the two groups upon stimulation with HA from other lineages. Cytokine production by stimulation with Furfurman was significantly reduced by this treatment whereas no such inhibition was detected in the cytokine production by LPS-stimulated BM-DCs. These results suggested that Syk may be a critical molecule in the delivery of Dectin-2-mediated signaling for development of the inflammatory response that is induced by IFV infection.

**Contribution of the sugar moiety to cytokine production by HA-stimulated BM-DCs**

Our data also suggested that HA may contain mannose polysaccharides and activate the Dectin-2-dependent signaling pathway. Therefore, we next treated BM-DCs with methyl-α-D-mannopyranoside (ManP), a competitive inhibitor for mannose binding site, and analyzed the effect of this treatment on the cytokine expression by HA-stimulated BM-DCs. As shown in Fig. 3A, IL-12p40 synthesis was significantly reduced in BM-DCs that were treated with ManP compared to untreated cells upon stimulation with HA derived from the A/H3N2 subtype, B/Victoria lineage, and B/Yamagata lineage. IL-6 produc-
Dectin-2 recognizes influenza HA

**Fig. 1** Cytokine production by BM-DCs upon stimulation with HA. BM-DCs from Dectin-2 KO mice (A), Mincle KO mice (B), and their WT control mice were co-cultured with the indicated doses of various types of HA, Furfurman (100 μg/mL), TDM (1 μg/mL), or LPS (1 ng/mL) for 24 h. IL-12p40 and IL-6 concentration in the culture supernatants was measured by ELISA. Each column represents the mean ± SD of triplicate cultures. Experiments were repeated twice with similar results. KO, knockout; TDM, trehalose-dimycolate; LPS, lipopolysaccharide; IL, interleukin; NS, not significant; *P < 0.05.
pression was either not detected or was weaker with the addition of D-glucose or D-galactose. A significant increase in IL-12p40 was found with the addition of D-glucose (5 μg/mL) and D-galactose (10 μg/mL) compared with the non-addition group in the HA stimulation derived from the A/H1N1 pdm09 subtype, and this significant increase was not found in other groups. The significant decrease in IL-12p40 production was found in HA stimulation derived from B/Yamagata lineage other than D-mannose (5 μg/mL) addition as compared with the non-addition group. This decrease was most remarkable with the addition of D-mannose (10 μg/mL). Furthermore, the addition of D-glucose or D-galactose showed higher suppression of IL-12p40 production than that by the addition of D-mannose alone in all HA stim-

Fig. 2 Involvement of Syk in the cytokine production by HA-stimulated BM-DCs. BM-DCs from WT mice were pre-treated with a Syk inhibitor BAY61-3606 (0.1 μM) or dimethyl sulfoxide as a vehicle control, for 1 h, followed by co-culturing with indicated doses of various types of HA (10 μg/mL), Furfurman (100 μg/mL), and LPS (1 ng/mL) for 24 h. After stimulation, IL-12p40 and IL-6 concentration in the culture supernatants was measured by ELISA. Each column represents the mean ± SD of triplicate cultures. Experiments were repeated twice with similar results. (−), vehicle control; LPS, lipopolysaccharide; IL, interleukin; NS, not significant; *P < 0.05.

Next, to examine the involvement of various sugar moieties, excess amounts of D-mannose, D-glucose, and D-galactose were added to the BM-DC culture that was stimulated with HA. As shown in Fig. 3B, the addition of D-mannose resulted in the dose-dependent suppression of IL-12p40 by BM-DCs stimulated with HA derived from the A/H3N2 subtype and B/Victoria lineage, although the suppression was either not detected or was weaker with the addition of D-glucose or D-galactose. A significant increase in IL-12p40 was found with the addition of D-glucose (5 μg/mL) and D-galactose (10 μg/mL) compared with the non-addition group in the HA stimulation derived from the A/H1N1 pdm09 subtype, and this significant increase was not found in other groups. The significant decrease in IL-12p40 production was found in HA stimulation derived from B/Yamagata lineage other than D-mannose (5 μg/mL) addition as compared with the non-addition group. This decrease was most remarkable with the addition of D-mannose (10 μg/mL). Furthermore, the addition of D-glucose or D-galactose showed higher suppression of IL-12p40 production than that by the addition of D-mannose alone in all HA stim-
Dectin-2 recognizes influenza HA

