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Dectin-2 Recognition of House Dust Mite Triggers Cysteiny1 Leukotriene Generation by Dendritic Cells

Nora A. Barrett, Akiko Maekawa, Opu M. Rahman, K. Frank Austen, and Yoshio Kanaoka

House dust mites are a significant source of airborne allergen worldwide, but there is little understanding of how they so potently generate allergic inflammation. We found that extracts from the house dust mites Dermatophagoides farinae (Df) and Dermatophagoides pteronyssinus and from the mold Aspergillus fumigatus stimulated a rapid and robust production of cysteinyl leukotrienes (cys-LTs), proinflammatory lipid mediators, from mouse bone marrow-derived dendritic cells (BMDCs). Con A affinity chromatography of the Df extract revealed that the relevant ligand is a glycan(s), suggesting stimulation via a dendritic cell (DC) lectin receptor. Cys-LT production in BMDCs from wild-type mice was inhibited by spleen tyrosine kinase (Syk) inhibitors and was abolished in BMDCs from FcRγ−/− mice, implicating either Dectin-2 or DC immunoactivating receptor. Transfection of each receptor in bone marrow-derived mast cells revealed that only Dectin-2 mediates cys-LT production by Df, Dermatophagoides pteronyssinus, and Aspergillus fumigatus. Lentiviral knockdown of Dectin-2 in BMDCs attenuated Df extract-elicited cys-LT generation, thereby identifying Dectin-2 as the receptor. Lung CD11c⁺ cells, but not peritoneal or alveolar macrophages, also generated cys-LTs in response to Df. These findings place Dectin-2 among the C-type lectin receptors that activate arachidonic acid metabolism and identify the Dectin-2/FcRγ/Syk/cys-LT axis as a novel mechanism by which three potent indoor allergens may activate innate immune cells to promote allergic inflammation.


Self/non-self discrimination is a cardinal feature of innate immunity, whereby conserved microbial signatures, pathogen-associated molecular patterns, are recognized by germline-encoded pattern recognition receptors (PRRs) on dendritic cells (DCs) and macrophages. These receptors, including TLRs, C-type lectin receptors (CLRs), and nucleotide-binding oligomerization domain (Nod)-like receptors, can regulate endocytosis and Ag presentation, activate innate immune response genes, or trigger the arachidonic acid cascade (1–3) to initiate an acute inflammatory response and subsequent adaptive immunity. Despite advances in understanding the role of PRRs in response to microbial constituents such as LPS, peptidoglycan (PGN), and zymosan, there is comparatively little information on the role of PRRs in response to allergens.
the in vitro loading of BMDCs with Df (21). This finding sug-
ggested that endogenously produced cys-LTs regulate DC function in
an autocrine fashion to promote allergic inflammation. We sought to
define the mechanism by which house dust mites elicit
cys-LT generation in DCs. In this study, we show that glycan(s) in
Df, Dermatophagoides pteronyssinus (Dp), and Aspergillus fu-
migatus (Af) are recognized by the DC CLR, Dectin-2, to generate
cys-LTs.

Materials and Methods

Reagents

Lyophilized extracts of Df, Dp, Af, and the cockroach species Periplaneta
americana (Pa) and Blatella germanica (Bg) were generated by the sup-
plier (Greer) as follows: source materials (whole bodies for Df, Dp, Pa,
and Bg and mycelia and spores harvested from Af cultures) were extracted with
0.01 M ammonium bicarbonate (1:20, w/v) overnight at 2–8°C. The crude
extract, recovered after centrifugation, was dialyzed against pyrogen-free
water, sterilized using a 0.2-μm membrane filter, and lyophilized under
aspect conditions. OVA (Grade V), LPS from Escherichia coli O55:BS,
PGN from Staphylococcus aureus, calcium ionophore A23187, and E64
(trans-e-poxysuccinyl-l-leucylamido-(4-guanidino)butane), and α-Mann-
ose were from Sigma-Aldrich. Lipopolysaccharide from Staphylococcus
aureus, FSL-1/Pam3CSK4, Pam3CSK4, MannN-ac-t-Ala-D-iso-
Glu (MDP), and murabutide were from InvivoGen. Proteinase K was from
Taconic Farms. LTC4S (M 2-ME, and 40 ng/ml) were purchased from Qiagen. Spleen tyrosine kinase
Gln (MDP), and murabutide were from InvivoGen. Proteinase K was from
Roche. DNase I and RNase A were from InvivoGen. Spleen tyrosine kinase
(Gly) inhibitors, 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihy-
Roche. DNase I and RNase A were from Qiagen. Spleen tyrosine kinase
Df

Extracts of lin, 100
aureus

were from Sigma-Aldrich. Lipoteichoic acid from

and resuspended at a concentration of 4

were diluted into RPMI 1640 medium at two

were harvested, washed, blocked in PBS containing 1

beads (Miltenyi Biotec) per the manufacturer's protocol.

