

# The C-type Lectin Receptor CLECSF8 (CLEC4D) Is Expressed by Myeloid Cells and Triggers Cellular Activation through Syk Kinase<sup>\*[5]</sup>

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**Background:** C-type lectins play important roles in immunity and homeostasis.

**Results:** CLECSF8 is expressed on neutrophils and monocytes and can mediate phagocytosis, the respiratory burst and inflammatory cytokine production, in part through association with a novel adaptor.

**Conclusion:** CLECSF8 can trigger cellular activation.

**Significance:** This study identifies a novel C-type lectin that can control immune cell function.

CLECSF8 is a poorly characterized member of the “Dectin-2 cluster” of C-type lectin receptors and was originally thought to be expressed exclusively by macrophages. We show here that CLECSF8 is primarily expressed by peripheral blood neutrophils and monocytes and weakly by several subsets of peripheral blood dendritic cells. However, expression of this receptor is lost upon *in vitro* differentiation of monocytes into dendritic cells or macrophages. Like the other members of the Dectin-2 family, which require association of their transmembrane domains with signaling adaptors for surface expression, CLECSF8 is retained intracellularly when expressed in non-myeloid cells. However, we demonstrate that CLECSF8 does not associate with any

known signaling adaptor molecule, including DAP10, DAP12, or the Fc $\gamma$  chain, and we found that the C-type lectin domain of CLECSF8 was responsible for its intracellular retention. Although CLECSF8 does not contain a signaling motif in its cytoplasmic domain, we show that this receptor is capable of inducing signaling via Syk kinase in myeloid cells and that it can induce phagocytosis, proinflammatory cytokine production, and the respiratory burst. These data therefore indicate that CLECSF8 functions as an activation receptor on myeloid cells and associates with a novel adaptor molecule. Characterization of the CLECSF8-deficient mice and screening for ligands using oligosaccharide microarrays did not provide further insights into the physiological function of this receptor.

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[5] This article contains supplemental Figs. 1–8 and Methods.

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C-type lectin receptors form a superfamily of molecules which contain at least one C-type lectin-like domain (CTLD)<sup>7</sup> (1). These receptors recognize a wide range of ligands varying from endogenous molecules to conserved (often carbohydrate-based) structures found in microbes called pathogen-associated molecular patterns (PAMPs) (2). C-type lectins function in diverse ways and have been found to play essential roles in both immunity and homeostasis. The transmembrane receptors may contain cytoplasmic signaling motifs that enable intracellular signaling upon ligand binding, yet receptors lacking these motifs can also trigger intracellular signaling by associating

<sup>7</sup> The abbreviations used are: CTLD, C-type lectin-like domain; DCIR, dendritic cell immunoreceptor; DCAR, dendritic cell immunostimulating receptor; BDCA, blood dendritic cell antigen; TLR, toll-like receptor; M1CL, myeloid inhibitory C-type lectin-like receptor; PE, phycoerythrin; APC, allophycocyanin; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin; PMN, polymorphonuclear leukocyte; NGL, neoglycolipid; mCLECSF8, murine CLECSF8; hCLECSF8, human CLECSF8; PBL, peripheral blood leukocyte; DC, dendritic cell(s).

with adaptors such as the FcR $\gamma$  chain, DAP10, or DAP12 (3, 4). The association with these adaptors is normally mediated by charged residues in or near the transmembrane domains of these CLRs (3).

Our research focuses on C-type lectins expressed by myeloid cells, particularly those of the Dectin-1 and Dectin-2 clusters (3, 5, 6). Of relevance here is the Dectin-2 cluster, comprising Dectin-2, DCIR, DCAR, BDCA-2, Mincle, and CLECSF8. All these type II transmembrane receptors are encoded by six exons and share a common structure consisting of a single extracellular CTLD, a stalk region of varying length, and a transmembrane region (3). Other than DCIR, which contains an immunoreceptor tyrosine-based inhibitory motif, all the other receptors have short cytoplasmic tails that lack signaling motifs. Mincle, Dectin-2, DCAR, and BDCA-2 have been shown to associate with the FcR $\gamma$  chain, an adaptor containing an immunoreceptor tyrosine-based activation motif (3).

Characterization of the receptors in the "Dectin-2 cluster" over the last few years has revealed exciting new insights into the functions of these molecules. Dectin-2, for example, has been shown to recognize fungi (7–10), whereas Mincle recognizes mycobacteria, fungi, and an endogenous ligand that is released during necrotic cell death (11–13). Importantly, both of these receptors signal through association with the FcR $\gamma$  chain and can directly induce innate inflammation and the development of adaptive immunity. CLECSF8 (CLEC4D), which also lacks signaling motifs in its short cytoplasmic tail and is presumed to associate with signaling adaptors, is the least characterized member of this family. Murine CLECSF8 (mCLECSF8) was first identified by differential display PCR and shown at the mRNA level to be expressed in a macrophage-restricted manner, with highest transcript levels found in peritoneal macrophages, bone marrow, and spleen and lower levels in lung and lymph nodes (14). Human CLECSF8 (hCLECSF8) also demonstrated a monocyte/macrophage-restricted expression pattern at the mRNA level, with transcript expression found to be down-regulated upon culture *in vitro* and up-regulated by IL6, TNF, IFN $\gamma$ , and IL10 (15). hCLECSF8 was additionally shown to be capable of mediating endocytosis (15), but the function of this receptor is still unknown. Here we have characterized the expression and function of murine and human CLECSF8 using both primary cells and transfected cell lines.

## EXPERIMENTAL PROCEDURES

**Primary Cells**—Peripheral blood leukocytes (PBLs) were isolated from healthy volunteers as described previously (16). For the generation of macrophages and DCs, human peripheral blood mononuclear cells were isolated by centrifugation over a Ficoll-Paque<sup>TM</sup> PLUS (GE Healthcare) gradient according to standard procedures. Day 0 monocytes were purified by adherence on gelatin-coated plates for 1 h, followed by extensive washing to remove non-adherent cells. After at least 12 h of incubation, monocytes (day 1) were harvested and then differentiated into day 4 and day 7 macrophages by culturing in X-VIVO medium (BioWhittaker) with 1% heat-inactivated autologous serum. To generate monocyte-derived DC, IL-4 (25 ng/ml, R&D Systems), and GM-CSF (50 ng/ml, R&D Systems)

in RPMI with 10% heat-inactivated FCS (RPMI medium) were added to day 1 monocytes and then analyzed on day 4. Maturation of the DC was induced by culturing the cells for a further 2 days in fresh medium and then into media containing 1 mg/ml *Salmonella typhimurium* LPS (Sigma) and then analyzed on day 8.

To isolate neutrophils from peripheral blood, leukocytes were separated by centrifugation over a two-layer (62 and 75%) Percoll Plus (GE Healthcare) gradient at 20 °C for 25 min (10 min at 200  $\times$  g and 15 min at 400  $\times$  g without interruption). The neutrophils were collected from the interface between the two Percoll solutions and washed with PBS containing 5 mM glucose and 0.2% BSA. After centrifugation for 7 min at 250  $\times$  g at 4 °C, contaminating erythrocytes were lysed by hypotonic treatment for 30 s with 0.2% NaCl followed by restoration of isotonicity with 1.6% NaCl. After centrifugation, neutrophils (~95% pure) were resuspended in RPMI and counted.

To test the effect of cytokines and TLR agonists on CLECSF8 expression, PBLs were plated at 5  $\times$  10<sup>5</sup> cells/well in 24-well plates and stimulated for 6 h with IL4 (20 ng/ml), IL6 (80 ng/ml), IL10 (20 ng/ml), TNF (10 ng/ml), IFN $\gamma$  (10 ng/ml), Pam3CSK4 (TLR1/2, 100 ng/ml), LPS (TLR4, 100 ng/ml), flagellin (TLR5, 20 ng/ml), and FSL-1 (TLR2/6, 20 ng/ml). All TLR agonists were from Invivogen, and cytokines were from R&D Systems. Cells were then analyzed by flow cytometry as described below.

