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Dectin-2 Recognition of House Dust Mite Triggers Cysteinyl Leukotriene Generation by Dendritic Cells[1]

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Abstract

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 FcR$\gamma^{-/-}$ 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)[3] 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

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[3]Abbreviations used in this paper: PRR, pattern recognition receptor; DC, dendritic cell; CLR, C-type lectin receptor; Nod, nucleotide-binding oligomerization domain; PGN, peptidoglycan; cys-LT, cysteinyl leukotriene; LT, leukotriene; 5-LO, 5-lipoxygenase; WT, wild type; *Df*, *Dermatophagoides farinae*; BMDC, bone marrow-derived DC; *Dp*, *Dermatophagoides pteronyssinus*; *Af*, *Aspergillus fumigatus*; *Pa*, *Periplaneta americana*; *Bg*, *Blattella germanica*; MHC class II, MHCII^{+}; DCAR, DC immunoactivating receptor; BMMC, bone marrow-derived mast cell; shRNA, short hairpin RNA; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; Syk, spleen tyrosine kinase; LTC_4S, LTC_4 synthase.

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 cysteinyl leukotrienes (cys-LTs), leukotriene (LT) C_4, LTD_4, and LTE_4, are peptide-conjugated lipids and potent inflammatory mediators derived from arachidonic acid through the 5-lipoxygenase (5-LO) pathway (4, 5). On cell activation, arachidonic acid is released from membrane phospholipids of the outer nuclear membrane by cytosolic phospholipase $A_2\alpha$ and converted to 5-hydroperoxyeicosatetraenoic acid and then to LTA_4 by the serial actions of 5-LO in the presence of 5-LO-activating protein. LTA_4 can be converted to LTB_4 by LTA_4 hydrolase or can be conjugated to reduced glutathione to generate LTC_4 by LTC_4 synthase (LTC_4S), the terminal enzyme in intracellular cys-LT synthesis (6, 7). LTC_4 then is exported in an energy-dependent step requiring multidrug resistance-associated proteins 1 or 4 (8, 9) and is metabolized extracellularly by the cleavage of glutamic acid and then glycine to provide LTD_4 and LTE_4, respectively. Cys-LTs are produced by hematopoietic cells such as mast cells, basophils, and macrophages after activation by FcεRI cross-linking or by microbial stimuli, including zymosan, PGN, and IgG-opsonized microbes (1, 10–12). The cys-LTs, acting through type 1 or 2 receptors, termed $CysLT_1$ or $CysLT_2$ (13, 14), are potent mediators of bronchial smooth muscle constriction, vascular permeability, and pulmonary inflammation in bronchial asthma (15–18).

We previously generated mice with a targeted deletion of LTC_4S (19) and demonstrated, in a model of OVA sensitization and intranasal challenge, that cys-LTs are required for the generation of Th2 cell-dependent pulmonary inflammation (20). Sensitized and challenged $LTC_4S^{-/-}$ mice had impaired induction of Th2 cytokine transcripts in lung and reduced generation of Th2 cytokines from parabronchial lymph nodes after ex vivo restimulation with Ag, compared with sensitized and challenged wild-type (WT) controls. Furthermore, in a mouse model using intranasal *Dermatophagoides farinae* (*Df*) extract-loaded bone marrow-derived DCs (BMDCs) for sensitization followed by intranasal challenge with *Df* extract, eosinophilic pulmonary inflammation was attenuated when a $CysLT_1$ receptor antagonist was added during the in vitro loading of BMDCs with *Df* (21). This finding suggested 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 fumigatus* (*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 *Blattella germanica* (*Bg*) were generated by the supplier (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 aseptic conditions. OVA (Grade V), LPS from *Escherichia coli* O55:B5, PGN from *Staphylococcus aureus*, calcium ionophore A23187, and E64 (trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane), and α-D-mannose were from Sigma-Aldrich. Lipoteichoic acid from *Staphylococcus aureus*, FSL-1/Pam2CGDPKHPKSF, Pam_3CSK4, MurNAc-L-Ala-D-iso-Gln (MDP), and murabutide were from InvivoGen. Proteinase K was from Roche. DNase I and RNase A were from Qiagen. Spleen tyrosine kinase (Syk) inhibitors, 3-(1-methyl-1H-

indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide; 2-(2-aminoethylamino)-4-(3-trifluoromethylanilino)-pyrimidine-5-carboxamide, dihydrochloride, dihydrate; 3,4-methyeledioxy-β-nitrostyrene; and piceatannol were from Calbiochem.

Mice

FcR$\gamma^{-/-}$ mice on a BALB/c background and BALB/c WT mice were purchased from Taconic Farms. $LTC_4S^{-/-}$ mice on a BALB/c background (N10) or WT littermate controls were generated and maintained in our laboratory (19). $TLR2^{-/-}$ mice and $MyD88^{-/-}$ mice on a C57BL/6 background were a gift from Dr. S. Akira (Osaka University, Suita, Japan). All studies were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute.

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 penicillin, 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 suspension was plated in each petri dish and cultured at 37°C in a 5% CO_2 incubator for 7–9 days. On day 3, 10 ml of complete medium with GM-CSF was added to each plate. On days 6 and 8, 10 ml of medium with GM-CSF was exchanged from each plate. The floating cell population harvested at days 7–9 contained 85–95% $CD11c^+$ MHC class II $(MHCII)^+$ cells, as assessed by flow cytometry. For stimulation, the cells were washed, resuspended in RPMI 1640 medium with 40 ng/ml GM-CSF at 2×10^6 cells/ml, plated in 96-well plates, and incubated at 37°C for 1 h. Extracts of *Df*, *Dp*, and *Af* were diluted into RPMI 1640 medium at two times concentration, warmed, and added to the cells for a final extract concentration of one times and a final cell concentration of 1×10^6 cells/ml. For Syk or mannose inhibition, the inhibitors were added for 30 min before stimulation with extract.

