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CLEC-2 Is a Phagocytic Activation Receptor Expressed on Murine Peripheral Blood Neutrophils¹

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CLEC-2 is a member of the “dectin-1 cluster” of C-type lectin-like receptors and was originally thought to be restricted to platelets. In this study, we demonstrate that murine CLEC-2 is also expressed by peripheral blood neutrophils, but only weakly by bone marrow or elicited inflammatory neutrophils. On circulating neutrophils, CLEC-2 can mediate phagocytosis of Ab-coated beads and the production of proinflammatory cytokines, including TNF- α , in response to the CLEC-2 ligand, rhodocytin. CLEC-2 possesses a tyrosine-based cytoplasmic motif similar to that of dectin-1, and we show using chimeric analyses that the activities of this receptor are dependent on this tyrosine. Like dectin-1, CLEC-2 can recruit the signaling kinase Syk in myeloid cells, however, stimulation of this pathway does not induce the respiratory burst. These data therefore demonstrate that CLEC-2 expression is not restricted to platelets and that it functions as an activation receptor on neutrophils. *The Journal of Immunology*, 2009, 182: 4150–4157.

Myeloid cells express an extensive collection of cell surface receptors that are involved in a diverse range of functions ranging from microbial recognition and activation of cellular responses to cell development, migration, proliferation, maturation, and survival. Of specific interest are the Group V C-type lectin-like receptors, which are type II transmembrane proteins, with a single extracellular carbohydrate recognition domain (CRD),⁴ a transmembrane region, and a cytoplasmic tail that may contain signaling motifs (1, 2). Within this group is a subgroup of receptors known as the “dectin-1 cluster,” which is encoded in a distinct genetic locus within the NK gene complex (3, 4). This subgroup consists of dectin-1, LOX-1 (lectin-like oxidized low-density lipoprotein-1), MICL (myeloid inhibitory C-type lectin-like receptor),

CLEC9A, CLEC12B, and CLEC-1 and 2. Unlike other Group V C-type lectins, which are predominantly expressed on NK and T cells (5), most of the receptors in the dectin-1 cluster are found on myeloid populations and function in homeostasis and immunity (4, 6–9).

CLEC-2 was originally identified through a computational screen for C-type lectin-like receptors (10). To date, surface expression of CLEC-2 has only been shown on platelets, although RT-PCR analysis has shown transcripts in PBMC, bone marrow cells, monocytes, dendritic cells, and granulocytes (10, 11). Podoplanin, a sialoglycoprotein involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation, has recently been identified as a physiological ligand for CLEC-2, and it has been suggested that their interaction may be involved in tumor growth and/or metastasis (12–14). Exposure of platelets to rhodocytin, a snake venom toxin that is also a ligand for CLEC-2, leads to tyrosine phosphorylation of a cytoplasmic ITAM-like motif and Syk-dependent platelet activation (11). In addition to Syk, Src and Tec family kinases, PLC γ , and Rac1 are also involved in the signaling pathway activated by CLEC-2 (15, 16). Furthermore, CLEC-2 has been identified as an HIV-1 attachment factor that may capture and transfer infectious HIV-1 in cooperation with DC-SIGN (17). There are two further splice variants of murine CLEC-2 (mCLEC-2), with different expression profiles and subcellular localization compared with full-length CLEC-2 and in addition, full-length CLEC-2 can be cleaved into a soluble homodimeric form (18).

We are interested in the dectin-1 cluster of receptors and wondered whether CLEC-2, like the other receptors in the cluster, was also expressed on myeloid cells. We show in this study that in addition to platelets, mCLEC-2 is expressed on peripheral blood neutrophils as well as on monocytes activated with selected TLR ligands. We demonstrate that this receptor can function as an activation receptor on these cells, inducing phagocytosis and proinflammatory cytokine production, but not the respiratory burst.

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⁴ Abbreviations used in this paper: CRD, carbohydrate recognition domain; CLEC, C-type lectin like receptor; mCLEC-2, murine CLEC-2; HA, hemagglutinin.

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Materials and Methods

Cell lines and growth conditions

NIH3T3 and HEK293T fibroblasts, RAW264.7 macrophages, EL4 T cells, A20 B cells, HEK293T-based Plat-E ecotropic retroviral packaging cells (a gift from Professor Kitamura, University of Tokyo, Tokyo, Japan), and Syk deficient (C35) and Syk-reconstituted (WT8) B cell lines (19), were maintained in DMEM or RPMI 1640 (Cambrex) supplemented with 10% FCS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Cambrex) and cultured at 37°C with 5% CO₂.