**Cell Lines and Growth Conditions**—NIH3T3 fibroblasts, RAW264.7 macrophages, A20 B cells, and Plat-E ecotropic retroviral packaging cells were maintained in DMEM or RPMI 1640 (Invitrogen) supplemented with 10% FCS (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Cambrex) and cultured at 37 °C with 5% CO<sub>2</sub>.

**Generation of Constructs and Transduced Cell Lines**—The complete mCLECSF8, hCLECSF8, Mincle, and Dectin-2 open reading frames were isolated by PCR and cloned into the pFBneo retroviral vector (Stratagene) containing an HA tag as described previously for myeloid inhibitory C-type lectin-like receptor (MICAL) (17). The receptor chimeras were generated using overlapping extension PCR so that the final constructs translated as follows: D1<sup>CTLD</sup>/SF8<sup>TM-cyto</sup> (SF8-76<sup>TGGTW</sup>SF8-80/D1-110<sup>QTTGG</sup>D1-113), SF8<sup>CTLD</sup>/D1<sup>TM-cyto</sup> (D1-112<sup>TGGFSQ</sup>D1-117/SF8-74<sup>GATGG</sup>SF8-78), Janus (SF8-215<sup>WKPSK</sup>SF8-219/D1-69<sup>FWRHN</sup>D1-73), D1<sup>CTLD</sup>/Min<sup>TM-cyto</sup> (MIN-80<sup>CPLNWK</sup>MIN-85/D1-113<sup>GGFSQS</sup>D1-118), Min<sup>CTLD</sup>/D1<sup>TM-cyto</sup> (D1-119<sup>CLPNWI</sup>D1-124/MIN-74<sup>GSVKN</sup>MIN-79), D1<sup>CTLD</sup>/D2<sup>TM-cyto</sup> (D2-80<sup>PNHWK</sup>D2-84/D1-113<sup>GGFSQSC</sup>D1-119), D2<sup>CTLD</sup>/D1<sup>TM-cyto</sup> (D1-119<sup>CLPNWI</sup>D1-124/D2-73<sup>EKMWGC</sup>D2-78), and D1<sup>CTLD</sup>/D2<sup>TM-cyto</sup> (D2-80<sup>PNHWK</sup>D2-84/D1-113<sup>GGFSQSC</sup>D1-119). DNA constructs encoding DAP10, DAP12, and FcR $\gamma$  were a kind gift from Phil Taylor (University of Cardiff) and NKG2D from Brian Rabinovich (University of Texas). All constructs were verified by sequencing. PCR, using Titanium Taq (Clontech), of hCLECSF8 and hG3DPH from human tissue cDNA panels (Clontech) was performed using standard procedures and the following primers: 5'-AAAGTGCTGAAGGGAGCACCTGG-3' and 5'-AATGGCGTCTGGTCCACCCAAC-3' for hCLECSF8. The hG3DPH primers were from Clontech.

## Characterization of CLECSF8

To generate stable cell lines, constructs were packaged into virions using Plat-E ecotropic cells, and the various cell lines were transduced as described previously (17). All cell lines were used as nonclonal populations to reduce founder effects and were generated at least twice to confirm phenotype.

**FACS Analysis**—FACS was performed on live cells according to conventional protocols at 4 °C in the presence of 2 mM NaN<sub>3</sub>. Cells were blocked with PBS containing 5 mM EDTA, 0.5% BSA, 5% heat-inactivated rabbit serum (murine cells) or murine serum (PBLs), and 50 μg/ml human IgG (*in vitro* cultured human cells). For permeabilization of cells prior to staining, cells were fixed with 1% formaldehyde at room temperature and permeabilized by the addition of 0.5% saponin. After staining with primary antibodies for 1 h at 4 °C, cells were washed and stained with the relevant secondary antibody. Cells were fixed with 1% formaldehyde prior to analysis.

The following antibodies were used in these assays: anti-HA (clone 16B12, Covance); anti-hCLECSF8 (MAB2806) and anti-hCLECSF8-APC (FAB2806A, R&D Systems); CD14-PE, CD16-FITC, CD3-FITC, CD4-FITC, CD8-FITC, and CD86-PE (all from Serotec); CD19-PE, CD56-PE, HLA-DR-FITC, and DC-SIGN-FITC (all from BD Biosciences); BDCA-2, CD1c, and BDCA-3 (all from Miltenyi Biotec); as well as irrelevant PE- or FITC-labeled or unlabeled mouse IgG<sub>1</sub> (BD Biosciences), IgG<sub>2b</sub>, and IgG<sub>3</sub> (Serotec) control antibodies. The secondary antibodies used were goat anti-mouse PE and donkey anti-mouse APC (both from Jackson ImmunoResearch Laboratories).

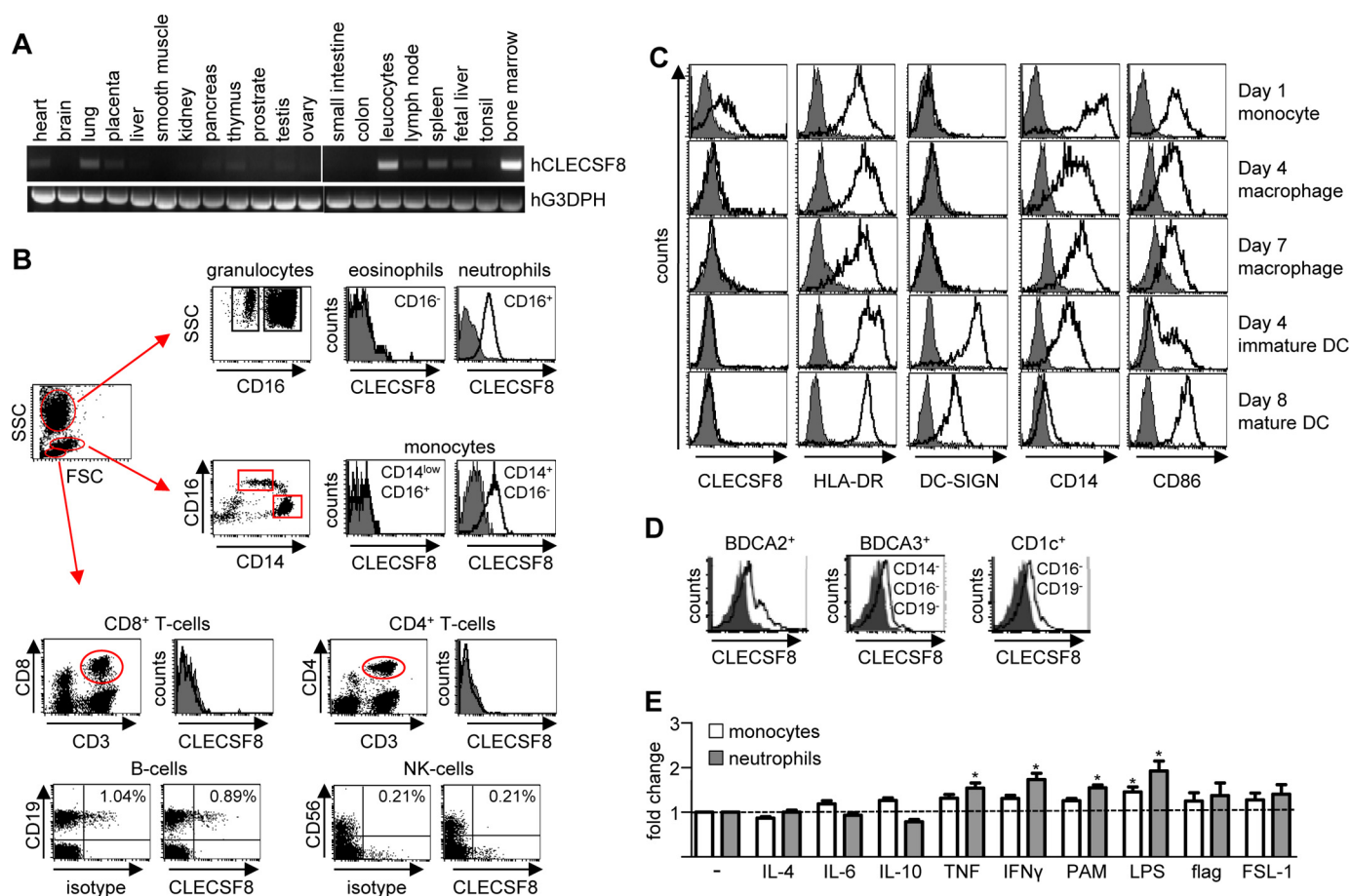
**Phagocytosis Assays**—Phagocytosis was quantified in transduced RAW264.7 cells by seeding cells at 5 × 10<sup>5</sup> cells/well in 12-well plates the day before the assay. Some cells were treated with 5 μM Cytochalasin D (Calbiochem) for 40 min before and throughout the assay to inhibit phagocytosis. After washing, FITC-zymosan (Molecular Probes) was added at a ratio of 10 particles/cell and left to settle for 1 h at 4 °C to facilitate synchronized phagocytosis. After washing to remove unbound particles, cells were incubated at 37 °C for 30 min. External zymosan was detected with anti-zymosan antibodies as described (18). For microscopy, cells were seeded onto acid-treated coverslips at 3 × 10<sup>4</sup> or 2 × 10<sup>5</sup> cells/well in 24-well plates the day prior to the experiment. FITC-zymosan binding and phagocytosis was allowed to take place as described above. Cells were fixed and permeabilized, and actin was stained with 1 μM tetramethylrhodamine B isothiocyanate-phalloidin (Sigma-Aldrich). Coverslips were mounted with Vectashield (Vector Laboratories), and cells were examined by fluorescence microscopy on a Zeiss Axiovert 40.