Pulmonary and peritoneal cell harvest

To obtain pulmonary $CD11c^+$ cells, mice were killed with 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 an overdose of i.p. pentobarbital, the neck was dissected, and the trachea was cannulated with a 22-gauge angiocatheter. Bronchoalveolar lavage with 0.75 ml of sterile PBS with 1 mM EDTA was performed three times. The cells were centrifuged, washed, and plated at 10^5 cells per well in a 24-well plate in DMEM with 10% FCS. The cells were allowed to adhere for 3 h, washed again, and stimulated with 100 μl of *Df* extract at 100 μg/ml for 60 min. To obtain peritoneal 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, according to the manufacturer's protocol, resulting in a concentration of <3 pg/μg OVA. LPS content was measured by *limulus* amebocyte assay (Bio-Whittaker). For cysteine 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-tert-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 DNase I in 5 mM $CaCl_2$ at 37°C for 1 h. For purification, 600 μl of *Df* extract at 20 mg/ml was treated as follows: the extract was diluted to 1 mg/ml in endotoxin-free PBS, mixed with three volumes of chloroform/methanol (2:1, v/v) in borosilicate tubes, 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 harvested with Pasteur pipettes, evaporated 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 mannoside/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 purified by chloroform:methanol extraction, or *Df* extract purified by chloroform: methanol followed by Con A chromatography) was added to BMDCs on day 8 for 30 min at a 1/200 dilution.

BMMC transfection

Plasmids for mouse FcRγ cDNA in pCMV-SPORT6 (clone MGC-36077) and for mouse Dectin-2 cDNA in pCMV-SPORT6 (clone MGC-35901) were purchased from American Type Culture Collection. To obtain mouse DC immunoactivating receptor (DCAR) cDNA, RT-PCR was performed with a C57BL/6 mouse lung RNA and the mouse DCAR-specific primers sense 5′-GGTCGACTGAGTTCTGGCCTGTTTG-3′ and antisense 5′-GAGTGATTCATAAGTTTATTTTCTTCATCTG-3′ under the following conditions: 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, 35 cycles. 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 expression in mammalian cells. Mouse bone marrow-derived mast cells (BMMCs), 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 FcRγ-chain, DCAR plus FcRγ-chain, Dectin-2 plus FcRγ-chain, Dectin-2 plus pCMV-SPORT6 vector. Cells were electroporated, moved into RPMI 1640 medium with IL-3, and incubated at 37°C with 5% CO_2. Eighteen h later, transfected BMMCs were harvested, washed, counted, and stimulated with *Df*, *Dp*, *Af,* or Con A-purified *Df* extracts at 100 μg/ml for 45 min at 37°C. For inhibition studies, mannose was added to cells for 30 min before stimulation 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 Biosciences) 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 Laboratories), 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/0.2 ml/10^6 cells, followed by donkey anti-rat-APC (Jackson ImmunoResearch Laboratories) at 1/100 dilution. 10 min before analysis on a

FACSCanto flow cytometer (BD Biosciences), propidium iodide (1 μg/ml/10^6 cells; BD Biosciences) was added to exclude dead cells. Analysis was conducted with FlowJo 7.2.

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 10^5 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 ng/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: CCGGGCGGTGTGTTTCAATAGTTTACTCGAGTAAACTATTGAAACACACCGCTTTTTG.

Statistical analysis

Results are expressed as means ± SEM. Data were analyzed using Graph-Pad 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 and harvested pulmonary CD11c$^+$ cells

To examine DC production of cys-LTs in response to an allergen, we generated BMDCs from WT and $LTC_4S^{-/-}$ 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 $LTC_4S^{-/-}$ BMDCs. Cys-LT production was robust by 2 min (750 ± 150 pg/10^6 cells) and peaked by 30 min (2118 ± 348 pg/10^6 cells) over four studies (Fig. 1*A*). BMDCs generated from $MyD88^{-/-}$ mice had no impairment in cys-LT production in response to *Df* (Fig. 1*B*) and neither LPS alone nor the combination LPS and OVA at 100 μg/ml elicited cys-LTs from WT BMDCs (Fig. 1*C*). 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, Pam_3CSK4) nor Nod agonists (MurNAc-L-Ala-D-isoGln and murabutide) elicited cys-LT generation from BMDCs (Fig. 1*D* 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$^+$ 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$^+$

cells generated 1848 ± 357 pg cys-LTs/10^6 cells in response to 100 μg *Df* extract in three experiments (Fig. 2*A*), alveolar macrophages (Fig. 2*B*), and peritoneal macrophages (Fig. 2*C*) 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 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. 3*A*). 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. 3*B*). 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 nonpolar lipids, had no activity. The aqueous phase, containing highly polar molecules and carbohydrates, retained activity at the same 1/200 dilution (Fig. 4*A*). 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. 4*B* shows that fractions eluted with 0.1 and 0.2 M α-methyl mannoside/α-methyl glucoside elicited 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 flow-through had no significant activity. To further characterize the relevant ligand, we preincubated BMDCs with mannose at varied concentrations and then stimulated them with either unfractionated *Df* extract (Fig. 4*C*) or the 0.2 M α-methyl mannoside/α-methyl glucoside elution fraction from Con A chromatography (Fig. 4*D*). Mannose inhibited both the unfractionated *Df* extract and the Con A-purified *Df* extract with IC_{50} 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 FcRγ-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 IC_{50} 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 FcRγ-chain. To establish whether the relevant receptor for these allergens used one of these adaptor proteins, we next generated BMDCs from mice with a targeted deletion of the FcRγ-chain and assessed the response to extracts from *Df*, *Dp*, and *Af*.

BMDCs from WT and FcR$\gamma^{-/-}$ 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 FcR$\gamma^{-/-}$ 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.

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. 7*B*) showed specific staining with the anti-DCAR Ab as compared with the isotype control Ab. Anti-DCAR Ab staining of cells transfected with the FcRγ-chain alone was <6% (Fig. 7*A*). Similarly, 27% of BMMCs cotransfected with Dectin-2 and the FcRγ-chain (Fig. 7*C*) showed specific staining with the anti-Dectin-2 Ab, while anti-Dectin-2 Ab staining of cells transfected with the FcRγ-chain alone was 3% (Fig. 7*A*). BMMCs transfected with Dectin-2 alone (Fig. 7*D*) 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 FcR$\gamma^{-/-}$ mice (44% of $CD11c^+$ $MHCII^{low}$ population) compared with WT controls (87% of $CD11c^+$ $MHCII^{low}$ 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. 7*E*). 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 FcRγ-chain with unfractionated *Df* extract and with the 0.2 M α-methyl mannoside/α-methyl glucoside elution fraction from the Con A-purified *Df* extract. Fig. 8*A* shows that both the unfractionated *Df* extract and the Con A-purified *Df* extract triggered similar generation of cys-LTs. Furthermore, cys-LT generation in response to both the unfractionated and Con A-purified *Df* extracts could be inhibited by mannose with IC_{50} values of 43 ± 10 mM mannose (Fig. 8*B*) and 31 ± 10 mM mannose (Fig. 8*C*), respectively. These findings confirm that the Con A-purified ligand in the *Df* extract is a glycan acting at the mannose-binding Dectin-2 receptor.