Pulmonary and peritoneal cell harvest

BMDC generation and allergen stimulation

BMDCs were generated in vitro according to Lutz et al. (22). In brief, bone
marrow was harvested from the femurs and tibiae, disaggregated, washed,
and resuspended at a concentration of 4 × 10^5 non-RBCs/ml in RPMI 1640
medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicil-
in, 100 μg/ml streptomycin, 2 mM l-glutamine, 50 μM 2-ME, and 40
ng/ml recombinant mouse GM-CSF (PeproTech). Then, 10 ml of this
suspending was plated in each petri dish and cultured at 37°C in a 5% CO2
incubator for 7–9 days. On day 3, 10 ml of complete medium with GM-
CSF was added to each plate. On days 4 through 9, 10 ml of medium
was exchanged at each plate. The floating cell population harvested
at days 7–9 contained 85–95% CD11c (HMC class II (MHCI)I) and
Noveml and peritoneal cell harvest

to obtain pulmonary CD11c^+ cells, mice were killed in isoflurane
inhalation. The lungs were perfused with sterile PBS with 1 mM EDTA,
removed, chopped with sterile scalpels, and digested for 30 min with 500
U/ml type IV collagenase (Worthington Biochemical) and 0.02 mg/ml
DNase I at 37°C. The digests were filtered, washed twice, and separated
with CD11c^+ beads (Miltenyi Biotec) per the manufacturer’s protocol.
CD11c^+ cells were plated in 96-well plates in RPMI 1640 medium with
GM-CSF, and 2 × 10^5 cells were stimulated with 200 μl of Df extract at
100 μg/ml for 60 min. To obtain alveolar macrophages, mice were killed with
isoflurane inhalation, the peritoneal cavity was lavaged with 5 ml sterile PBS with 1 mM EDTA, and the cell suspension was processed as above.

OVA detoxification and Df inhibition, digestion, and purification

Endotoxin Detoxi-Gel (Pierce) was used to deplete LPS from OVA. Ac-
cording to the manufacturer’s protocol, resulting in a concentration of <3
pg/ml. LPS content was measured by limulus amebocyte assay (Bio-
Whittaker). For cattle protease inhibition, Df extract was treated with 50
mM E64 at a 100:1 inhibitor to ligand ratio for 15 min at 37°C in 1 mM
DTT. Inhibition of Df protease activity was verified in an assay using the
fluorogenic peptide substrate N-tet-butoxycarbonyl (Boc)-Gln-Ala-Arg-7-
amino-4-methyl-coumarin (Bachem). For digestion, Df extract was treated with 1 mg/ml proteinase K, with 20 μg/ml RNase A, or with 20 μg/ml
EDTA (1 mg/ml for 5 mM CaCl_2 at 37°C for 1 h). LTC4S extracted with
Df extract at 20 μg/ml was treated as follows: the extract was diluted to
1 mg/ml in endotoxin-free PBS, mixed with three volumes of chloroform/
(2:1, v/v) was added, vortexed, and centrifuged at 500 × g for 10 min to separate the mixture into an upper highly polar
aqueous phase, a denatured protein interface, and a lower organic phase.
The aqueous and organic layers were washed with Pasteur pipettes, evap-
orated overnight, and resuspended in 600 μl PBS. When indicated, the
entire aqueous phase was further separated on a 1 ml Con A Sepharose
column (Sigma-Aldrich) according to the manufacturer’s instructions,
washed, and eluted sequentially with 0.1, 0.2, and 0.4 M α-methyl man-
noside/α-methyl glucoside (1:1, w/w, Sigma-Aldrich) and finally with 10% ethanol. Fractions were evaporated overnight and resuspended in 600 μl
PBS. Each 600 μl preparation (unfractionated Df extract, Df extract puri-
fiy for chloroform: methanol extraction, or Df extract purified by chloro-
form:methanol followed by Con A chromatography) was added to BMDCs
on day 8 for 30 min at a 1/200 dilution.

BMDC isolation

Plasmids for mouse Fcγ receptor CD40 (CD40), CD86, and CD197 were
amplified from American Type Culture Collection to obtain mouse DC
immunactivating receptor (DCAR) cDNA. RT-PCR was performed with
a C57BL/6 mouse lung RNA and the mouse DCAR-specific primers
sense 5′-GGTCGTCAGTGGTCTGCCTG-3′ and antisense 5′-GATGAGTCAAGGTGTTTTCAT-3′. The amplified
fragments were ligated into a PCR-Script vector (Stratagene) and were
sequenced. The full-length cDNA for DCAR was then ligated into a
pcMV-Sport6 vector for transient transfection in 293T cells. Mouse
masquerade-derived mast cell (MMCs), generated as previously described
(19), were harvested between wk 6–8, washed, and counted in serum-free
RPMI 1640 medium. Then, 5 × 10^6 cells were incubated with 20 μg total
plasmid (10 μg for each plasmid) on ice for 15 min for each cotransfection.
Groups were as follows: pcMV-Sport6 vector plus Fcγ receptor, DCAR
plus Fcγ-receptor, Dectin-2 plus Fcγ-receptor, Dectin-2 plus pcMV-
SPORT6, or Con A purified Df extract at 100 μg/ml for 45 min at 37°C.
For inhibition studies, mannose was added to cells for 30 min before stim-
ulation with extract.