Generation of constructs and transduced cell lines

The complete murine CLEC-2 and a hemagglutinin (HA)-tagged version of the receptor were amplified from mouse spleen cDNA by PCR. The CLEC-2/dectin-1 chimera was also generated by PCR, such that the chimeric sequence translated as CLEC-2¹⁻²⁷/dectin-1⁴⁴⁻²⁴⁴, generating a chimeric receptor consisting of the cytoplasmic tail of CLEC-2 and the transmembrane, stalk, and CRD of dectin-1. We also generated a chimeric ITAM mutant in which the tyrosine in the ⁵DxYxxL¹¹ cytoplasmic motif was mutated to a phenylalanine (referred to in the text as Y7F). All constructs were cloned into the pFBneo (Stratagene) retroviral vector, packaged into virions using Plat-E ecotropic packaging cells, and the various cell lines were transduced as previously described (20). All cell lines were used as nonclonal populations to reduce founder effects and were generated at least twice to confirm phenotype. A purified soluble fusion protein, Fc-CLEC-2, containing the CRD and stalk region of CLEC-2, fused to human IgG1 Fc, was generated by PCR and cloned into the pSecTag2 vector, essentially as described for dectin-1 (21, 22).

Primary cells and stimulations

Six- to 12-wk-old BALB/c, 129/Sv, or C57BL/6 mice, obtained from the specific pathogen-free animal unit at the University of Cape Town (UCT), were used in these experiments. All procedures were approved by the UCT animal ethics committee. Bone marrow and peripheral blood was collected as described previously (23). Thioglycollate-elicited peritoneal neutrophils were isolated by standard procedures and identified by their high expression of Gr-1. To isolate neutrophils, peripheral blood was collected by cardiac puncture into a final concentration of 10 mM EDTA, and leukocytes were separated by centrifugation over a 3-layer (78, 66, and 54%) Percoll Plus (GE Healthcare) gradient at 4°C. Neutrophils were harvested from the 77/66% interface and the remaining erythrocytes lysed using Gey's solution. Cell viability was generally ≥95% as determined by trypan blue staining.

To test the effect of TLR agonists on CLEC-2 surface expression, PBLs were plated in 24-well plates and stimulated for 6 h with Pam3CSK4 (TLR2/1, 100 ng/ml), LPS (TLR4, 100 ng/ml), flagellin (TLR5, 20 ng/ml), and FSL-1 (TLR2/6, 20 ng/ml) (all TLR agonists from Invivogen). Cells were then analyzed by flow cytometry, as described below.

Generation and purification of polyclonal and mAbs against CLEC-2

A purified polyclonal Ab, specific for CLEC-2, was affinity purified from the serum of Wistar rats after immunization with Fc-CLEC-2. mAb production was performed at Cancer Research U.K., Wistar rats were immunized three to four times with RBL-2H3 cells expressing mouse CLEC-2 fused to an HA epitope. Fusion of splenocytes with the rat myeloma cell line Y3 was conducted using standard procedures. Hybridoma screening was conducted as previously reported (9).

FACS analysis

FACS was performed on live cells according to conventional protocols at 4°C in the presence of 2 mM NaN₃, as previously described (23). The Abs used in these studies include: anti-CD61-biotin (BD Pharmingen), PE donkey anti-rat IgG (Jackson Immunoresearch Laboratories), anti-HA (clone 16B12, Covance), 2A11 (anti-dectin-1) (24), 5C6-FITC (anti-CD11b) (25), Gr-1-biotin, 7/4-FITC, and F4/80-biotin were produced in house. Isotype-control Abs were either obtained from the same suppliers or produced in house. Biotinylated Abs were detected using streptavidin-allophycocyanin (BD Pharmingen).

Phagocytosis assays

Phagocytosis was quantified in NIH3T3 and RAW264.7 cells as previously described (26, 27). In brief, transduced cells were seeded at 2.5 × 10⁵ cells/well the day before the assay. To inhibit phagocytosis, some

cells were pretreated with 10 μM Cytochalasin D (Calbiochem) for 40 min before and throughout the assay. After washing, FITC-zymosan (Molecular Probes, 1 to 5 particles/cell) or anti-CLEC-2 coated Dynabeads (Invitrogen, 1 bead/cell) labeled with FITC, were added and allowed to settle for 1 h at 4°C. After washing to remove unbound particles, cells were incubated at 37°C for various times, as indicated. External zymosan was detected with anti-zymosan Abs, as described (26). For the detection of external anti-CLEC-2 coated FITC-labeled Dynabeads, cells were incubated with a PE-conjugated anti-rat Ab. FITC⁺ cell populations which had bound or internalized beads were gated, and the percentage of phagocytosis was determined by comparing the PE⁻ to the PE⁺ cell populations.