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. 9*A*). 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. 9*B*). 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. 9*C*), 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/FcRγ/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 $CysLT_1$ receptor, and cys-LTs can specifically modulate human and murine DC migration (39, 40) and cytokine production including IL-10 and MCP-1 (21, 41, 42). Furthermore, the presence of a $CysLT_1$ 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 $CysLT_1$ receptor-mediated priming of DCs (21). LTD_4 signaling through $CysLT_1$ receptor can transactivate c-*Kit* in mouse mast cells to augment their stem cell

factor-dependent viability and proliferation (43). Krishnamoorthy et al. (44) recently showed that DC c-*Kit* 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-*Kit*-mutant mice ($Kitl^{Sl/Sld}$) or $Kit^{W/Wv}$ mice. Thus, transactivation of DC c-*Kit* 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 FcRγ-chain leading to phosphorylation of ITAMs on the FcRγ-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-elicited immediate arachidonic acid release in human monocyte-derived DCs can be inhibited by coordinate 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. 1*C*); from the TLR2 agonists lipoteichoic acid, FSL-1/Pam2CGDPKHPKSF, or Pam_3CSK4 (data not shown); from the Nod2 agonist MurNAc-L-Ala-D-isoGln (Fig. 1*D*); or from the Nod1/2 agonist murabutide (Fig. 1*D*). Furthermore, cys-LT production in response to the *Df* extract was not diminished in BMDCs generated from $MyD88^{-/-}$ mice (Fig. 1*B*). 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-elicited 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 Ca^{2+} 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-elicited 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 is expressed on some $F4/80^+$ tissue macrophage populations, on thioglycollate-elicited peritoneal macrophages, and on two APC populations in mouse lung, $CD11c^{low}CD68^+$ macrophages, and a subset of $CD11c^{high}$ 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 FcRγ-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 $CD11c^{high}$ pulmonary DCs (50) is not unexpected given the preferential expression of Dectin-2 on a subset of $CD11c^{high}MHCII^{low}$ cells in BMDC cultures (data not shown) and the maturation-dependent expression of other CLRs (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-elicited cys-LT production was not dependent on *Df* protease activity or intact protein. Cys-LT generation elicited by unfractionated and Con A-purified *Df* extract was inhibited by mannose with nearly identical IC_{50} values in both BMDCs and BMMC transfectants expressing Dectin-2 and the FcRγ-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- or 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 (55), and Der p 1 can bind to other CLRs, including surfactant proteins-A and –D, the mannose receptor, and DC-specific ICAM-3 grabbing nonintegrin (56–58), but whether the critical glycan(s) binding to Dectin-2 is one of the many recognized glycoproteins in house dust mite remains to be determined. Other nonmammalian glycans from plants, arthropods, and helminths can promote allergic inflammation. Chitin, a glycan with repeating units of *N*-acetyl-β-D-glucosamine that is part of the house dust mite exoskeleton, activates pulmonary macrophages via an unknown receptor to generate LTB_4 and recruit eosinophils and basophils to the lung in a BLT1 receptor-dependent fashion (59). The immunodominant peanut glycoprotein Ara h 1 activates DCs to generate IL-4- and IL-13-producing $CD4^+$ T cells, a process which is mediated by DC-SIGN and abrogated by deglycosylation of Ara h 1 (60). Similarly, the adjuvant activity of *Schistosoma mansoni* egg Ag, which elicits Th2 cytokines and Ag-specific IgE, is mediated by DC-SIGN and liver/lymph node-SIGN and lost on deglycosylation of the egg Ag (61–63). Furthermore, when the critical *Schistosoma 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, *Df*, *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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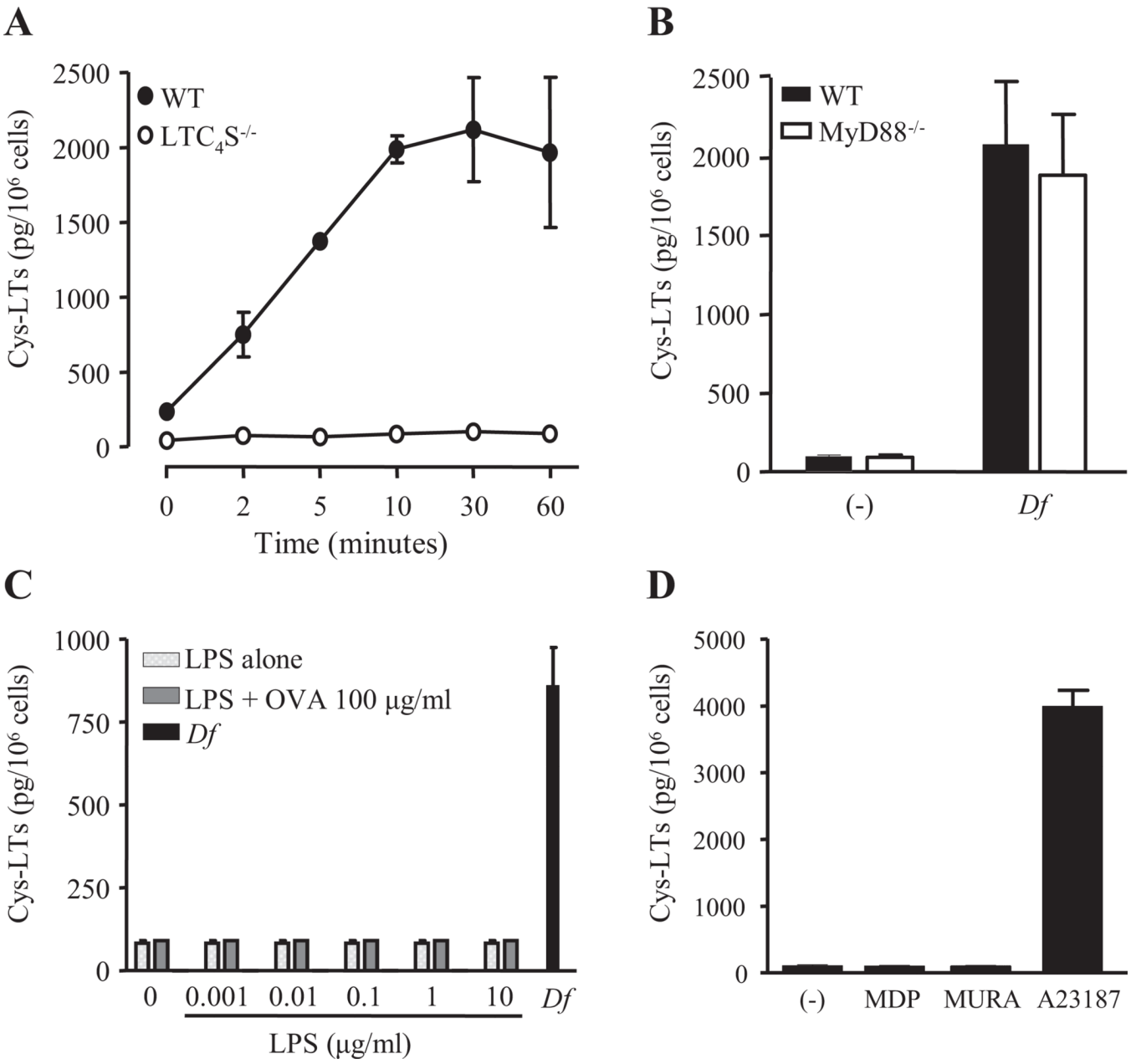