Cys-LT measurement

Cys-LTs in the supernatants of stimulated cells were measured by enzyme
immunoassay according to the manufacturer’s protocol (Amersham Bio-
sciences) with a lower limit of detection at 60 pg/ml.

Flow cytometry

To verify Dectin-2 and DCAR expression in transfection and knockdown
experiments, cells were harvested, washed, blocked in PBS containing 1
mM EDTA and 10% donkey serum (Jackson ImmunoResearch Laborato-
ries), and serially stained with rat anti-DCAR (R&D Systems), rat anti-
Dectin-2 (clone D2.11E4, AbCAM), or rat IgG2a isotype control Ab (BD
Biosciences) at a concentration of 1 μg/ml/10^6 cells, followed by don-
key anti-rat-APC (Jackson ImmunoResearch Laboratories) at 1/100 dilu-
tion. 10 min before analysis on a FACScanto flow cytometer (BD Bio-
sciences), propidium iodide (1 μg/ml/10^6 cells; BD Biosciences) was added
to exclude dead cells. Analysis was conducted with FlowJo 7.2.
Of culture and stimulated with 100 μg/ml Df extract for the indicated times. The concentrations of cys-LTs in the supernatants were measured by enzyme immunoassay. Results are means ± SEM from four experiments. Significance was determined using a paired Student’s t test. B, BMDCs from WT or MyD88<sup>−/−</sup> mice were stimulated with 100 μg/ml Df extract for 60 min. Results are means ± SEM from four experiments. C and D, BMDCs from WT mice were stimulated with LPS, LPS plus OVA, or 100 μg/ml Df for 60 min (C) or with 10 μg/ml MDP. 10 μg/ml murabutide (MURA), or the calcium ionophore A23187 at 5 μM for 60 min (D). Results are means ± SEM from three experiments.

**Lentiviral knockdown**

Five short hairpin RNA (shRNA) constructs for mouse Dectin-2 in a pLKO1 lentiviral vector (TRCN0000066783, TRCN0000066784, TRCN0000066785, TRCN0000066786, TRCN0000066787) were purchased from Open Biosystems. Each hairpin construct had a 21-bp sense and antisense stem and a 6-bp loop. Infectious viral particles were prepared by cotransfection of 293T cells with the pLKO1 construct, a packing vector (pSPAX2, Addgene), and an envelope vector (pMD2.G, Addgene), according to the manufacturer’s protocol. Viral stocks were titered in HT1080 cells (American Type Culture Collection). Mouse BMDCs were cultured as described above, and adherent and nonadherent cells at day 6 were harvested, washed, and replated at 10<sup>5</sup> cells/ml. After 2 h, viral stocks were added to achieve a multiplicity of infection of 5. At 24 h and again at 48 h, 1 ml of RPMI 1640 medium with 10% FCS and 40 μg/ml GM-CSF was added. Cells were harvested at 72 h for functional study and flow cytometry. The shRNA construct TRCN0000066785 was the most effective, as analyzed by flow cytometry expression of Dectin-2, and was used for further functional studies. The sequence for TRCN0000066785 is as follows: CCGGGC GTGTGTTCATATGTTACTCGAGTAAACTATTGAAACACAC CGCTTTTTG.

**Statistical analysis**

Results are expressed as means ± SEM. Data were analyzed using GraphPad Prism. The unpaired Student’s t test was used for statistical analysis, except where noted. A value of p < 0.05 was considered significant.