Phagocytosis by neutrophils was similarly determined, except that FITC-labeled Dynabeads (Invitrogen) coated with either anti-hCLECSF8 or an isotype control were added at 2 beads/cell and permitted to bind at 10 °C for 30 min. Cells were treated with 5 μM Cytochalasin D or 50 μM piceatannol (Sigma) for 40 min prior to and during the assay where indicated. Phagocytosis was allowed to occur at 37 °C for 30 min. External beads were detected with a goat anti-mouse-PE antibody. Cells that had bound or internalized FITC-beads were gated, and the percentage of phagocytosis was determined by comparing the PE<sup>-</sup> and the PE<sup>+</sup> populations as described previously (19).

**Fluorescent Zymosan Binding and Cytokine Production Assays**—Fluorescent zymosan binding and TNF production by transduced RAW264.7 cells were determined as described previously (20). Soluble β-glucan (100 μg/ml), piceatannol (50 μM), or R406 (0.5–1 μM) were added prior to the assay where indicated. Synergism between TLR4 and CLECSF8 was similarly determined, except that the transduced RAW264.7 cells were stimulated with either 100 μg/ml particulate β-glucan or 10 ng/ml LPS alone or in combination, as indicated. After incubation for 3 h at 37 °C, TNF in the supernatants was measured by ELISA (BD Biosciences). Analysis of cytokine production following cross-linking with anti-HA (Covance) or isotype control-coated anti-mouse beads (Dynabeads, Invitrogen), prepared as described above, was performed similarly.

**Respiratory Burst Assays**—For analysis of the respiratory burst in transduced RAW264.7 macrophages, cells were loaded with 20 μM dihydrorhodamine 123 (Sigma) and incubated with 50 μg/ml particulate β-glucan for the indicated length of time. In neutrophils, reactive oxygen species production was measured by cross-linking CLECSF8 on the cell surface. Briefly, on the day prior to the experiment, 24-well poly-L-lysine plates (BD Biocoat) were activated with 2.5% glutaraldehyde (Sigma) followed by coating with 50 μg/ml cross-linking sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) and subsequent blocking of free aldehyde groups with 0.2 M glycine. 10 μg/ml anti-hCLECSF8 or isotype control antibody was then added, allowed to bind overnight, and washed before use. Freshly isolated neutrophils were loaded with 20 μM dihydrorhodamine 123, added to the wells, and incubated at 37 °C for 30 min. Reactive oxygen species production was determined by measuring the conversion of dihydrorhodamine 123 to rhodamine using flow cytometry.

**Cell Lysates, Immunoprecipitations, and Western Blotting**—Signaling complexes were immunoprecipitated from transduced RAW264.7 cells by coating 2 × 10<sup>7</sup> cells/time point with 10 μg/ml anti-HA for 2 h with rotation at 4 °C. Cells were then stimulated with pervanadate for 1 or 3 min and lysed as described previously (21). Lysates were added to pan mouse IgG Dynabeads (Invitrogen), preblocked with PBS containing 0.5% BSA, and rotated for 2 h at 4 °C. After extensive washing, immunoprecipitates were analyzed by Western blotting. To confirm transductions with the adaptor molecules and association of the adaptors with the relevant receptor, A20 cell lysates were precleared by rotation with protein A-Sepharose beads (GE Healthcare) for 30 min at 4 °C, and then the beads were removed by centrifugation for 10 min. Anti-FcRγ, anti-DAP10, or anti-DAP12 were added to the lysates and rotated for 2 h at 4 °C before addition of protein A-Sepharose beads for one additional hour. Beads were washed extensively with lysis buffer, and immunoprecipitates were analyzed by Western blotting. To prepare cellular extracts, cells were lysed and prepared as described previously (21). For Western blotting, proteins were detected with anti-phosphotyrosine (clone 4G10), anti-Syk, anti-phospho-Syk (Cell Signaling Technology), anti-HA (clone 16B12, Covance), anti-DAP10 and DAP12 (kind gift from Toshiyuki Takai, Tohoku University), or anti-FcRγ (Millipore), followed by appropriate HRP-linked secondary antibodies (Jackson ImmunoResearch Laboratories).



**FIGURE 1. CLECSF8 expression on human peripheral blood leukocytes.** *A*, PCR of hCLECSF8 and hG3DPH (loading control) on commercial cDNA preparations isolated from various human tissues and cells, as indicated. *B*, flow cytometric analysis of peripheral blood showing the gating, based on side scatter (SSC) and forward scatter (FSC) profiles, of granulocytes, monocytes, and lymphocytes and staining with specific cell markers and anti-hCLECSF8, as indicated. See also supplemental Fig. 1. *C*, analysis of hCLECSF8 expression on monocyte-derived macrophages and DC. HLA-DR, CD14, DC-SIGN, and CD86 were included as markers of macrophage and DC maturation as described in the text. DCs were matured for 2 days using 1  $\mu$ g/ml LPS as described under "Experimental Procedures." The data are representative of at least five independent donors. *D*, analysis of hCLECSF8 expression on various DC subpopulations in isolated PBMCs. The filled gray histograms represent the isotype controls, and the black histograms represent specific antibody staining, as indicated. The data are representative of at least three independent donors. *E*, freshly isolated peripheral blood monocytes (open bars) and neutrophils (gray bars) were treated with various cytokines and TLR agonists for 6 h, and hCLECSF8 expression was analyzed by flow cytometry. The data were normalized to the untreated control value and shown as the mean  $\pm$  S.E. of pooled data from at least five independent donors. \*,  $p < 0.05$ . Flag, flagellin.