For microscopy, cells were fixed and permeabilized, and actin was stained with 1 μM tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma-Aldrich). Syk recruitment was detected with anti-phospho-Syk (Cell Signaling Technologies), followed by cyanine (Cy)-3-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories). Coverslips were mounted with Vectashield (Vector Laboratories) containing Hoechst nuclear dye and cells were observed by confocal laser scanning microscopy on a Zeiss LSM 510 META confocal microscope. Images were processed using Adobe Photoshop version 6.0.

Phagocytosis in PBLs was determined similarly, except, FITC labeled Dynabeads, coated with anti-CLEC-2, 2A11 or isotype control Abs, were added at a ratio of two beads/cell. Following incubation with rotation for 1 h at 4°C, unbound beads were removed by centrifugation over a two layer Percoll Plus gradient (40 and 70%) and the cells harvested following centrifugation. Phagocytosis, in the presence or absence of 5 μM Cytochalasin D, was allowed to occur at 37°C for 45 min. External beads were detected with a PE-conjugated anti-rat Ab, as described above, and granulocytic FITC⁺ cell populations which had bound or internalized beads were gated, and the percentage of phagocytosis was determined by comparing the PE⁻ to the PE⁺ cell populations.

Fluorescent zymosan binding, respiratory burst, and cytokine production assays

Zymosan binding and TNF-α production by transduced RAW264.7 cells in the presence or absence of soluble β-glucan was determined as previously described (24). To examine cytokine production from primary cells, murine neutrophils were purified as described and plated at 3 × 10⁵ cells/well. Cells were left unstimulated or stimulated with 15 μg/ml rhodocytin, purified as described previously (28), or 1 μg/ml LPS (Sigma-Aldrich), for 6 h at 37°C. TNF-α in supernatant was measured using the OptEIA mouse TNF-α ELISA set (BD Biosciences). For the analysis of the Syk-sufficient and Syk-deficient B-cells, 2 × 10⁶ transduced cells were stimulated with various concentrations of unlabelled zymosan (Sigma-Aldrich) for 16 h at 37°C and IL-2 secreted into the supernatants was quantified by OptEIA mouse IL-2 ELISA set (BD Biosciences).

For analysis of the respiratory burst, cells were loaded with dihydrorhodamine 123 (Sigma-Aldrich) at a final concentration of 2 μM. After incubation for 1 h at 37°C with zymosan (25 particles/cell), 15 μg/ml rhodocytin, or 1 mg/ml PMA, the conversion of dihydrorhodamine 123 to rhodamine was assessed by flow cytometry.

Immunoprecipitations

Immunoprecipitations from RAW macrophages were performed as previously described (6), except pervanadate-stimulated RAW264.7 cell lysates were added to streptavidin beads precoupled with tyrosine phosphorylated or unphosphorylated biotinylated CLEC-2 signaling peptides (25 μM; MQDEDGYITLNIKPR; Cancer Research U.K. Peptide Synthesis Laboratory). The dectin-1 peptides have been described previously (29). For immunoprecipitations from A20 cells expressing a HA-tagged version of CLEC-2, 1 × 10⁷ cells were precoated with anti-HA Ab and then stimulated with pervanadate and lysed as previously described (6). Cell lysates were added to sheep anti-rat IgG coated Dynabeads and rotated for 2 h at 4°C. Beads were washed extensively before analysis by Western blotting. Proteins in the immunoprecipitates were detected with anti-phosphotyrosine (clone 4G10) or anti-Syk (Santa Cruz Biotechnology), followed by appropriate HRP-linked secondary Abs (Jackson Immunoresearch Laboratories).