**Results**

**Extract from the house dust mite Df elicits cys-LT generation from cultured BMDCs**

To examine DC production of cys-LTs in response to an allergen, we generated BMDCs from WT and LTC<sub>4</sub>S<sup>−/−</sup> mice and stimulated them with extract from the house dust mite Df, or with the model Ag used in immunologic studies, OVA, with LPS. In a time-dependent study, Df extract at a concentration of 100 μg/ml elicited cys-LTs from WT BMDCs, but not from LTC<sub>4</sub>S<sup>−/−</sup>-BMDCs. Cys-LT production was robust by 2 min (750 ± 150 pg/10<sup>6</sup> cells) and peaked by 30 min (2118 ± 348 pg/10<sup>6</sup> cells) over four studies (Fig. 1A). BMDCs generated from MyD88<sup>−/−</sup> mice had no impairment in cys-LT production in response to Df (Fig. 1B) and neither LPS alone nor the combination LPS and OVA at 100 μg/ml elicited cys-LTs from WT BMDCs (Fig. 1C). These findings demonstrate that the LPS that contaminates Df extracts was neither sufficient nor required for cys-LT production from BMDCs. We also tested other purified TLR and Nod agonists that could potentially provide an adjuvant activity in the Df extract. Neither TLR2 agonists (lipoteichoic acid, FSL-1/Pam2CGDPKHPKSF, Pam3CSK4) nor Nod agonists (MurNAc-L-Ala-D-isoGln and murabutide) elicited cys-LT generation from BMDCs (Fig. 1D and data not shown), despite an intact response to the calcium ionophore A23187. These results imply that the relevant Df ligand does not act at a TLR2, TLR4, or Nod-like pattern-recognition receptor.

To assess the relevance of Df extract-elicited cys-LTs to the response to inhaled Ags, we examined whether Df extract could elicit cys-LTs from pulmonary APCs. CD11c<sup>+</sup> cells from the lung were obtained by enzymatic digestion and magnetic bead isolation and stimulated with either medium or Df extract for 60 min. For comparison, macrophages were obtained by bronchoalveolar lavage and peritoneal lavage. Although pulmonary CD11c<sup>+</sup> cells generated 1848 ± 357 pg cys-LTs/10<sup>6</sup> cells in response to 100 μg Df extract in three experiments (Fig. 2A), alveolar macrophages (Fig. 2B), and peritoneal macrophages (Fig. 2C) did not respond, despite an intact response to the calcium ionophore A23187, suggesting that Df does not activate the major macrophage population in the lung.

**Extracts from the house dust mite Dp and the mold Af elicit cys-LTs from BMDCs**

Because of the robust response of BMDCs to Df, we next examined whether extracts from other indoor allergens could elicit a

![Image](http://www.jimmunol.org/DownloadedFrom/1121/1121.html)
similar response. Extracts of the related house dust mite Dp triggered cys-LT generation in a dose- and time-dependent manner with the peak cys-LT production of 5382 ± 501 pg/10^6 cells at 30 min in response to 100 µg/ml Dp extract (Fig. 3A). The mold Af similarly elicited cys-LTs in a dose- and time-dependent fashion reaching 3993 ± 688 pg/10^6 cells at 30 min in response to 100 µg/ml Af extract (Fig. 3B). Higher concentrations of Dp and Af extracts did not further augment cys-LT generation (data not shown). Extracts from two cockroach species, Pa and Bg, did not elicit cys-LT generation at 1, 10, or 100 µg/ml (data not shown).

Con A purification of the Df extract demonstrates that the relevant ligand for cys-LT production is a glycan

Group 1 allergens from Df and Dp, termed Der f 1 and Der p 1, respectively, are cysteine proteases. They have enhanced immunogenicity attributed to their disruption of intercellular tight junctions in the respiratory mucosa, increasing submucosal exposure to Ag, and to cleavage of immunoregulatory proteins, including CD25, CD23, α-1 antitrypsin, and CD40 (23–25). Dust mite extracts also contain prominent serine protease activity (Der p 1, Der p 3, Der p 6, Der p 9) (26), which can activate airway protease-activated receptor-2 to produce TNF-α and allergic pulmonary inflammation (27). To determine whether this protease activity was essential to Df elicitation of cys-LTs from BMDCs, we subjected the extract to enzyme inhibition and various digestions and assessed for loss of cys-LT generating function. Cysteine protease inhibition with 5 mM E64, heat inactivation by boiling at 98°C for 30 min, and digestions with proteinase K, DNase I, and RNase A did not diminish the extract activity (data not shown), suggesting that the ligand was not a protein or large-sized nucleic acid.

We then separated the Df extract by chloroform/methanol into aqueous and organic phases, evaporated the fractions, reconstituted them in medium, and added them to BMDCs to assess for cys-LT generating activity. The organic phase, containing non-polar lipids, had no activity. The aqueous phase, containing highly polar molecules and carbohydrates, retained activity at the same 1/200 dilution (Fig. 4A). We further purified the aqueous phase with Con A affinity chromatography, which has a particular affinity for terminal α-D-mannose and α-D-glucose residues. Fig. 4B shows that fractions eluted with 0.1 and 0.2 M α-methyl mannoside/α-methyl glucoside elicted peak cys-LT generation, with residual activity in the fractions eluted with 0.4 M α-methyl mannoside/α-methyl glucoside and 10% ethanol. The 0.4 M α-methyl mannoside/α-methyl glucoside elution buffer and the column flowthrough had no significant activity. To further characterize the relevant ligand, we preincubated BMDCs with mannose at varied concentrations and then stimulated them with either unfraccionated Df extract (Fig. 4C) or the 0.2 M α-methyl mannoside/α-methyl glucoside elution fraction from Con A chromatography (Fig. 4D). Mannose inhibited both the unfraccionated Df extract and the Con A-purified Df extract with IC50 values of 8.9 ± 1.9 and 10.5 ± 0.2 mM mannose, respectively. These findings indicated that the relevant ligand for cys-LT generation in the Df extract is likely to be a glycan, and that the receptor could be a mannose-binding CLR.