**Statistics**—Comparisons between two groups were performed using the Student's *t* test. Comparisons between multiple groups were performed using one-way analysis of variance with Dunnett's post test. All experiments were performed in duplicate or triplicate and independently repeated at least twice (\*,  $p < 0.05$ ).

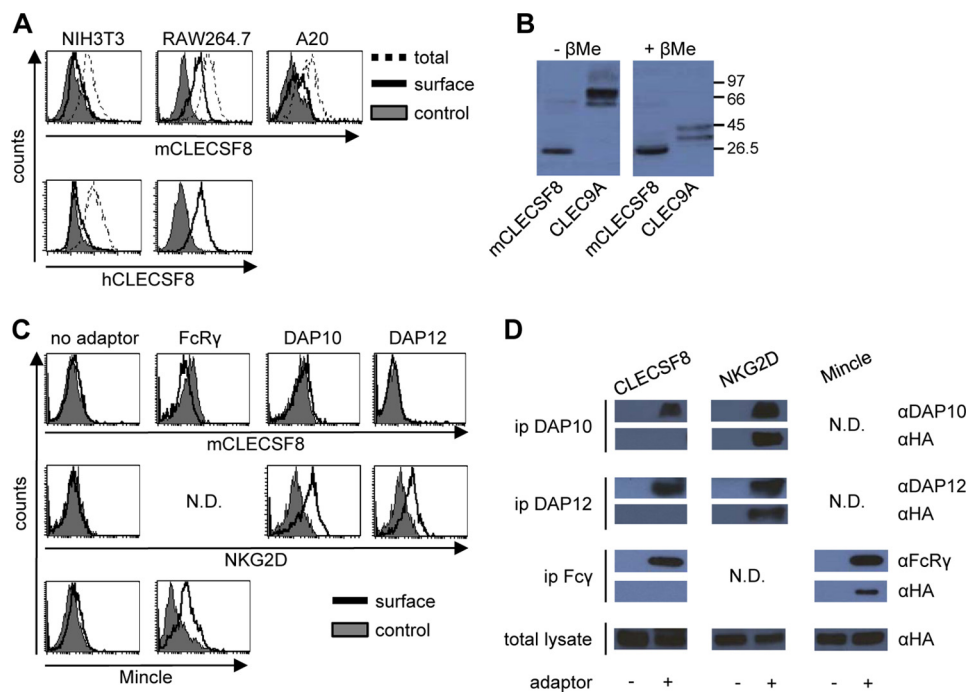
## RESULTS

**Expression of CLECSF8 on Human Peripheral Blood Cells**—To characterize CLECSF8, we first examined the expression profile of this receptor in various tissues by RT-PCR. We observed that CLECSF8 was expressed in many tissues, including the heart, lung, placenta, thymus, and lymph nodes, but was qualitatively more highly expressed in the bone marrow, spleen, and PBLs, as described previously (15) (Fig. 1A). Given its high level of expression on PBLs, we next characterized the cellular expression profile of this receptor on these cells by flow cytometry. For these analyses, we made use of a commercially available mAb to hCLECSF8 that specifically stained transduced cells expressing this receptor (supplemental Fig. 1A). Using this

antibody as well as discriminating cells on the basis of their size, granularity, and with lineage-specific markers, we detected hCLECSF8 expression on several cellular populations in peripheral blood, including CD16<sup>+</sup> neutrophils as well as on the CD14<sup>+</sup>CD16<sup>−</sup> inflammatory monocytes (22) (Fig. 1B and supplemental Fig. 1B). However, hCLECSF8 was not detected on CD14<sup>low</sup>CD16<sup>+</sup> monocytes, CD16<sup>−</sup> eosinophils, CD8<sup>+</sup> or CD4<sup>+</sup> T cells, CD19<sup>+</sup> B cells nor on CD56<sup>+</sup> NK cells.

We then investigated changes in hCLECSF8 expression upon differentiation of monocytes into macrophages and DCs *in vitro* (16). The differentiation of monocytes into macrophages, which can be characterized by the down-regulation of CD14 and up-regulation of HLA-DR, resulted in the loss of expression of hCLECSF8 (Fig. 1C). Similarly, CLECSF8 expression was not detected on monocytes cultured with GM-CSF and IL4 to induce a DC-like phenotype (characterized by increased DC-SIGN and decreased CD14 expression), nor on DCs matured with LPS (characterized by increased CD86 and loss of CD14 expression). However, a detailed analysis demonstrated that

## Characterization of CLECSF8



**FIGURE 2. CLECSF8 does not associate with known adaptor molecules.** *A*, anti-HA flow cytometric analyses of NIH3T3, RAW264.7, and A20 B cells transduced with HA-tagged murine or human CLECSF8. *Gray filled histograms* represent vector control cells, *solid black histograms* represent surface expression, and *dashed histograms* represent total (intracellular and surface) expression. *B*, anti-HA Western blot analyses of RAW264.7 cells expressing HA-tagged mCLECSF8 or hCLEC9A under non-reducing (-βMe) and reducing (+βMe) conditions. *C*, flow cytometric analyses of A20 B cells transduced with HA-tagged mCLECSF8, Mincle, or NKG2D with or without Fcγ, DAP10, or DAP12. *Gray filled histograms* represent vector control cells and *solid black histograms* represent surface expression. *N.D.*, not done. *D*, Western blot analyses of anti-Fcγ, anti-DAP10, or anti-DAP12 immunoprecipitations (*i.p.*) from the transduced A20 B cells showing presence of the adaptor molecules and the C-type lectin receptors (probed with anti-HA) as indicated.

this receptor was weakly expressed by the three major DC subsets in peripheral blood (Fig. 1D).

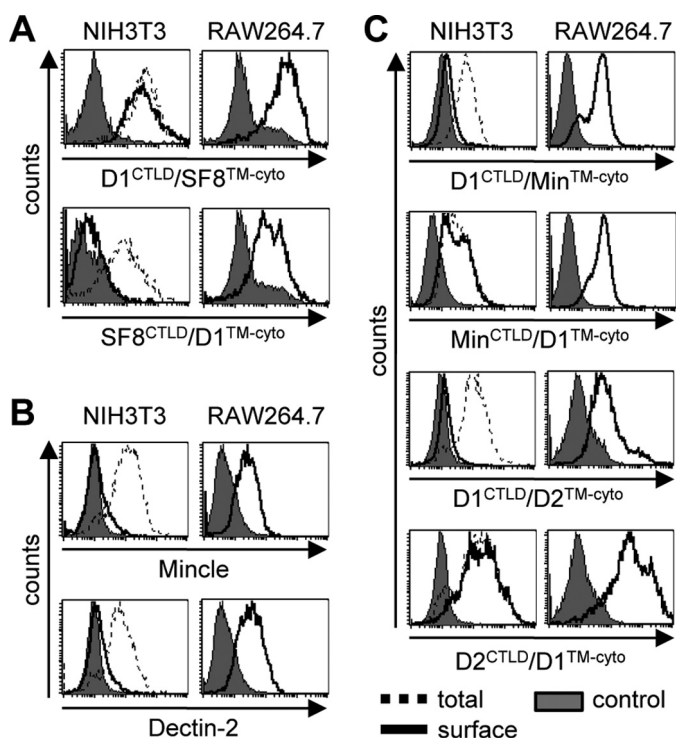
Expression of hCLECSF8 mRNA was shown previously to be modulated by cytokines and other agents (15), so we examined the effects of a variety of cytokines and TLR agonists on hCLECSF8 expression in both monocytes and neutrophils following a 6-h stimulation of PBL *in vitro*. Although we found a significant increase in hCLECSF8 expression on monocytes treated with LPS and on neutrophils treated with TNF, IFNγ, PAM<sub>3</sub>CSK<sub>4</sub>, and LPS, the effect of these agents on receptor levels were relatively modest (< 2-fold) (Fig. 1E). Overall, these data demonstrate that hCLECSF8 is highly expressed on a population of circulating monocytes and neutrophils whose expression can be marginally influenced by certain proinflammatory cytokines and microbial components but that is down-regulated following differentiation of monocytes into macrophages or DCs.