**Fig. 3** Effect of ManP and various monosaccharides on the cytokine production by BM-DCs stimulated with HA. (A) BM-DCs from WT mice were co-cultured with various types of HA (10 μg/mL), α-mannan (3 mg/mL), and CpG1826 (1 μg/mL) in the presence or absence of ManP (62.5 mM) for 24 h. IL-12p40 and IL-6 concentration in the culture supernatants was measured by ELISA. (B) BM-DCs from WT mice were co-cultured with each HA, including IFV-A (a) and IFV-B (b), in the presence or absence of indicated doses (5, 10 mg/mL) of D-mannose, D-glucose, D-galactose, D-mannose plus D-glucose, D-mannose plus D-galactose, or D-glucose plus D-galactose for 24 h. IL-12p40 concentration in the culture supernatants was measured by ELISA. Each column represents the mean ± SD of triplicate cultures. Experiments were repeated twice with similar results. (−), untreated sample; ManP, methyl-α-D-mannopyranoside; Man, D-mannose; Glu, D-glucose; Gal, D-galactose; IL, interleukin; NS, not significant; *P < 0.05.
ulations. These data suggested that the mannose moiety of HA may be involved in cytokine production by BM-DCs, and that Dectin-2 may be a candidate receptor by interacting at least partially with this sugar moiety, although other sugars may also support this interaction.

**Effect of mannose-depletion on cytokine production by HA-stimulated BM-DCs**

To confirm whether HA contained mannose polysaccharides, each HA was incubated with ConA-immobilized sepharose beads to deplete mannose polysaccharides. BM-DCs were stimulated with HA that was treated or untreated with ConA-immobilized sepharose beads, and the inflammatory cytokines in the culture supernatants were measured. As shown in Fig. 4A, IL-12p40 production by BM-DCs was significantly reduced when stimulated with all types of HA that were treated with ConA-immobilized sepharose beads compared to untreated HA. IL-6 production showed similar results, except for stimulation with the A/H1N1pdm09 subtype. As a positive control, cytokine production by BM-DCs stimulated with α-mannan was diminished after treatment with ConA-immobilized sepharose beads. However, in CpG1826 stimulation, no influence was shown on IL-12p40 and IL-6 production by the ConA-sepharose beads treatment. These results suggest that Dectin-2 ligand in HA may be a ConA-bound fraction that includes mannose polysaccharides.

To address this possibility, we examined whether ConA-bound materials in HA showed similar activity to induce cytokine production by BM-DCs. For this purpose, IL-12p40 production was examined in the culture supernatants of BM-DCs that were stimulated with the materials extracted from the ConA-bound fraction of HA. As shown in Fig. 4B, IL-12p40 synthesis was restored when stimulated with the ConA-bound materials that were eluted by ManP in all types of HA, whereas this cytokine synthesis was completely diminished when treated with RPMI1640 (medium) instead of ManP. These data suggested that mannose polysaccharides of HA may be involved in the initiation of inflammatory responses that are mediated by Dectin-2.

**NFAT-GFP reporter assay**

Finally, to investigate whether representative CLR s were triggered by viral HA, GFP expression by the reporter cells that were transfected with the Dectin-1, Dectin-2, or Mincle gene was examined upon stimulation with various types of HA in a flow-cytometric analysis. As shown in Fig. 5A and C, all types of HA failed to trigger signaling that was mediated by Dectin-1 and Mincle in this reporter assay. However, HA derived from the A/H3N2 subtype alone induced GFP expression in the Dectin-2 reporter cells whereas other types of HA did not show such activity (Fig. 5B). These results suggested that IFV may interact with Dectin-2, but not with Dectin-1 and Mincle, through HA that is expressed on their surface, although there may be differences in its activity among the strains or the content of mannose polysaccharides in HA of this virus.