BMDC cys-LT generation in response to Df, Dp, and Af extracts is dependent on Syk and FcγR-chain signaling

To characterize the relevant BMDC receptor for Df, Dp, and Af that mediates cys-LT production, we first examined whether signaling through Syk, a downstream component of many immune receptors, was required for production of cys-LTs in response to these extracts. Syk inhibition reduced cys-LT production in response to Df, Dp, and Af extracts in a dose-dependent manner with IC50 values of 0.26, 0.30, and 0.26 µM, respectively (Fig. 5). Syk inhibition did not reduce the response of BMDCs to the calcium ionophore A23187, indicating that the cys-LT biosynthetic pathway was intact. Syk is a signaling intermediate recruited to the cytoplasmic side of activated ITAM-bearing receptors or ITAM-bearing adaptor proteins, such as DAP12 and the Fcγ-chain. To establish whether the relevant receptor for these allergens used one of these adaptor proteins, we next generated BMDCs from mice...
FIGURE 4. Cys-LT generation in response to purified Df extract and inhibition by mannose. A. Df extract was separated by chloroform/methanol extraction, evaporated, and reconstituted with RPMI 1640 medium. BMDCs were stimulated with the aqueous phase (AQ) and with unfractionated Df extract for 30 min. Results are means ± SEM from three experiments. B. The aqueous phase was then subjected to Con A affinity chromatography. The bound materials were eluted sequentially with elution buffer containing 0.1, 0.2, and 0.4 M of α-methyl mannoside/α-methyl glucoside and subsequently with 10% ethanol. Fractions were evaporated, reconstituted with RPMI 1640 medium, and added to day 8 BMDCs for a 30-min stimulation. Cys-LTs in the supernatant were measured by enzyme immunoassay. Values are means ± half-range (n = 2). Results are representative of three independent experiments. C and D. BMDCs were incubated with mannose for 30 min before stimulation with Df (C) or with the 0.2 M α-methyl mannoside/α-methyl glucoside elution fraction from Con A-purified Df (D). Values are means ± SEM from three experiments.

with a targeted deletion of the FcRγ-chain and assessed the response to extracts from Df, Dp, and Af.

BMDCs from WT and FcRγ−/− mice had no significant differences in expression of MHCII, CD80, and CD86, as assessed by flow cytometry (data not shown). Fig. 6 shows that 100 µg/ml Df, Dp, and Af extracts elicited robust cys-LT production at 60 min in WT BMDCs, but Df-, Dp-, and Af-elicited cys-LT production was reduced by 85–92% in BMDCs from FcRγ−/− mice, despite comparable cys-LT production in response to the calcium ionophore A23187. This finding demonstrated that the relevant BMDC receptor(s) for these allergens requires the FcRγ-chain and Syk activation for signaling.

FIGURE 5. Effect of Syk inhibition on Df, Dp, and Af extract-elicited cys-LT generation by BMDCs. Day 8 BMDCs from BALB/c WT mice were incubated with varied concentrations of a Syk inhibitor, 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide, for 30 min at 37°C before stimulation with 100 µg/ml of either Df, Dp, or Af extract. 5 µM A23187 was used as a positive control to show the integrity of the cys-LT biosynthetic pathway. Supernatants were harvested 30 min after stimulation, and cys-LTs were measured by enzyme immunoassay. Results are representative of three independent experiments.

FIGURE 6. Effect of FcRγ deficiency on Df, Dp, and Af extract-elicited cys-LT generation by BMDCs. Day 8 BMDCs from BALB/c WT or FcRγ−/− mice were stimulated with Df, Dp, or Af extract at 100 µg/ml. 5 µM A23187 was used as a positive control. Supernatants were harvested 60 min after stimulation, and cys-LTs were measured by enzyme immunoassay. Results are means ± SEM from three experiments. * p = 0.0001; † p = 0.001 as compared with WT BMDCs.
Dectin-2 is a receptor for Df, Dp, Af, and Con A-purified Df extracts

DCs express numerous endocytic and pattern recognition receptors, several of which have been reported to signal through the FcRγ-chain (28). Therefore, we next transfected two candidate DC CLRs that signal through the FcRγ-chain, DCAR (29), and Dectin-2 (30), into BMMCs, which do not generate cys-LTs in direct response to these allergens, to test the ability of the transfectants to signal in response to extracts of Df, Dp, and Af.