**CLECSF8 Does Not Associate with Known Adaptors for Expression at the Cell Surface**—Many of the Dectin-2 clusters of receptors associate with the Fcγ signaling adaptor for transport to the cell surface (3). Thus, we next examined the expression profile of HA-tagged human and murine CLECSF8 in transduced NIH3T3 fibroblasts, A20 B-cells, and RAW264.7 macrophages. As described for other receptors in this cluster, we observed that both human and murine CLECSF8-HA were retained intracellularly when expressed in NIH3T3 or A20 cells but were located on the cell surface when expressed in RAW264.7 macrophages (Fig. 2A). This suggested that CLECSF8 associates with a myeloid-expressed adaptor for expression at the cell surface. Western blot analyses of lysates

from the transfected RAW264.7 cells indicated that murine CLECSF8 was expressed as a monomer of ~24 kDa (equivalent to its predicted molecular weight (14), Fig. 2B). Cells expressing the unrelated human C-type lectin CLEC9A, which dimerizes through disulfide linkages (21), were included as a control.

To determine which adaptor was involved in mediating the surface expression of CLECSF8, we cotransduced mCLECSF8-HA-expressing A20 cells along with murine DAP10, DAP12, or the Fcγ chain. As positive controls for these experiments we included Mincle, which associates with the Fcγ chain, or NKG2D, which associates with DAP10 or DAP12 (13, 23). None of the CLR receptors were detected on the cell surface of transduced A20 cells in the absence of an adaptor molecule (Fig. 2C). However, although coexpression with the appropriate adaptor induced surface expression of Mincle and NKG2D, as expected (13, 23), none of the adaptors resulted in surface expression of mCLECSF8-HA. Western blot analyses of immunoprecipitations of the adaptors from the various cell lysates confirmed expression of the various constructs as well as their association with the relevant receptors (Fig. 2D). These results suggest that, unlike other members of this family, CLECSF8 does not associate with DAP10, DAP12, or Fcγ.

**The Intracellular Retention of CLECSF8 Requires the CTLD**—The association of the signaling adaptors with other members of the Dectin-2 cluster involves charged residues in the transmembrane domain or cytoplasmic tail (3). As CLECSF8 lacks these residues and did not associate with any of the known adaptors, we sought to determine which domain of CLECSF8 was responsible for its intracellular retention in



**FIGURE 3. The intracellular retention of CLECSF8 is dependent on the CTLD.** A, anti-HA flow cytometric analyses of the HA-tagged mCLECSF8 chimeras expressed in RAW264.7 and NIH3T3 cells, as indicated. B, anti-HA flow cytometric analyses of NIH3T3 and RAW264.7 cells expressing Mincle or Dectin-2, as indicated. C, flow cytometric analyses of the various Mincle/Dectin-1 and Dectin-2/Dectin-1 chimeras in NIH3T3 and RAW264.7 cells, as indicated. Gray filled histograms represent vector control cells, solid black histograms represent surface expression, and dashed histograms represent total (intracellular and surface) expression.

NIH3T3 cells. For these experiments, we generated chimeric receptors whereby various domains of mCLECSF8 were replaced with that of an unrelated C-type lectin, Dectin-1 (schematic representations of these chimeras are shown in supplemental Fig. 2). We chose to use Dectin-1 for these experiments, as this receptor does not associate with, nor require, an adaptor for cell surface expression (24). Furthermore, the specificity of the Dectin-1 CTLD for  $\beta$ -glucans has been well documented (24–26), allowing the chimeric receptors to be used in functional analyses (see below).

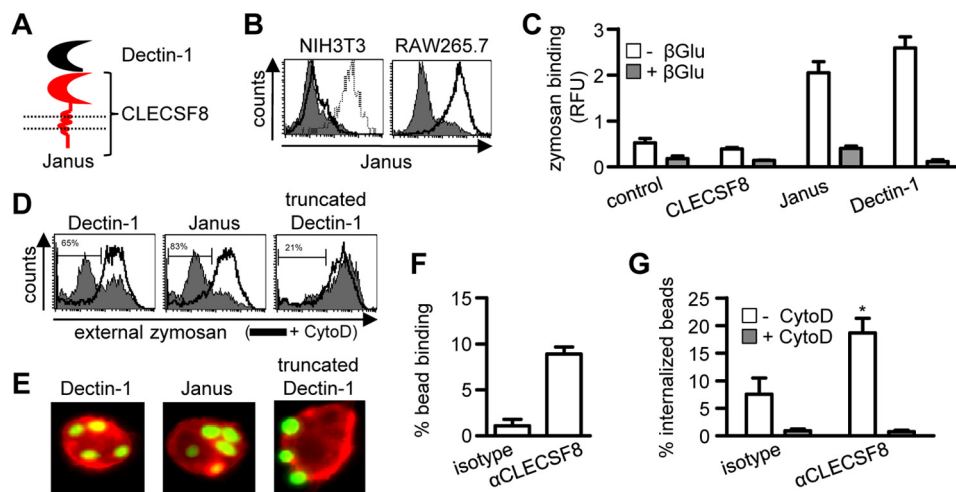
We generated two chimeras; the first comprising the CTLD of Dectin-1 coupled to the transmembrane and intracellular regions of CLECSF8 (termed  $D1^{CTL D}/SF8^{TM-cyto}$ ) and the second consisting of the CTLD of CLECSF8 coupled to the transmembrane and intracellular regions of Dectin-1 (termed  $SF8^{CTL D}/D1^{TM-cyto}$ ). The expression of both of these chimeras was then examined in transduced NIH3T3 and RAW264.7 cells. We found that the chimera  $SF8^{CTL D}/D1^{TM-cyto}$ , containing the CTLD of mCLECSF8, displayed the same expression profile as full-length mCLECSF8 in that it was retained intracellularly in NIH3T3 cells but expressed at the cell surface of RAW264.7 cells (Fig. 3A). In contrast, the chimera  $D1^{CTL D}/SF8^{TM-cyto}$  was expressed on the surface of both cell types. As expected (24), full-length Dectin-1 was expressed on the surface of both NIH3T3 and RAW264.7 cells (data not shown). These findings demonstrate that the CTLD of mCLECSF8 is responsible for its intracellular retention in NIH3T3 cells.

As intracellular retention has not been shown previously to depend on the CTLD, we wondered whether any other members of the Dectin-2 cluster of receptors possessed similar characteristics. We therefore generated HA-tagged full-length Mincle and Dectin-2 as well as chimeras similar to those described above (see supplemental Fig. 2) and examined the expression profile of each of these receptors in transduced NIH3T3 and RAW264.7 cells. Full-length Mincle and Dectin-2 displayed the same profile as full-length mCLECSF8 in that they were expressed on the surface of RAW264.7 macrophages but were retained intracellularly in NIH3T3 cells (Fig. 3B). However, in contrast to CLECSF8, chimeras containing the CTLD of Dectin-1 coupled to the transmembrane and intracellular regions of Mincle ( $D1^{CTL D}/Min^{TM-cyto}$ ) or Dectin-2 ( $D1^{CTL D}/D2^{TM-cyto}$ ) were retained intracellularly in NIH3T3 cells, whereas chimeras consisting of the transmembrane and intracellular regions of Dectin-1 coupled to the CTLD of Mincle ( $Min^{CTL D}/D1^{TM-cyto}$ ) or Dectin-2 ( $D2^{CTL D}/D1^{TM-cyto}$ ) were expressed at the cell surface (Fig. 3C). All chimeras were expressed on the surface of RAW264.7 cells (Fig. 3C). These results, therefore, demonstrate that the intracellular retention of CLECSF8 occurs through a novel mechanism involving its CTLD.