**DISCUSSION**

In the present study, the main findings are as follows: 1) production of pro-inflammatory cytokines caused by BM-DCs upon stimulation with HA from various types of IFV was significantly reduced under Dectin-2-deficient conditions, but not under Mincle-deficient conditions; 2) addition of a Syk inhibitor led to reduced production of these cytokines; 3) depletion of ConA-bound molecules from HA resulted in significant reduction in its ability to induce cytokine production by BM-DCs; 4) ConA-bound molecules that were eluted by ManP caused the production of cytokines by BM-DCs; and 5) in the NFAT-GFP reporter assay, HA derived from the A/H3N2 subtype, but not from other subtypes, including the A/H1N1pdm09 subtype, B/Victoria lineage, and B/Yamagata lineage, was found to trigger the activation signals via Dectin-2 whereas HA from any subtype of IFV failed to trigger Mincle-dependent activation signals.

Viral recognition by DCs and macrophages initiates the host immune response against IFV infection. TLRs are known to be involved in sensing viral components and initiating the inflammatory response. TLR3 and TLR7 essentially contribute to the host protective immunity against IFV infection (Diebold et al. 2004; Le Goffic et al. 2007). TLR3 is involved in producing pro-inflammatory cytokines and recruiting immune cells to the inflamed site (Le Goffic et al. 2006). However, these receptors are expressed in the cytoplasm or in the endosome of APCs, and it is thus difficult for these receptors to contact the viral surface molecules to induce host immune responses. In extracellular interactions with IFV, TLR4 has been reported to be involved in triggering IFV-induced inflammatory responses. TLR4 is also associated with acute lung injury that is caused by H5N1 avian influenza whereas there is no evidence that IFV activates directly TLR4-mediated signaling (Imai et al. 2008). Based on these reports,
Dectin-2 recognizes influenza HA