Fig. 7 shows the transfection efficiency, as assessed by flow cytometry. Twenty-seven percent of BMMCs cotransfected with DCAR and the FcRγ-chain (Fig. 7B) showed specific staining with the anti-DCAR Ab (green) and by flow cytometry. E, Each transfectant was stimulated with medium or with Df, Dp, or Af extract at 100 μg/ml for 45 min, and cys-LTs in the supernatant were measured by enzyme immunoassay. Results are means ± SEM from three experiments. *, p < 0.005; †, p < 0.001 as compared with BMMCs transfected with FcRγ alone.

FIGURE 7. Df, Dp, and Af extract-elicited cys-LT generation by BMMCs transfected with Dectin-2. BMMCs were transfected with FcRγ alone (A), DCAR and FcRγ (B), Dectin-2 and FcRγ (C), or Dectin-2 alone (D). Eighteen hours later, each transfectant was stained with the Dectin-2 Ab (blue), DCAR Ab (green), or isotype control Ab (black) and analyzed by flow cytometry. E, Each transfectant was stimulated with either medium or with Df, Dp, or Af extract at 100 μg/ml for 45 min, and cys-LTs in the supernatant were measured by enzyme immunoassay. Results are means ± SEM from three experiments. *, p < 0.005; †, p < 0.001 as compared with BMMCs transfected with FcRγ alone.

with Dectin-2 alone (Fig. 7D) had little expression of Dectin-2 protein (12% of cells were positive with a low mean fluorescence intensity), consistent with a requirement for the FcRγ-chain for surface expression of Dectin-2 in these transfectants. Although BMMCs express endogenous FcRγ, it is preferentially paired with FcεRI in BMMCs, and our requirement to specifically cotransfect FcRγ is consistent with reports by other investigators assessing FcRγ-dependent receptor expression in BMMCs (31, 32). The expression of Dectin-2 was also reduced in BMDCs from FcRγ−/− mice (44% of CD11c+ MHCIIlow population) compared with WT controls (87% of CD11c+ MHCIIlow population) (data not shown).

BMMCs cotransfected with Dectin-2 and the FcRγ-chain generated cys-LTs in response to 100 μg/ml Df, Dp, and Af extract (Fig. 7E). In contrast, there was little production of cys-LTs in BMMCs transfected with Dectin-2 alone, with DCAR and the FcRγ-chain, or with the FcRγ-chain alone. This finding established that the Dectin-2/FcRγ-chain pathway can mediate...
production of proinflammatory cys-LTs in response to unfractionated extracts.

To confirm that Dectin-2 is a receptor for the ligand(s) purified by Con A affinity chromatography, we stimulated BMMCs transfected with Dectin-2 and the FcR-chain were incubated with mannose for 30 min before stimulation with Con A-purified ligand. A representative histogram (A) and quantitation (B) of Dectin-2 expression on BMMCs infected with vector alone (n = 7) and BMMCs infected with viral particles containing Dectin-2 shRNA (n = 3) is shown. *, p = 0.01 as compared with BMMCs infected with control virus. C, Control (n = 6) and Dectin-2 knockdown (KD, n = 3) BMMCs at 72 h were also stimulated with Df at 100 μg/ml for 60 min to assess cys-LT production. ‡, p = 0.004 as compared with BMMCs infected with control virus. Values in B and C are means ± SEM from three experiments.

FIGURE 8. Mannose inhibition of cys-LT generation from Dectin-2 transfectants stimulated with unfractionated Df and Con A-purified Df. A, BMMCs transfected with Dectin-2 and the FcR-chain were stimulated with unfractionated Df extract or the 0.2 M α-methyl mannoside/α-methyl glucoside elution fraction from Con A-purified Df (Con A-Df) for 30 min. Cys-LTs were measured in the supernatant by enzyme immunoassay. Results are means ± SEM from three experiments. **, p < 0.01 compared with medium alone. B and C. BMMCs transfected with Dectin-2 and the FcR-chain were incubated with mannose for 30 min before stimulation with Df (B) or Con A-Df (C). Values are means ± SEM from three experiments.

Dectin-2 mediates allergen-elicited cys-LTs in BMDCs

To obtain evidence for Dectin-2 recognition of these allergens in primary cells, we used lentiviral knockdown of Dectin-2 in BMDCs and assessed subsequent Df extract-elicited cys-LTs. BMDCs were infected on day 6 of culture with viral particles containing either Dectin-2 shRNA in the pLKO1 lentiviral vector or the empty vector alone. Seventy-two hours later, cells were harvested for assessment of Dectin-2 expression by flow cytometry and for Df stimulation. 20% of BMDCs infected with control viral particles expressed high levels of Dectin-2 and 60% expressed low levels (Fig. 9A). These levels are similar to expression levels in uninfected BMDC cultures (data not shown). BMDCs infected with viral particles containing the Dectin-2 shRNA construct had diminished Dectin-2 expression, with 2% of cells expressing high levels of Dectin-2 and 50% expressing low levels. The mean fluorescence intensity of Dectin-2 staining in the Dectin-2 shRNA group over three independent experiments was significantly reduced by 75%, compared with cells infected with viral control particles (Fig. 9B). Cys-LT production by BMDCs infected with Dectin-2 shRNA was significantly reduced by 50%, as compared...
with cells infected with control viral particles (Fig. 9C), establishing that Dectin-2 is a BMDC receptor for Df that elicits cys-LTs.