**CLECSF8 Is a Phagocytic Receptor**—As the ligand of CLECSF8 is currently unknown, we set about characterizing the functions of this receptor using a chimeric receptor containing the CTLD of the  $\beta$ -glucan receptor Dectin-1. Although we have used this strategy successfully to characterize the functions of other C-type lectins, such as CLEC9A (21), the expression profile of CLECSF8 (discussed above) suggested that the CTLD of this receptor was important for its intracellular retention and may be involved in its association with any putative signaling adaptor. Thus, we generated a novel chimeric receptor termed Janus, consisting of the HA-tagged CTLD of Dectin-1 fused to the entire mCLECSF8 receptor (Fig. 4A). As we had shown for full-length CLECSF8, we could demonstrate that Janus was retained intracellularly when expressed in NIH3T3 cells and on the surface of RAW264.7 cells (Fig. 4B).

Although phagocytic ability is usually best demonstrated in normally non-phagocytic cells lines, such as NIH3T3 fibroblasts, this was not possible here because of the intracellular retention of CLECSF8 and of Janus within these types of cells. To circumvent this problem, we assessed the ability of RAW264.7 macrophages transduced with Janus, CLECSF8, or Dectin-1 to bind and internalize the  $\beta$  glucan-rich particle zymosan. As expected, the expression of Dectin-1 on these cells conferred the ability to bind zymosan in a  $\beta$  glucan-dependent manner (Fig. 4C). Similar levels of zymosan binding were also obtained by the Janus-expressing cells, demonstrating functionality of the chimeric receptor. Cells expressing full-length CLECSF8 or vector-only control cells showed background levels of zymosan binding (Fig. 4C). We then evaluated the ability of these cells to internalize these particles by flow cytometry and fluorescence microscopy in the presence or absence of cytochalasin D to inhibit actin polymerization (Fig. 4, D and E). Using these methods, we could demonstrate that RAW264.7 cells expressing Dectin-1 were able to internalize zymosan particles in an actin-dependent manner, as expected (18), and we

## Characterization of CLECSF8



**FIGURE 4. CLECSF8 is a phagocytic receptor.** *A*, schematic representation of Janus consisting of the CTLD of Dectin-1 coupled to full-length CLECSF8. *B*, flow cytometric analysis of Janus expression in transduced RAW264.7 and NIH3T3 cells. *Gray filled histograms* represent vector control cells, *solid black histograms* represent surface expression, and *dashed histograms* represent total (intracellular and surface) expression. *C*, quantification of zymosan binding by RAW264.7 cells transduced with vector control, CLECSF8, Janus, or Dectin-1 in the presence (*gray bars*) or absence (*white bars*) of  $\beta$ -glucan ( $\beta$ Glu) as indicated. *RFU*, relative fluorescence units. *D*, FACS-based phagocytosis assay showing the extent of zymosan internalization (*gray histograms*) by RAW264.7 cells expressing Dectin-1, Janus, or truncated Dectin-1. The *bars* indicate the percentage of cells with internalized zymosan. Cytochalasin D (*CytoD*, *unfilled histograms*) was included as a control to inhibit phagocytosis. *E*, fluorescence microscopy showing the interaction of TRITC-phalloidin stained RAW264.7 cells expressing Dectin-1, Janus, or truncated Dectin-1 with FITC-labeled zymosan (*green particles*). *F*, binding of anti-hCLECSF8 or isotype-coated FITC Dynabeads to peripheral blood neutrophils in the presence (*gray bars*) or absence (*white bars*) of cytochalasin D (*CytoD*). Shown are the mean  $\pm$  S.D., and the data are representative of at least three independent experiments. *\**,  $p < 0.05$ .

also observed a similar uptake of particles with cells expressing the Janus chimeric receptor. As a control for these experiments, we included RAW264.7 cells expressing a truncated version of Dectin-1, which lacks the cytoplasmic tail and signaling motifs (18), and observed that these cells were able to bind, but not to internalize, the zymosan particles (Fig. 4, *D* and *E*, and data not shown). The failure of the truncation mutant to mediate zymosan phagocytosis demonstrates that the uptake of zymosan by Janus is not merely being facilitated by enhanced particle binding but that it also involves intracellular signaling from the cytoplasmic tail of this receptor. Therefore these results indicate that CLECSF8 can function as a phagocytic receptor.

To examine whether CLECSF8 could function similarly on primary cells, we explored the ability of PMNs to ingest anti-hCLECSF8-coated, FITC-labeled,  $\sim 4.5$ - $\mu$ m Dynabeads. We could demonstrate that these beads bound specifically to RAW264.7 cells expressing hCLECSF8 (Fig. 4*F*). Furthermore, these beads were bound and internalized by PMNs to a much greater extent than the isotype control beads. As we had observed for the RAW264.7 cells, internalization of these beads occurred in an actin-dependent manner, as it could be inhibited by treatment with cytochalasin D (Fig. 4*G*). Overall, these data, therefore, indicate that CLECSF8 can function as a phagocytic receptor.

CLECSF8 induces the production of TNF. Many C-type lectin receptors can induce the production of cytokines (3, 6). Thus, we next determined if the stimulation of CLECSF8 would also induce this type of response. For these experiments, we made use of the RAW264.7 macrophages expressing Janus and explored their ability to induce the production of the proinflammatory cytokine TNF following stimulation with zymosan, using an approach we had used previously to characterize Dectin-1 (20) and CLEC9A (21). In comparison to the vector-only

controls, the expression of Janus was found to induce TNF in response to zymosan (Fig. 5*A*, *gray bars*). The ability of soluble  $\beta$ -glucans to inhibit this response (*black bars*) demonstrated the dependence on the Dectin-1 CTLD for ligand recognition in the chimeric receptor. Cells expressing full-length Dectin-1 were also able to induce TNF, as expected (20), and served as a control in these experiments.

We and others have shown previously that the production of TNF by macrophages in response to zymosan requires collaborative signaling between Dectin-1 and TLR2 (20, 27, 28). To determine whether cytokine production induced through the chimeric CLECSF8 receptor also involved collaborative signaling with the TLRs, we stimulated Janus-expressing RAW264.7 cells with combinations of specific receptor agonists. Stimulation with particulate  $\beta$ -glucan (to target the Dectin-1 CTLD of the chimera) or LPS (to target TLR4) alone, induced low levels of TNF. However, when added together, the stimuli were strongly synergistic (Fig. 5*B*). Furthermore, costimulation of mCLECSF8-expressing RAW264.7 cells with antibody-coated beads and LPS also resulted in the induction of TNF (Fig. 5*C*). Thus, these data show that stimulation of CLECSF8 can induce proinflammatory cytokine production, particularly when acting cooperatively with the TLRs.