Fig. 4 Involvement of mannose polysaccharide in cytokine production by BM-DC. (A) ConA-immobilized sepharose beads were mixed with various types of HA (10 μg/mL), α-mannan (3 mg/mL), and Cpg1826 (1 μg/mL) for 15 min. After centrifugation, the supernatants (shown as "sup") were collected and used for stimulation of BM-DCs from WT mice for 24 h. IL-12p40 and IL-6 concentration in the culture supernatants was measured by ELISA. (B) Each type of HA was treated with ConA-immobilized sepharose beads for 15 min, followed by centrifugation, and the obtained supernatants were described as "sup". The sedimented beads were treated with excess amounts of ManP for 15 min, followed by centrifugation, and the obtained supernatants were described as "ConA + ManP". As sham treatment, the same procedures were conducted using the culture medium, which did not contain ManP (shown as "ConA + medium"). BM-DCs from WT mice were co-cultured with the above supernatants for 24 h, and IL-12p40 concentration was measured by ELISA. Each column represents the mean ± SD of triplicate cultures. Experiments were repeated twice with similar results. ConA, concanavalin A; (−), untreated sample; ManP, methyl-α-D-mannopyranoside; NS, not significant; *, P < 0.05.
Fig. 5 NFAT-GFP reporter assay. The reporter cells expressing Dectin-1 (a), Dectin-2 (b), or Mincle (c) were cultured with various types of HA (10 μg/mL) for 20 to 24 h, and GFP expression was analyzed using a flow cytometer. The cells without expression of these receptors were used as controls. As positive controls, dzymosan, which is a Dectin-1 ligand, Furfurman, which is a Dectin-2 ligand, and TDM, which is a Mincle ligand, were used. Representative data from independent experiments are shown. Experiments were repeated twice with similar results. shaded area, medium; GFP, green fluorescent protein; dzymosan, hot alkali-treated zymosan; TDM, trehalose-dimycolate.
we focused on viral HA, including some polysaccharides, and addressed the possible molecular mechanism via mannose binding receptors, including Dectin-2 and Mincle, to elucidate the relationship between CLRs and IFV-associated PAMPs on their surface. Our results showed that HA derived from the A/H3N2 subtype, but not others, bound to Dectin-2 by reporter assay (Fig. 5B). Further, the reactiv-ness with BM-DCs was stronger in the type B lineage than the type A subtype, and the A/H1N1pdm09 subtype in particular had a weaker response. The difference in cytokine response due to the HA strain may be associated with the structural difference of the carbohydrate chain of HA. Previous reports showed that the proportion of polysaccharides, including HA, varies greatly among strains (Collins et al. 1978; Basak et al. 1981). HA derived from the A/H1N1pdm09 subtype has less polysaccharides than the A/H1N1 subtype that was previously prevalent, and there are few polysaccharides compared with the A/H3N2 subtype and type B lineage (York et al. 2019). HA that was used in our experiments may be different in respect to the content of man- nose-containing polysaccharides among the origin of IFV strains, which suggests the induction of different levels of immune responses by HA derived from distinct strains. In this context, as the binding of TDM to Mincle was completely inhibited by CRD mutation (Ishikawa et al. 2009), the binding difference may become clear due to CRD mutation in Dectin-2. Furthermore, a previous report showed that glycerol monomycolate included in the cell wall of M. tuberculosis is recognized by human Mincle, but not mouse Mincle (Hattori et al. 2014). Addi-tional examination is necessary to confirm the interaction between human Dectin-2 and each HA, because the reactivity to HA may differ among animal species. In contrast, not all HA induced the activation of Mincle reporter cells (Fig. 5B). Because Mincle recognizes sugar and lipid compounds (Ishikawa et al. 2009), it is not likely to be involved in the recognition of IFV, which is not assumed to include these compounds on the surface (Suzuki et al. 1988). As for the cytokine synthesis by BM-DCs via Dectin-1 in Fig. 1C, IL-12p40 and IL-6 production was significantly decreased in Dectin-1 KO mice. These results suggest the possibility that Dectin-1 interacts slightly with HA. On the contrary, however, no binding activity with Dectin-1 was found in any of the HA stimulations by reporter assay (Fig. 5A). Thus, HA may be recognized by Dectin-1 in collaboration with other PRRs. As β-glucan, which is a specific ligand of Dectin-1, is not includ-
be involved in the recruitment of innate lymphoid cells (ILCs), including NK cells, and host protection against IFV infection.

Among viral polysaccharides, the HA polysaccharides mostly contain mannose, and they also contain other polysaccharides, including α-galactose, β-galactose, fucose, and N-acetylgalactosamine (Mirshekari et al. 1997). Our results support these previous findings because mannose depletion from viral HA by ConA-sepharose beads or blocking of the mannose-binding sites on BM-DCs by an excess amount of mannose resulted in a significant reduction in cytokine production (Figs. 3, 4). This study has potential limitations, however. In Fig. 4, it is not possible to determine if HA was totally bound to ConA, because we did not have any method to accurately quantify HA. Based on these results, however, we believe that at least part of the activity in HA was bound to ConA. Furthermore, D-glucose may be associated with the initiation of the immune response by HA as well as D-mannose because ConA also binds to D-glucose. A previous report showed that the main polysaccharide included in HA is D-mannose, not D-glucose (York et al. 2019). The possibility that the presence of D-glucose may affect this interaction by some mechanism remains to be elucidated, although we assumed that D-mannose is directly involved in the activation of BM-DCs via Dectin-2. To clarify the interaction between other polysaccharides besides mannose and Dectin-2, more detailed analysis is necessary to test the effect by deglycosylation using enzymatic treatment, such as PNGase F, O-glycosidase, neuraminidase, β-1,4-galactosidase, or β-N-acetylgalactosaminidase.