**Discussion**

House dust mites are a significant source of aeroallergens worldwide, and sensitization to house dust mite is a risk factor for the development of asthma (33). Mouse models of allergic pulmonary inflammation have also shown that Ags from Df and Dp are potent immunogens that can sensitize mice when administered to the lung without use of an adjuvant (34–36). This observation is in distinction to model proteins such as OVA, which elicit respiratory tolerance and the generation of regulatory T cells (37). Abrogation of respiratory tolerance to OVA occurs when pulmonary DCs encounter the Ag with low-dose LPS in a TLR4-dependent fashion (38), demonstrating the importance of DC PRR signaling in regulating the response to inhaled Ags. In this study, we report that the myeloid CLR Dectin-2 on BMDCs and pulmonary CD11c+ cells recognizes a glycan(s) in three important indoor allergens, Df, Dp, and Af, and that the Dectin-2/FCγR/Syk signaling pathway elicits generation of cys-LTs. This finding provides a mechanism by which clinically relevant house dust mite and Aspergillus allergens can elicit pulmonary inflammation in concert with Ag presentation.

Both human DCs and mouse BMDCs express CysLT1 receptor, and cys-LTs can specifically modulate human and murine DC migration (39, 40) and cytokine production including IL-10 and MCP-1 (21, 31, 42). Furthermore, the presence of a CysLT1 receptor antagonist during the in vitro loading of BMDCs with Df extract inhibited the ability of BMDCs to sensitize mice on intranasal transfer, demonstrating that Df elicited both cys-LT generation and CysLT1 receptor-mediated priming of DCs (21). LT∆4 signaling through CysLT1 receptor can transactivate c-Kir in mouse mast cells to augment their stem cell factor-dependent viability and proliferation (43). Krishnamoorthy et al. (44) recently showed that DC c-Kir signaling in response to house dust mite is critical for both BMDCs and pulmonary DCs to produce IL-6 and promote Th2 and Th17 cell development, which is abrogated in DCs from stem cell factor/c-Kir-mutant mice (Kit±±/Syk±) or KitW/Wv mice. Thus, transactivation of DC c-Kir by cys-LTs could be another mechanism by which cys-LTs produced in response to house dust mite can modulate DC function in an autocrine fashion to promote allergic inflammation.

Dectin-2 contains a short amino-terminal cytoplasmic tail without its own ITAM, but binds and activates the FCγR-chain leading to phosphorylation of ITAMs on the FCγR chain, endocytosis of ligand, activation of NF-κB, and production of TNF-α and IL-1 receptor antagonist in transfected RAW 264.7 macrophages (30). Our study describes Dectin-2 function in primary cells and highlights a novel function for this receptor in the immediate activation of DCs. Activation of the arachidonic acid cascade by CLRs has a precedent. Zymosan stimulates arachidonic acid release in macrophages and mast cells, a process that is dependent on Dectin-1 (1, 45). Similarly, zymosan-elicted immediate arachidonic acid release in human monocyte-derived DCs can be inhibited by co-culture pretreatment with Abs to Dectin-1 and to DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (2). We could not elicit cys-LT production from the TLR4 agonist LPS (Fig. 1C); from the TLR2 agonists lipoteichoic acid, FSL-1/Pam2CGDHPKSF, or Pam3CSK4 (data not shown); from the Nod2 agonist MureNAc-l-Ala-D-isoGln (Fig. 1D); or from the Nod1/2 agonist murabutide (Fig. 1D). Furthermore, cys-LT production in response to the Df extract was not diminished in BMDCs generated from MMD8+-/- mice (Fig. 1B). We were able to elicit cys-LTs from DCs stimulated with PGN, a TLR2/6 agonist, but found that this was not dependent on TLR2 signaling, as cys-LT production was intact in BMDCs from TLR2-/- mice (data not shown). These findings are consistent with data in human polymorphonuclear leukocytes where PGN-elicted arachidonic acid release is independent of TLR2 signaling (46), and are in agreement with findings in RAW 264.7 macrophages showing that activation of the 5-LO pathway requires a sustained elevation in intracellular Ca2+ that cannot be achieved by TLR agonism alone (47). Activation of the arachidonic acid cascade is, thus far, a specific feature of CLR signaling.