Results

Murine CLEC-2 is expressed on peripheral blood neutrophils

To explore the expression of CLEC-2, we generated affinity purified polyclonal and a mAb to this receptor. The specificity of these

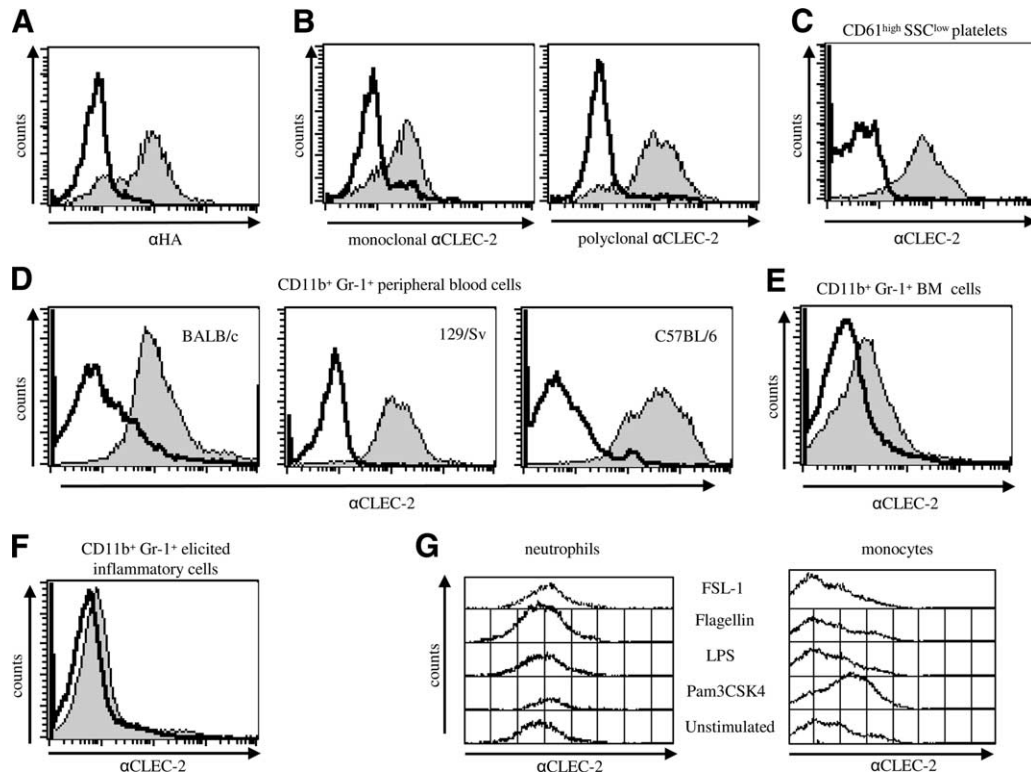


FIGURE 1. mCLEC-2 is expressed on the surface of peripheral blood neutrophils. *A*, Anti-HA staining of EL4 cells, as determined by flow cytometry, showing expression of HA-tagged mCLEC-2 (filled histogram) at the cell surface. The vector-only control cells are indicated by the unfilled histogram. *B*, The monoclonal and affinity-purified polyclonal Ab raised against CLEC-2 specifically recognize EL4 cells transduced with HA-tagged mCLEC-2 (filled histogram), but not vector-only control cells (unfilled histogram) as determined by flow cytometry. *C*, mCLEC-2 is expressed on the surface of CD61^{high}SSC^{low} platelets (filled histogram). *D*, Expression of mCLEC-2 (filled histogram) on CD11b⁺Gr-1⁺ peripheral blood neutrophils from BALB/c, C57BL/6, and 129/Sv mice, as indicated. *E*, mCLEC-2 is only weakly expressed on CD11b⁺Gr-1⁺ bone marrow cells and *F*, elicited inflammatory neutrophils. *C–F*, The polyclonal Ab was used for staining and isotype control staining is indicated by the unfilled histogram. The data shown are representative of at least three independent experiments. *G*, Regulation of mCLEC-2 expression on neutrophils and monocytes following stimulation with various TLR agonists, as detected with monoclonal anti-CLEC-2 by flow cytometry. The data show results of PBLs pooled from 18 mice.

Abs was demonstrated by staining EL4 cells transduced with HA-tagged CLEC-2 or vector-only controls (Fig. 1*A*). Analysis by flow cytometry demonstrated that both the polyclonal and mAbs specifically recognized CLEC-2 expressed on these cells (Fig. 1*B*).