**Cellular Responses Mediated by CLECSF8 Do Not Require the CTLD**—Our results indicate that CLECSF8 associates with a novel adaptor to induce its intracellular signaling, which possibly involves an interaction with its CTLD. To directly determine whether the CTLD of CLECSF8 is required, we examined the ability of the D1<sup>CTL</sup>/SF8<sup>TM-cyto</sup> chimera to mediate phagocytosis and TNF production in response to zymosan. This construct was expressed at the cell surface in RAW264.7 cells (see Fig. 3*A*) and could mediate zymosan binding (results not shown). Furthermore, we could demonstrate that cells express-

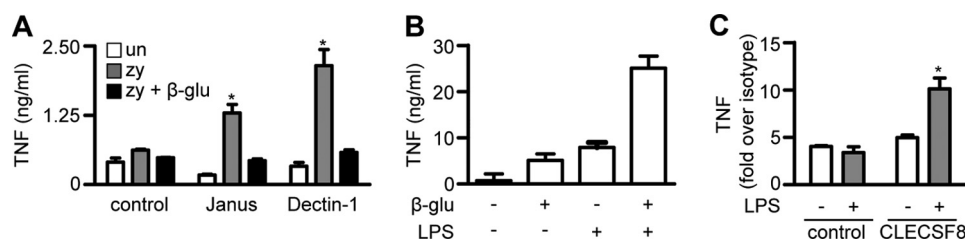


FIGURE 5. **CLECSF8 can induce proinflammatory cytokine production.** *A*, induction of TNF in RAW264.7 cells transfected with vector control, Janus, or Dectin-1 following stimulation with zymosan (zy) in the presence (black bars) or absence (gray bars) of  $\beta$ -glucan ( $\beta$ -glu). Unstimulated (un) cells are included as a control. \*,  $p < 0.05$  compared with control cells. *B*, TNF production by RAW264.7 cells transfected with Janus and stimulated with 100  $\mu$ g/ml  $\beta$ -glucan or 10 ng/ml LPS alone or in combination, as indicated. The data shown are mean  $\pm$  S.D. and are representative of at least three independent experiments. *C*, relative TNF levels produced by RAW264.7 cells transfected with HA-tagged mCLECSF8 stimulated with anti-HA- or isotype-coated beads with or without LPS, as indicated. The data shown are mean fold difference  $\pm$  S.D. of the anti-HA TNF responses versus those of the isotype controls. The data are representative of two independent experiments.

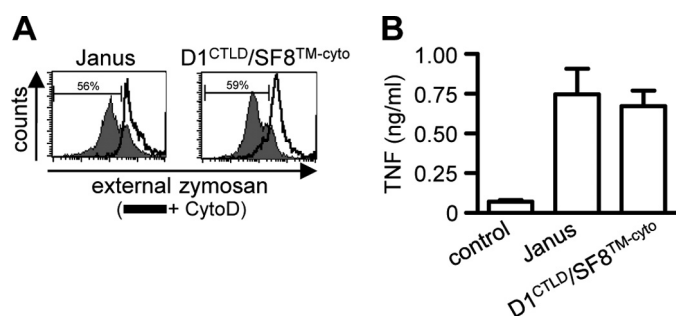


FIGURE 6. **The CTLD of CLECSF8 is not required for signaling.** *A*, FACS-based phagocytosis assay showing the extent of zymosan internalization (gray histograms) by RAW264.7 cells expressing Janus or the D1<sup>CTLD</sup>/SF8<sup>TM-cyto</sup> chimera. The bars indicate the percentage of cells with internalized zymosan. Cytochalasin D (CytoD, unfilled histograms) was included as a control to inhibit phagocytosis. *B*, induction of TNF in RAW264.7 cells transfected with vector control, Janus, or the D1<sup>CTLD</sup>/SF8<sup>TM-cyto</sup> chimera following stimulation with zymosan. \*,  $p < 0.05$  compared with control cells. The data are representative of at least two independent experiments.

ing the D1<sup>CTLD</sup>/SF8<sup>TM-cyto</sup> chimera were able to phagocytose zymosan (Fig. 6*A*) and induce TNF (*B*) in response to these particles to a level comparable with that induced by the Janus-expressing cells. Thus, these data show that transmembrane region and cytoplasmic tail, but not the CTLD, of CLECSF8 are sufficient to mediate intracellular signaling.

CLECSF8 can signal via Syk kinase. We have shown previously that the collaborative signaling between the TLRs and Dectin-1 requires Syk kinase (27). Thus, we explored the possibility that this kinase was also involved in the signaling mediated by CLECSF8. To this end, we first examined the effect of piceatannol, a Syk kinase inhibitor, on CLECSF8-mediated TNF production (in transduced RAW264.7 macrophages) and phagocytosis (in PMNs), and in both cases we observed that the presence of piceatannol significantly inhibited these responses (Fig. 7*A*). Similarly, we could show specific inhibition of zymosan-induced, but not LPS-induced, TNF production following treatment of Janus-expressing RAW264.7 macrophages with another Syk inhibitor, R406 (supplemental Fig. 3*A*).

To demonstrate that CLECSF8 was capable of signaling via Syk-kinase more directly, we isolated signaling complexes from RAW264.7 cells expressing hCLECSF8, mCLECSF8, or Dectin-1 by immunoprecipitation following treatment of the cells with or without pervanadate. Probing Western blot analyses of the immunoprecipitates with an anti-phosphotyrosine antibody revealed a tyrosine-phosphorylated molecule of  $\sim$ 70 kDa in both CLECSF8 and Dectin-1 precipitates from pervanadate-

stimulated cells that was identified to be Syk kinase (Fig. 7*B* and supplemental Fig. 3*B*). Furthermore, probing duplicate blots with an anti-phospho-Syk antibody demonstrated that the active form of this kinase was associated with these receptors following pervanadate stimulation (Fig. 7*B*). We could also directly demonstrate Syk activation during the uptake of zymosan in Janus-expressing RAW264.7 macrophages by immunofluorescence microscopy (Fig. 7*C*). However, despite considerable effort, we were unable to verify the association of Syk with CLECSF8 in PMNs (data not shown), a failure which we attribute to the very low levels of expression of this receptor on these primary cells. In fact, we were also unable to demonstrate immunoprecipitation of CLECSF8 itself from these cells because there is no appropriate antibody available for this analysis. Thus, these results suggest that CLECSF8 is able to mediate intracellular signaling via Syk kinase.

CLECSF8 can induce the respiratory burst. The ability of neutrophils to induce the respiratory burst is an important antimicrobial mechanism that can be stimulated by some C-type lectins, including Dectin-1 (29, 30). We therefore examined the ability of CLECSF8 to induce the respiratory burst by stimulating RAW264.7 macrophages expressing Janus (the CLECSF8 chimera) or Dectin-1 with zymosan and observed that the stimulation of these cells resulted in a robust respiratory burst response (Fig. 8*A*). In contrast, vector-only control cells or cells expressing full-length CLECSF8 failed to respond in this manner to zymosan.

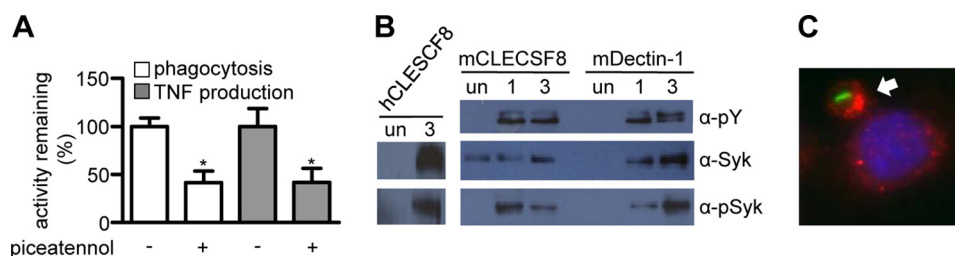
To demonstrate that CLECSF8 was able to induce this response in primary cells, we cultured purified PMN on anti-hCLECSF8 immobilized on plastic to determine whether specific stimulation of this receptor was able to induce a respiratory burst. Indeed, we observed that cross-linking of this receptor resulted in greatly increased reactive oxygen species production when compared with the isotype control (Fig. 8*B*). Thus, these results demonstrate that CLECSF8 is capable of inducing the respiratory burst in leukocytes.