FIGURE 1.

Generation of cys-LTs by BMDCs in response to *Df* extract. *A*, BMDCs from BALB/c WT or $LTC_4S^{-/-}$ mice were harvested after 9 days 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. $p < 0.01$ compared with $LTC_4S^{-/-}$. Significance was determined using a paired Student's *t* test. *B*, BMDCs from WT or $MyD88^{-/-}$ 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.

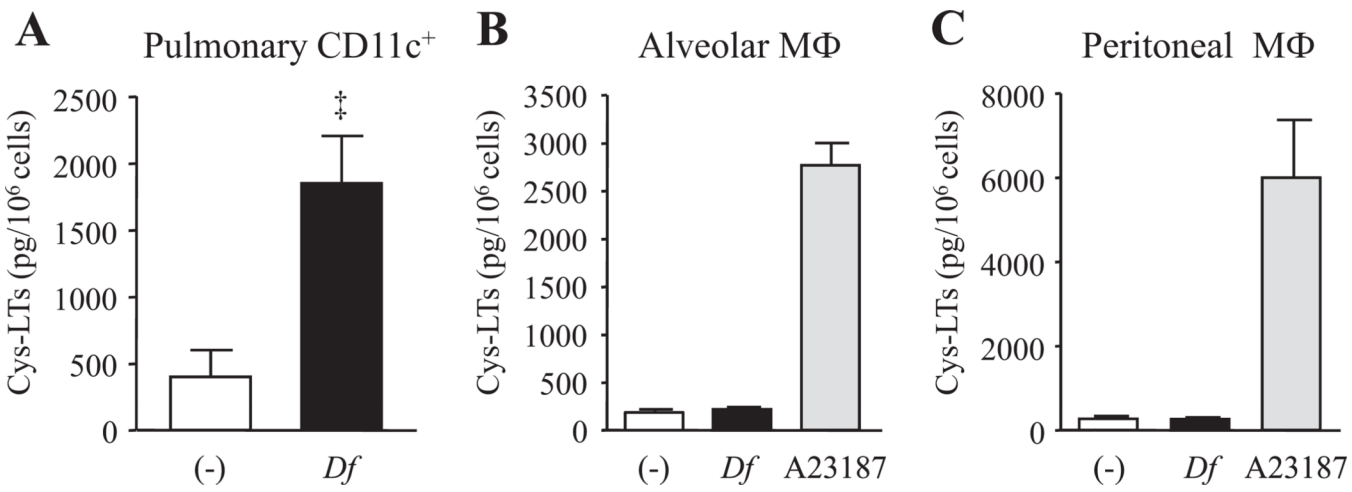

FIGURE 2.

Generation of cys-LTs by pulmonary CD11c$^+$ cells, alveolar macrophages, and peritoneal macrophages. Pulmonary CD11c$^+$ cells (*A*), alveolar macrophages (*B*), and peritoneal macrophages (*C*) were harvested and stimulated by medium (−) or 100 μg/ml *Df* extract for 60 min. Calcium ionophore A23187 at 5 μM was used as a positive control. The concentrations of cys-LTs in the culture supernatants were measured by enzyme immunoassay. Results are means ± SEM from three experiments. ‡, $p = 0.005$ compared with medium control.