Using these Abs, we first confirmed CLEC-2 expression on platelets, and could detect expression of this receptor on the surface of CD61^{high}SSC^{low} cells, as previously described (11) (Fig. 1*C*). To determine whether CLEC-2 was also expressed on other cell types, we then examined PBLs from BALB/c mice using a variety of markers to distinguish the various cellular populations (30), and could clearly detect expression of CLEC-2 on the surface of CD11b⁺Gr-1^{high} neutrophils (Fig. 1*D* and data not shown). The expression of this receptor on these cells was not dependent on the mouse strain, as similar levels of expression were also detected in other strains including C57BL/6 and 129/Sv mice (Fig. 1*D*). We did not detect CLEC-2 on any other cell population in the blood (data not shown). Thus, these data demonstrate that expression of CLEC-2 is not restricted to platelets, and that this receptor is also expressed by peripheral blood neutrophils.

Under normal conditions, the majority of neutrophils are located in the bone marrow, and only a small fraction of these cells is released into the blood, from where they can be recruited to sites of inflammation (31). However, when we characterized CLEC-2 expression in the bone marrow or on 18-h thioglycollate elicited peritoneal neutrophils, we found that expression of this receptor was much lower on these cells (Fig. 1, *E* and *F*). Similar findings

were obtained in all mouse strains examined (data not shown). Thus, these results suggest that expression of CLEC-2 appears to be up-regulated upon neutrophil emigration from the bone marrow into the peripheral blood, but down-regulated again following recruitment to sites of inflammation.

Regulation of CLEC-2 expression

As CLEC-2 expression was down-regulated on recruited inflammatory neutrophils (Fig. 1*F*), we determined whether stimulation of peripheral blood neutrophils with microbial agonists could also induce regulation of this receptor, as has been described for other dectin-1 cluster molecules, such as MICL (32). We examined CLEC-2 expression by flow cytometry following a 6 h stimulation of PBLs with a variety of TLR agonists, but did not observe any significant regulation of surface expression of neutrophil-expressed CLEC-2 (Fig. 1*G*). However, CLEC-2 expression was observed to increase on monocytes defined by FSC and SSC profiles (30), following stimulation with Pam3CSK4, a TLR2/TLR1 agonist (Fig. 1*G*). Thus, these results suggest CLEC-2 is not directly regulated on neutrophils following microbial stimulation, but that these conditions can induce up-regulation of the receptor on other leukocytes.

CLEC-2 mediates phagocytosis

Having identified CLEC-2 on neutrophils, we next wished to determine the function of this receptor on these cells. As CLEC-2