## DISCUSSION

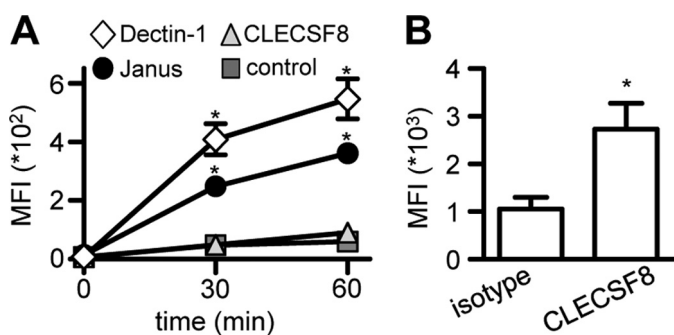
Recent interest in the Dectin-2 cluster of C-type lectins has provided significant new insights into the underlying mechanisms of immunity and homeostasis (3). Here we have characterized CLECSF8, one of the least well understood members of this group, and provide evidence that this receptor is expressed only by selected populations of myeloid cells and is capable of



## Characterization of CLECSF8



**FIGURE 7. CLECSF8 can signal via Syk kinase.** *A*, characterization of the effect of piceatannol on the ability of CLECSF8 to mediate phagocytosis in PMNs (white bars, see Fig. 4) and cytokine production in Janus expressing RAW264.7 cells (gray bars, see Fig. 5). The data shown are mean  $\pm$  S.E. normalized to the untreated cells and are pooled data from at least two independent experiments.  $^*p < 0.05$  compared with control cells. *B*, Western blot analyses of anti-HA immunoprecipitates from RAW264.7 cells transduced with hCLECSF8, mCLECSF8, or Dectin-1. Cells were either unstimulated (un) or stimulated for 1 or 3 min with pervanadate. Blots were probed with anti-phosphotyrosine ( $\alpha$ -pY), anti-Syk, and anti-phospho-Syk ( $\alpha$ -pSyk), as indicated. See supplemental Fig. 3B for an example of loading controls. *C*, fluorescence microscopy showing pSyk association (red) with phagosomes following the uptake of FITC-labeled zymosan (green) by RAW264.7 cells expressing Janus. Nuclei are stained with DAPI (blue).



**FIGURE 8. CLECSF8 can induce the respiratory burst.** *A*, RAW264.7 cells transfected with the vector control, Janus, mCLECSF8, or Dectin-1 were stimulated with zymosan for the indicated amount of time, and the respiratory burst was measured by assessing the conversion of dihydrorhodamine 123 to rhodamine. *B*, stimulation of peripheral blood neutrophils for 30 min on anti-CLECSF8 or isotype coated plates. The data are expressed as mean fluorescent intensity (MFI)  $\pm$  S.E. of triplicates pooled from three different donors.  $^*p < 0.05$  versus control.

triggering cellular activation. Originally described as a monocyte/macrophage-restricted receptor (14, 15), we observed that expression of CLECSF8 was limited to CD14<sup>+</sup> CD16<sup>-</sup> monocytes in peripheral blood, which are the subset thought to be recruited to sites of inflammation where they differentiate into macrophages and DC (31). Interestingly, CLECSF8 was expressed at low levels on the major circulating DC subsets, and we found that expression of this receptor became down-regulated upon differentiation of monocytes into macrophages or DCs *in vitro*. CLECSF8 was also expressed on circulating neutrophils, but we did not detect this receptor on any other PBL population.

Using a variety of approaches in both primary cells and in transfected cell lines, we demonstrated that CLECSF8 can induce phagocytosis, cytokine production, and the respiratory burst. Thus, like several other members of the Dectin-2 cluster, CLECSF8 is capable of triggering cellular activation. How this receptor mediates these activities is still unclear, as CLECSF8 lacks recognizable signaling motifs in its cytoplasmic tail. The transmembrane/cytoplasmic tail regions of the other Dectin-2 cluster activation receptors associate with the FcR $\gamma$  chain, which triggers intracellular signaling through Syk kinase (3, 4). Although we could demonstrate that signaling from CLECSF8 required similar domains of this receptor and involved Syk kinase, this receptor did not associate with the FcR $\gamma$  chain nor other adaptors that have been implicated in C-type lectin sig-

naling, such as DAP12 or DAP10 (2). This suggests that CLECSF8 does not associate with a typical adaptor protein. However, given the unusual mechanism of intracellular retention (discussed below), it is possible that CLECSF8 does associate with these adaptors in myeloid cells. However, we did not detect association of any of these adaptors with CLECSF8 following immunoprecipitation of this receptor from transfected RAW264.7 macrophages (data not shown). These observations need to be confirmed in adaptor knockout macrophages.

These adaptors, in addition to mediating intracellular signaling, are required for expression of the C-type receptors at the cell surface (3). With most C-type lectins, this association is mediated by a positively charged residue in the transmembrane domain (13, 32, 33). However, with Dectin-2, the association with FcR $\gamma$  requires a membrane-proximal region of the cytoplasmic tail (34). Although we found that the transmembrane and cytoplasmic regions were sufficient to mediate intracellular signaling, surface expression of CLECSF8 involved the CTLD. This suggests that CLECSF8 may interact with a novel adaptor for transport to the cell surface. Although the nature of this protein is still unclear, it could be another C-type lectin, reminiscent of the association between NGK2A and CD94, that is required for efficient surface expression of this complex (35).

Despite the novel insights into the functions of CLECSF8 described here, the ligands and physiological role of this receptor are still unclear. All of the Dectin-2 cluster of receptors, except CLECSF8, are "classical" C-type lectins possessing the conserved residues required for binding carbohydrates. CLECSF8, on the other hand, retains the residues required for calcium coordination, but lacks the conserved triplet motif that is normally associated with sugar recognition (3). Indeed, screening a carbohydrate microarray using a soluble CLECSF8-Fc fusion protein as a probe did not reveal any ligands for this receptor (supplemental Fig. 4). Given the diverse repertoire of molecules that have been identified as ligands for C-type lectin receptors in general and the Dectin-2 cluster of receptors in particular, it is impossible to predict the nature of the ligand(s) of CLECSF8.

Similar to other members of the family, which have been shown to recognize fungi, bacteria, and viruses, CLECSF8 may play a role in pathogen recognition (8–12, 36). Expression of CLECSF8 on circulating neutrophils, in particular, suggests a role in innate immunity because these short-lived cells play

crucial roles in the first line of defense against invading pathogens. Indeed, the ability of CLECSF8 to induce proinflammatory cytokine production, phagocytosis, and the respiratory burst are all suggestive of a role in pathogen recognition and clearance. However, we have extensively characterized the CLECSF8<sup>-/-</sup> mice available from the Consortium for Functional Glycomics and have not found any defects in their ability to resist infection with pathogenic fungi (*Candida albicans*), extracellular bacteria (*Staphylococcus aureus*), intracellular bacteria (*Listeria monocytogenes*), or nematodes (*Nippostrongylus brasiliensis*) (supplemental Fig. 5).

Another possibility is that CLECSF8, like other members of the Dectin-2 family, recognizes endogenous ligand(s) and functions in controlling homeostasis. Mincle, for example, recognizes a nuclear protein (SAP130) released from necrotic cells, resulting in the production of proinflammatory cytokines and infiltration of neutrophils to the site of necrosis (13). Furthermore, the inhibitory receptor DCIR has been shown to play a role in preventing autoimmune arthritis by controlling DC expansion, although the endogenous ligand of this receptor remains unknown (37). However, we observed no effect of CLECSF8 deficiency in murine models of sterile peritonitis (supplemental Fig. 6), response to necrotic cell death (supplemental Fig. 7), experimental autoimmune uveoretinitis (supplemental Fig. 8), or the development of spontaneous autoimmune arthritis (data not shown). Thus, despite extensive analysis, we still have no insights into the physiological role of CLECSF8.

In summary, we have demonstrated that surface expression of CLECSF8 involves novel interactions with its CTLD and that it functions as an activation receptor by triggering intracellular signaling through an unidentified adaptor molecule. The identification of this adaptor and the ligands and physiological role of CLECSF8 are the immediate issues that require further attention and are currently under investigation.

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