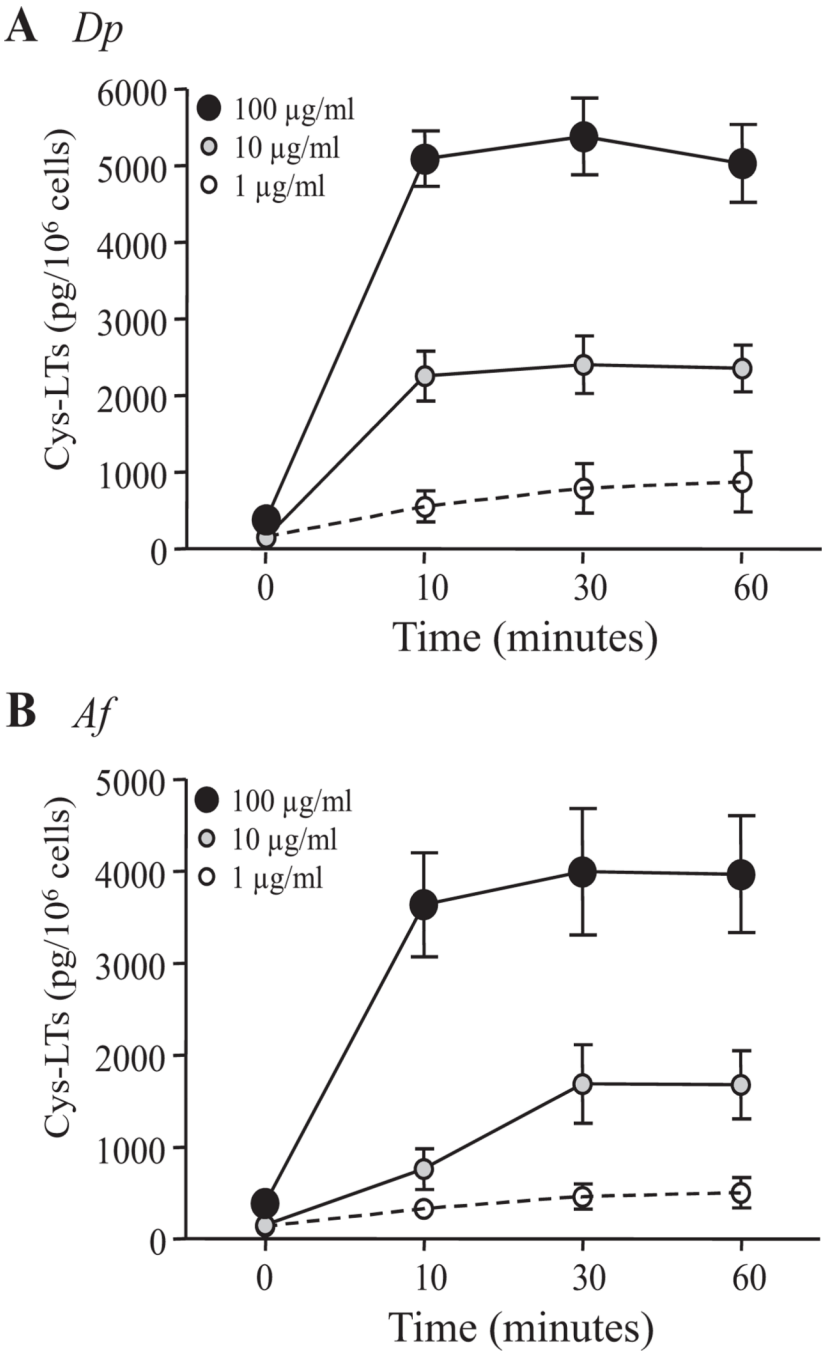

FIGURE 3.

Dose- and time-dependent generation of cys-LTs by BMDCs in response to extracts of *Dp* and *Af*. Day 8 BMDCs from BALB/c WT mice were stimulated with *Dp* (*A*) or *Af* (*B*) at 1, 10, and 100 µg/ml for the indicated times. The concentrations of cys-LTs in the supernatants were measured by enzyme immunoassay. Results are means ± SEM from four experiments. $p = 0.001$ for *Dp* concentration and *Af* concentration, $p = 0.0001$ for *Dp* and *Af* time-dependence. Significance was determined using two-way ANOVA.

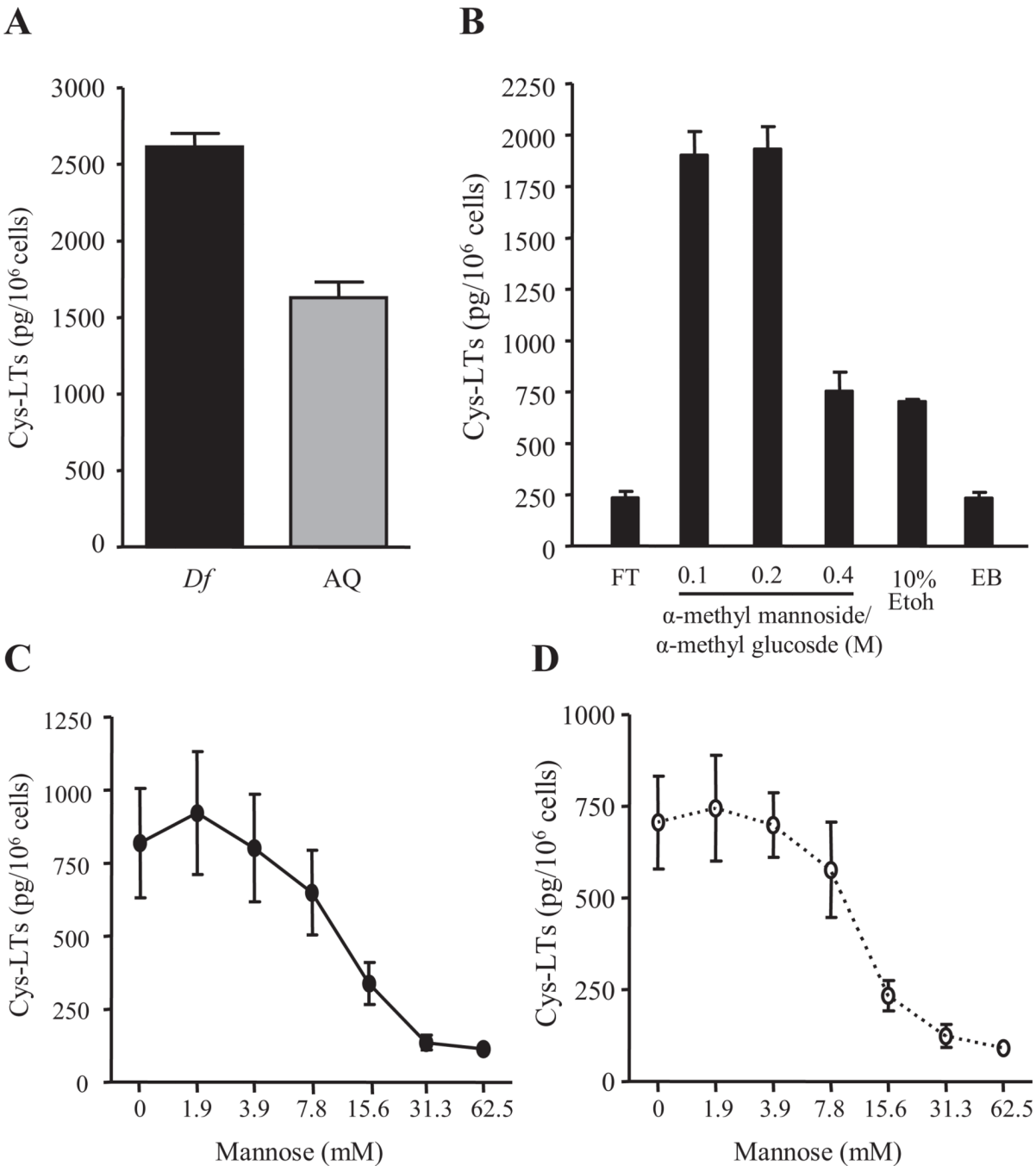

FIGURE 4.