Cytokine production by BM-DCs that are stimulated with HA that is derived from the type B strain of IFV was significantly decreased in Dectin-2 KO mice compared to WT mice (Fig. 1A) whereas these HA rarely triggered Dectin-2-reporter cells (Fig. 5B). CLR proteins are known to be coordinately involved in initiating inflammatory responses with other PRRs. For example, O-antigen containing α-linked mannose in most Gram-positive bacteria enhances immune responses by cross-talk between Dectin-2 and TLR4 signaling via phosphorylation of the Syk molecule (Wittmann et al. 2016). The possibility that Dectin-2 cooperates with other CLR proteins has also been reported. DC-SIGN functions as a receptor for the intracellular invasion of A/H1N1pdm09 and A/H3N2 subtype virus in a sialic acid-independent manner (Grayson et al. 2007; Gillespie et al. 2016). DC-SIGN may collaborate with Dectin-2 and induce the inflammatory response, because DC-SIGN is associated with the invasion of IFV through the high mannose structure in HA (Londrigan et al. 2011). Therefore, we assumed that Dectin-2 may be involved mainly in the secretion of pro-inflammatory cytokines, and that the possible cross-talk of Dectin-2 with other PRRs may also be responsible for the recognition of viral HA.

The present study demonstrated that Dectin-2 is involved in the recognition of HA derived from IFV and contributes to the production of pro-inflammatory cytokines by BM-DCs. A previous investigation reported that CARD9 plays essential roles in dampening influenza pneumoniae (Uematsu et al. 2015). Furthermore, the increase in the sugar content in HA is related to the impairment of viral virulence or the attenuation of secondary bacterial pneumoniae (McCullers et al. 2014). Therefore, we assumed that the pneumoniae symptom caused by IFV is relieved in Dectin-2-deficient mice. It is important to examine how Dectin-2 is involved in the host immune response in influenza pneumoniae. Recently, various vaccine adjuvants targeting innate immune receptors, such as TLRs, have been developed (Lewnard et al. 2018). Because lethal ARDS is a serious clinical problem in novel-type influenza pandemics (Kawachi et al. 2009; To et al. 2010), it is an urgent issue to establish an effective way to regulate this hyperinflammatory condition of the lungs. Our findings could help to improve our understanding of the precise mechanism of IFV infection–related ARDS and to develop novel influenza preventive and therapeutic methods by targeting Dectin-2-dependent inflammatory responses. Further investigations are needed to clarify the complex immune responses and the regulation of the pathogenic mechanism based on Dectin-2 in IFV infection.

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Supporting data 1  CD11c expression on BM-DCs. Bone-marrow cells from WT mice were co-cultured with GM-CSF (20 ng/mL) for 8 days and non-adherent cells were collected. The obtained cells were stained with phycoerythrin (PE)-labeled anti-mouse CD11c antibody and CD11c expression was analyzed using a flow cytometer. shaded area, isotype-matched IgG
Supporting data 2  Involvement of Dectin-1 in the cytokine production by HA-stimulated BM-DCs. BM-DCs from Dectin-1 KO mice, Dectin-1/Dectin-2 KO mice, and their WT control mice were co-cultured with the indicated doses of various types of HA, dzymosan (60 μg/mL), Furfurman (100 μg/mL), or CpG1826 (1 μg/mL) for 24 h. IL-12p40 and IL-6 concentration in the culture supernatants was measured by ELISA. Each column represents the mean ± SD of triplicate cultures. Experiments were repeated twice with similar results. KO, knockout; dzymosan, hot alkali-treated zymosan; IL, interleukin; NS, not significant; *P < 0.05.