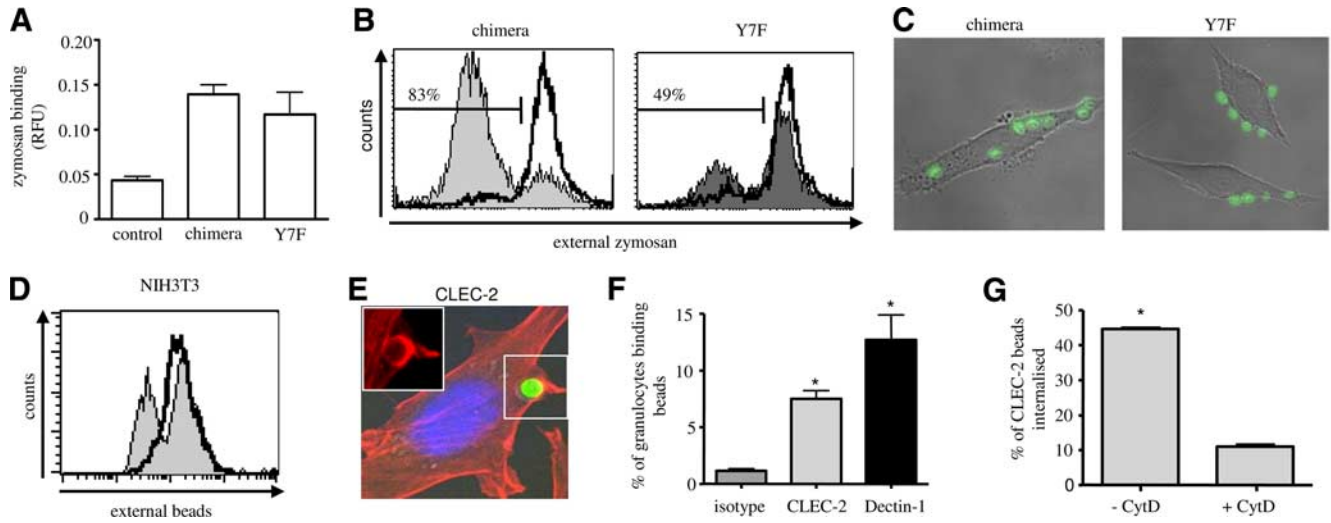


FIGURE 2. mCLEC-2 mediates phagocytosis. *A*, Quantitation of zymosan binding by transduced NIH3T3 fibroblasts. *B*, FACS-based analysis of phagocytosis, showing the extent of zymosan internalisation (gray histograms) by NIH3T3 cells expressing the constructs, as indicated. Cytochalasin D (unfilled histograms) was used to inhibit actin polymerization and served as a control in this assay. The histograms shown are representative of at least three independent experiments, and the bars indicate the percentage of cells with internalized particles. *C*, Confocal image demonstrating FITC-zymosan uptake by RAW264.7 cells expressing the chimera, but not by cells expressing the Y7F chimera. *D*, FACS-based analysis demonstrating uptake of anti-CLEC-2 coated Dynabeads (gray histogram) by NIH3T3 cells expressing the chimeric receptor. Cytochalasin D treated cells (black histogram) were included as a control. *E*, Confocal image showing an NIH3T3 cell expressing mCLEC-2 internalising a FITC-labeled anti-CLEC-2 coated dynabead. *Inset* shows the presence of an actin-based (red) phagocytic cup around the particle. *F*, In comparison to isotype-coated beads, granulocytes specifically recognize anti-CLEC-2 and anti-dectin-1 coated dynabeads. *G*, Cells binding the anti-CLEC-2 coated dynabeads internalize these particles in an actin dependent fashion. *, $p < 0.05$ vs control (Student's *t* test).

contains a tyrosine-based ITAM-like sequence, which is similar to that used to mediate phagocytosis by dectin-1 (15, 26), we explored the possibility that CLEC-2 could also mediate particle uptake. For these experiments, we initially examined the phagocytic potential of CLEC-2 using a chimeric receptor consisting of the extracellular and transmembrane regions of dectin-1, fused to the cytoplasmic tail of CLEC-2. This chimeric receptor would allow us to trigger CLEC-2 signaling using zymosan, a defined particulate ligand for the CRD of dectin-1 (33), and is a strategy we have successfully used previously to characterize the phagocytic potential of other receptors in the dectin-1 cluster (6, 20).

We generated NIH3T3 fibroblast cell lines stably expressing the chimeric receptor (data not shown) and examined the ability of these normally nonphagocytic cells to bind and internalize zymosan. Where indicated, cytochalasin D was included to inhibit actin polymerization, and hence particle uptake. As expected, expression of the chimeric receptor in the NIH3T3 cells conferred an ability to bind FITC-labeled zymosan, (Fig. 2*A*). Furthermore, the cells were able to internalize these particles, in an actin-dependent manner (Fig. 2*B*).

To evaluate whether the cytoplasmic tyrosine of CLEC-2 contributes to this activity, we generated a chimeric receptor construct in which the tyrosine within the cytoplasmic ITAM-like motif was mutated to a phenylalanine (Y7F). Expression of the Y7F construct in NIH3T3 fibroblasts was comparable to wild-type chimeric receptor (data not shown) and it was equally capable of conferring the ability to bind zymosan (Fig. 2*A*). However, mutation of the cytoplasmic tyrosine significantly reduced the ability of these cells to internalize the zymosan particles (Fig. 2*B*). Similar results were also obtained when these chimeric receptors were expressed in RAW264.7 macrophages (Fig. 2*C*). Thus, these data demonstrate that the cytoplasmic tail of CLEC-2 can mediate phagocytosis and that this activity is largely mediated through the cytoplasmic ITAM-like motif.