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.

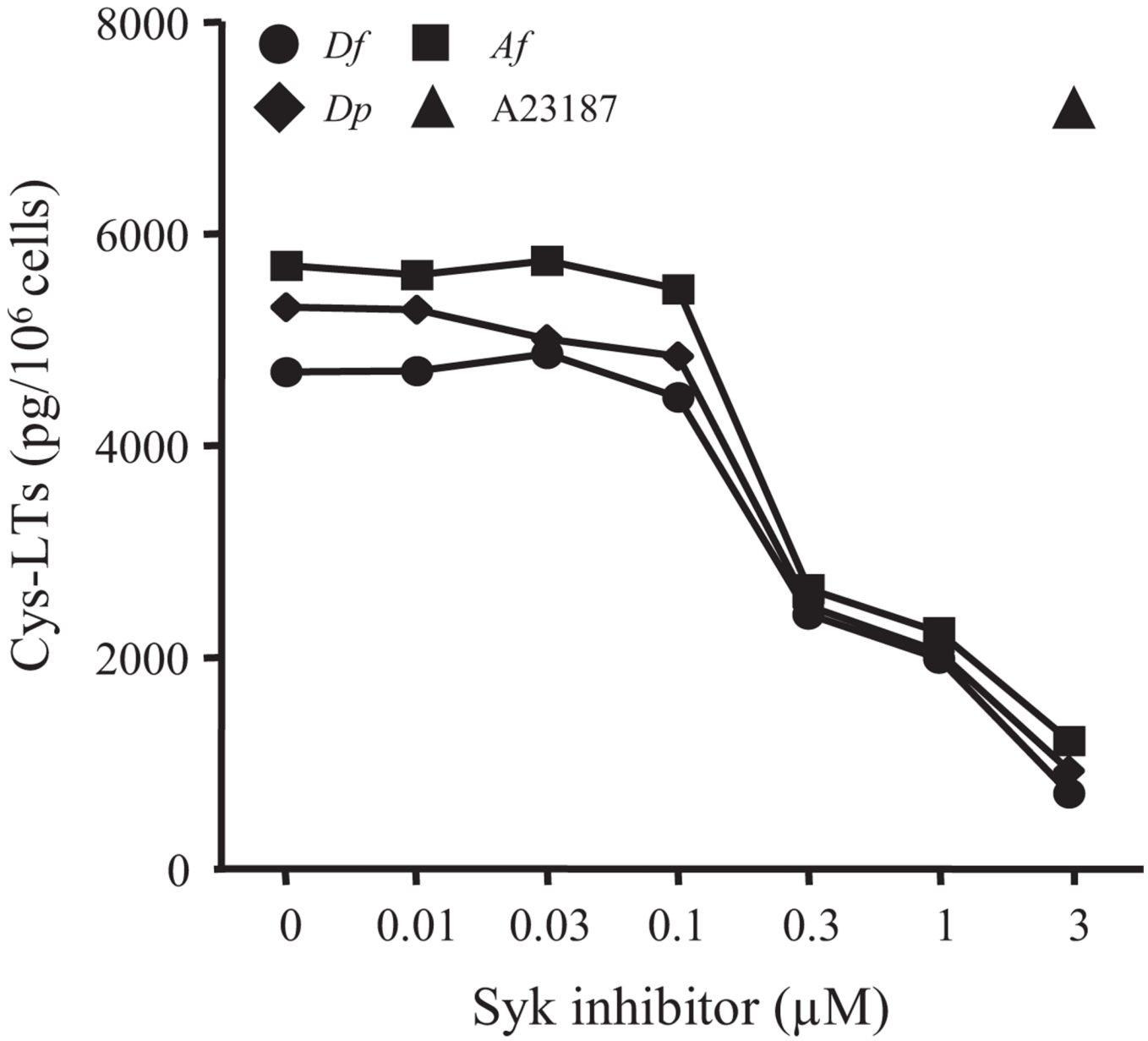

FIGURE 5.

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.