Although pulmonary CD11c+ cells displayed Df extract-elicted cys-LT production, resident CD11c+ alveolar macrophages (and resident peritoneal macrophages) could not generate cys-LTs in response to Df extract, despite an intact response to the calcium ionophore A23187. Although murine Dectin-2 was initially cloned from a BALB/c neonatal Langerhans cell line and identified as a DC specific receptor (48), it has been expressed on some F4/80+ tissue macrophage populations, on thioglycollate-elicited peritoneal macrophages, and on two APC populations in mouse lung, CD11clowCD68+ macrophages, and a subset of CD11chigh DCs (49, 50). The failure of the alveolar macrophages to respond to Df extract, despite cell surface expression of Dectin-2 by flow cytometry (data not shown) and an intact response to the calcium ionophore A23187, may derive from a lack of effective coupling to the FCγR-chain, the requirement for another adaptor protein in Dectin-2 activation in alveolar macrophages, or the presence of a paired ITIM-containing inhibitory receptor such as DC immunoreceptor, which has been found for other members of the myeloid CLR family (51). The restricted expression of Dectin-2 to a subset of CD11chigh pulmonary DCs (50) is not unexpected given the preferential expression of Dectin-2 on a subset of CD11chighMHCIilow cells in BMDC cultures (data not shown) and the maturation-dependent expression of other CLR (3). Alternatively, Dectin-2 expression may be restricted to one of three well-characterized CD11c+ lung DC populations: myeloid, plasmacytoid, or CD11c+CD11b+CD103+ DCs.

Proteases are thought to be central to house dust mite immunogenicity (52, 53). To our initial surprise, we found that Df extract-elicted cys-LT production was not dependent on Df protease activity or intact protein. Cys-LT generation elicited by unfracticated and Con A-purified Df extract was inhibited by mannose with nearly identical IC50 values in both BMDCs and BMMC transfectants expressing Dectin-2 and the FCγR-chain. These findings indicate that the Dectin-2 ligand in Df extract is a glycan acting at the mannose-binding Dectin-2 receptor. A mannose-glucose-rich ligand in the Df extract is consistent with data from a glycan array showing that the carbohydrate recognition domain of Dectin-2 recognizes high-mannose structures (Man9GlcNAc2) (54). Der f 1 and Der p 1 have sites for N-linked glycosylation (34). Although pulmonary CD11c+ cells displayed Df extract-elicted cys-LT production, resident CD11c+ alveolar macrophages (and resident peritoneal macrophages) could not generate cys-LTs in response to Df extract, despite an intact response to the calcium ionophore A23187. Although murine Dectin-2 was initially cloned from a BALB/c neonatal Langerhans cell line and identified as a DC specific receptor (48), it has been expressed on some F4/80+ tissue macrophage populations, on thioglycollate-elicited peritoneal macrophages, and on two APC populations in mouse lung, CD11clowCD68+ macrophages, and a subset of CD11chigh DCs (49, 50). The failure of the alveolar macrophages to respond to Df extract, despite cell surface expression of Dectin-2 by flow cytometry (data not shown) and an intact response to the calcium ionophore A23187, may derive from a lack of effective coupling to the FCγR-chain, the requirement for another adaptor protein in Dectin-2 activation in alveolar macrophages, or the presence of a paired ITIM-containing inhibitory receptor such as DC immunoreceptor, which has been found for other members of the myeloid CLR family (51). The restricted expression of Dectin-2 to a subset of CD11chigh pulmonary DCs (50) is not unexpected given the preferential expression of Dectin-2 on a subset of CD11chighMHCIilow cells in BMDC cultures (data not shown) and the maturation-dependent expression of other CLR (3). Alternatively, Dectin-2 expression may be restricted to one of three well-characterized CD11c+ lung DC populations: myeloid, plasmacytoid, or CD11c+CD11b+CD103+ DCs.

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is mediated by DC-SIGN and liver/lung node-SIGN and lost on deglycosylation of the egg Ag (61–63). Furthermore, with the critical S. mansoni egg Ag glycan, lacto-N-fucopentaose III, is conjugated to human serum albumin, it promotes Th2 cytokine production and human serum albumin-specific IgE (64).

The DC and macrophage PRRs activated by microbial Ags have profound influences on subsequent adaptive immunity, but there is less information on the role of these receptors and their signaling pathways in the genesis of allergic responses. In this study, we identify Dectin-2 as a PRR for three potent indoor allergens, Dp, and Af, and report that activation of this receptor generates cys-LTs. In doing so, we have identified a novel non-IgE-mediated pathway by which allergens can elicit pulmonary cys-LT generation and suggest that nonmammalian glycans in some allergens may contain shared molecular patterns to generate allergic disease.

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Disclosures
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References

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