To prove that CLEC-2 can mediate phagocytosis in primary cells, we made use of Ab-coated $\sim 4.5\text{-}\mu\text{m}$ FITC-labeled Dynabeads, following a similar approach used recently to demonstrate the phagocytic potential of another C-type lectin, CD302 (34). We confirmed that beads coated with anti-CLEC-2 Abs bound specifically to transduced NIH3T3 fibroblasts expressing full length CLEC-2 and that these particles were internalized in an actin dependent fashion (Fig. 2*D* and data not shown). Confocal images of these cells clearly show the presence of actin-based phagocytic

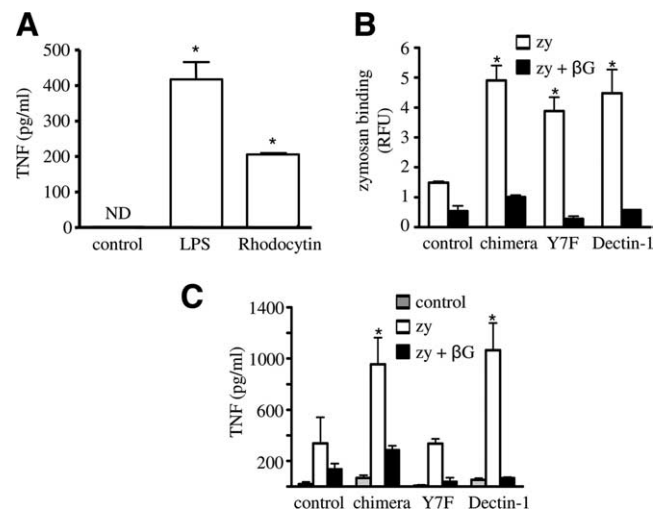


FIGURE 3. mCLEC-2 can induce proinflammatory cytokine production. *A*, Addition of rhodocytin or LPS to purified neutrophils induces the production of TNF- α . *B*, Quantitation of zymosan (zy) binding and *C*, zymosan induced TNF- α production, by transduced RAW264.7 macrophages, in the presence or absence of soluble β -glucan (β G), as indicated. The data shown are the mean \pm SD and are representative of at least three independent experiments. *, $p < 0.05$ vs control (Student's *t* test).

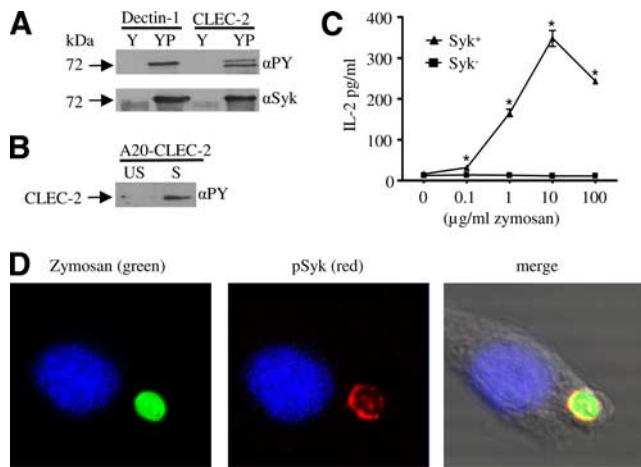


FIGURE 4. mCLEC-2 can recruit and signal via Syk kinase. *A*, Western blotting of immunoprecipitates from RAW macrophages using phosphotyrosylated (YP) and unphosphorylated peptides (Y), corresponding to the cytoplasmic tail of mCLEC-2 or dectin-1. *B*, Western blotting of anti-HA immunoprecipitates from A20 cells expressing HA-tagged mCLEC-2. Cells were either unstimulated (US) or stimulated with pervanadate (S). Blots were probed with anti-phosphotyrosine (α PY) and anti-Syk as indicated. *C*, IL-2 production following zymosan stimulation of Syk-deficient (Syk^-) and Syk-sufficient (Syk^+) B-cells, transduced with the chimeric receptor. The data shown are the mean \pm SD and are representative of at least three independent experiments. *, $p < 0.05$ vs C35 Syk^- cells (Student's t test). *D*, Confocal image demonstrating the recruitment of phospho-Syk (red) by RAW264.7 macrophages expressing the chimeric receptor following stimulation with FITC-zymosan. The nucleus is stained with Hoechst dye (blue).

cups around ingested beads (Fig. 2E). Furthermore, we could demonstrate that these beads bound specifically to peripheral blood granulocytes, as beads coated with isotype control Abs did not bind to these cells (Fig. 2F). Anti-dectin-1 Ab coated beads were included as a positive control and also bound to peripheral blood granulocytes, as expected (23). Upon incubation at 37°C, these beads were internalized by the granulocytes in an actin dependent fashion, as uptake could be inhibited by the addition of cytochalasin D (Fig. 2G and data not shown). Collectively these results demonstrate that CLEC-2 can function as a phagocytic receptor.