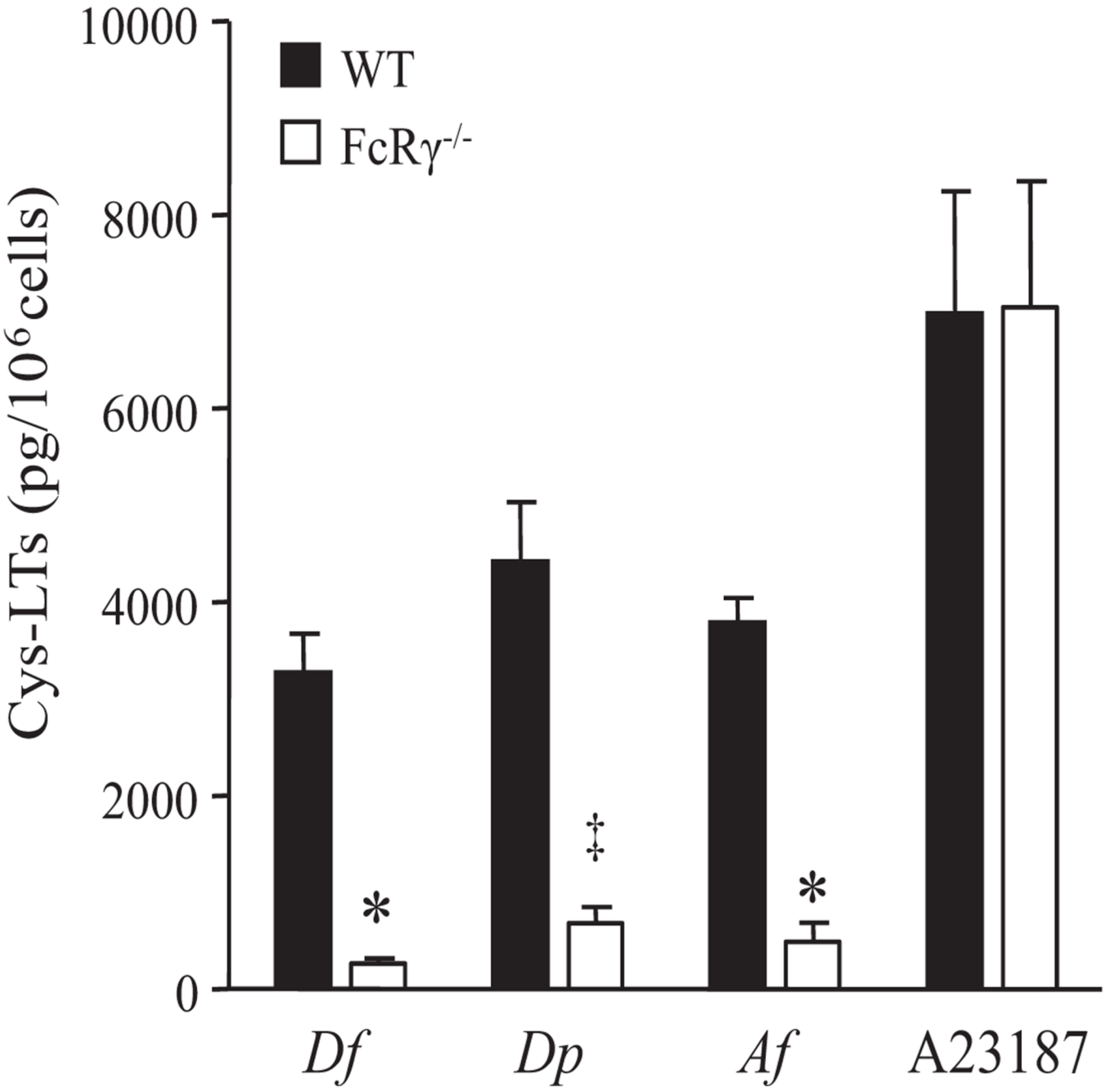

FIGURE 6.

Effect of FcRγ deficiency on *Df*, *Dp*, and *Af* extract-elicited cys-LT generation by BMDCs. Day 8 BMDCs from BALB/c WT or FcR$\gamma^{-/-}$ 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.

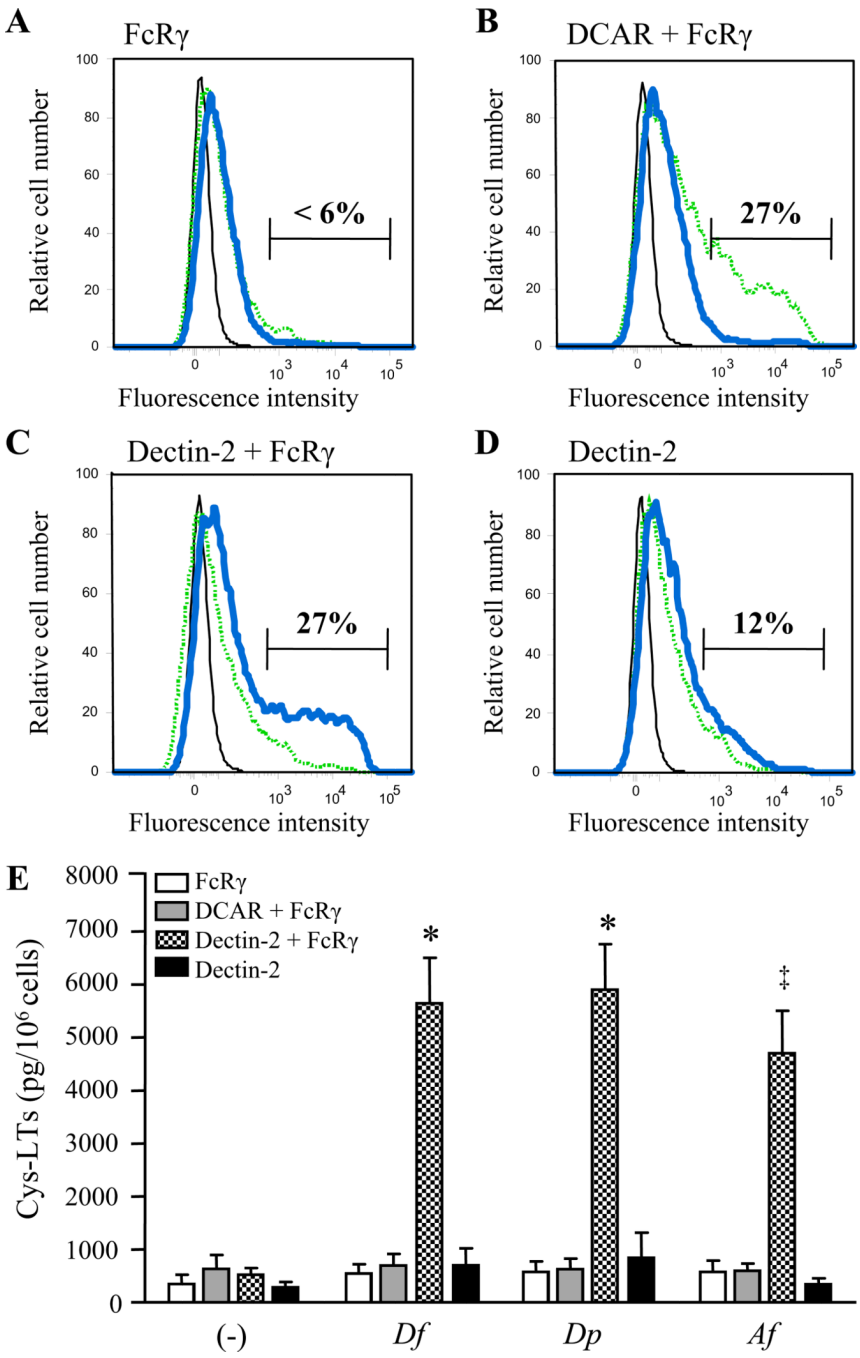

FIGURE 7.

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.

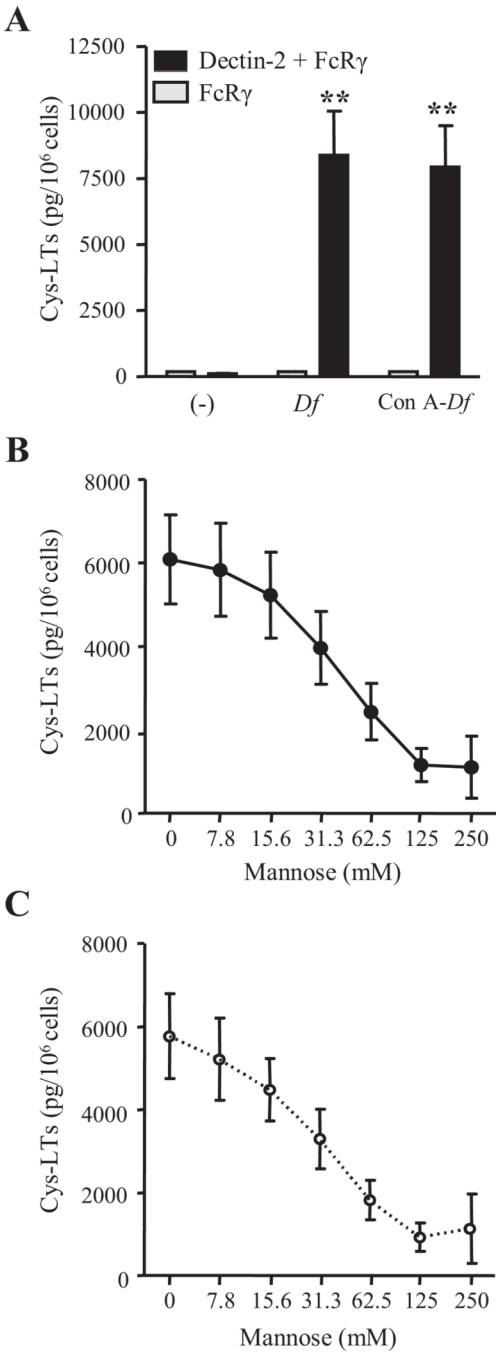

FIGURE 8.

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 FcRγ-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 FcRγ-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.

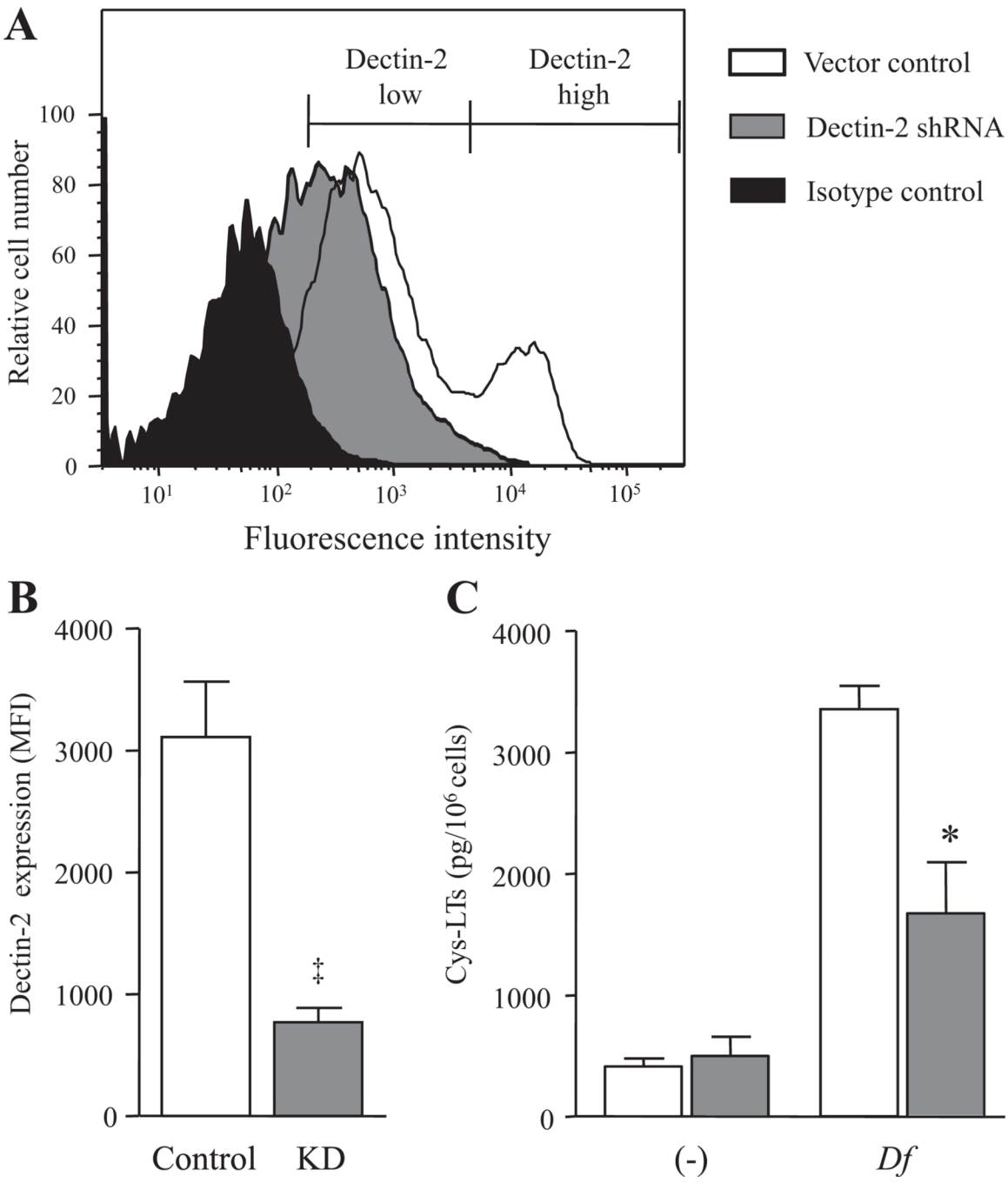

FIGURE 9.

Df extract-elicited cys-LT generation in BMDCs with knockdown of Dectin-2. Day 6 BMDCs from BALB/c WT mice were harvested and infected with viral particles containing Dectin-2 shRNA or vector alone to achieve a multiplicity of infection of 5. After 72 h, Dectin-2 expression was assessed by flow cytometry. A representative histogram (*A*) and quantitation (*B*) of Dectin-2 expression on BMDCs infected with vector alone ($n = 7$) and BMDCs infected with viral particles containing Dectin-2 shRNA ($n = 3$) is shown. ‡, $p = 0.01$ as compared with BMDCs infected with control virus. *C*, Control ($n = 6$) and Dectin-2 knockdown (KD, $n = 3$) BMDCs 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 BMDCs infected with control virus. Values in *B* and *C* are means ± SEM from three experiments.