FIGURE 5. mCLEC-2 does not induce the respiratory burst. *A*, The respiratory burst in various RAW264.7 transfectants, measured by assessing the conversion of dihydrorhodamine 123 to rhodamine by flow cytometry, following stimulation with zymosan. The data are expressed as mean fluorescent intensity (MFI). The data shown are the mean \pm SD and are representative of at least three independent experiments. *, $p < 0.05$ vs control (Student's t test). *B*, The respiratory burst in peripheral blood neutrophils, determined as in *A*, following stimulation with rhodocytin or PMA. Number indicates mean fluorescent intensity.

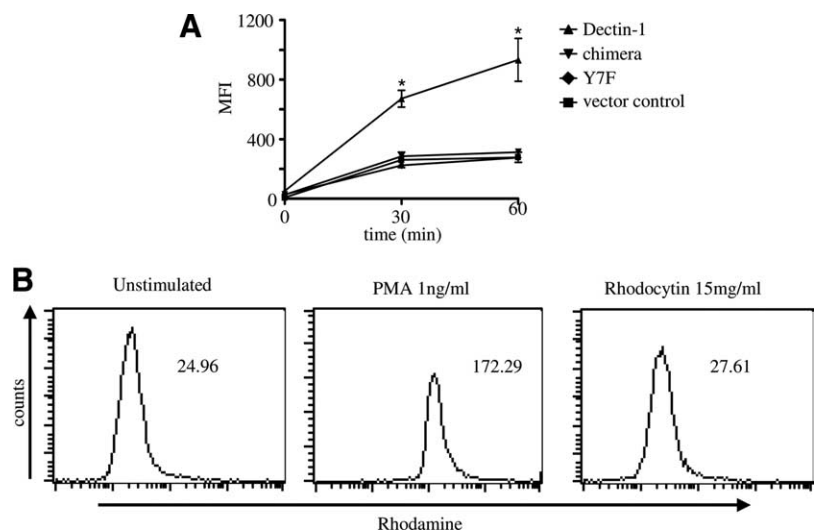
CLEC-2 induces production of TNF- α

In addition to phagocytosis, the cytoplasmic ITAM-like motif of dectin-1 can induce the production of cytokines, including TNF- α (24, 35–37). To investigate whether signaling via CLEC-2 can similarly induce cytokine production in murine neutrophils (38), we stimulated these cells for 6 h with the CLEC-2 ligand, rhodocytin (14), or LPS, and found that both stimuli induced the release of TNF- α (Fig. 3A). Although stimulation with rhodocytin suggests that CLEC-2 can mediate cytokine production on primary neutrophils, rhodocytin is not only a ligand for CLEC-2 and is known to be recognized by several other receptors, which could potentially be contributing to the cytokine inducing activity we observed (11). We therefore attempted to stimulate cells using Ab cross-linking, and Ab-coated dynabeads, but were unable to demonstrate specific responses in this manner due to high background levels of cytokine production in our control samples (data not shown).

Therefore, to specifically demonstrate that signaling from CLEC-2 is able to induce cytokine production, we used our chimeric dectin-1/CLEC-2 receptor constructs, described above, expressed in heterologous murine cell lines (data not shown). Comparable expression of the full length and Y7F mutant chimeric constructs in RAW264.7 macrophages conferred the ability to bind zymosan in these cells, which could be inhibited by the addition of soluble β -glucan (Fig. 3B). Furthermore, in response to zymosan, the full length chimeric receptor induced high levels of TNF- α , comparable to those induced by dectin-1, included here as a positive control (Fig. 3C). In contrast, the level of TNF- α produced from cells expressing the Y7F mutant chimera in response to zymosan was comparable to the levels from the vector-only transduced cells (Fig. 3C). Thus, these data demonstrate that CLEC-2 can induce cytokine production and that this activity is dependent on the ITAM-like motif in the cytoplasmic tail of the receptor.

CLEC-2 can signal via Syk kinase in myeloid cells

CLEC-2 signals via Syk kinase in platelets (11, 15), and we wanted to confirm that this pathway was being used by this receptor in myeloid cell types. We first performed immunoprecipitations from RAW264.7 lysates using tyrosine phosphorylated or unphosphorylated peptides, corresponding to the cytoplasmic tails of murine CLEC-2 or dectin-1. Subsequent Western blot analysis demonstrated that the phosphorylated CLEC-2 and dectin-1 peptides could associate with Syk from the macrophage cell extracts (Fig.



4A). We also confirmed that cellular activation results in the phosphorylation of CLEC-2, by immunoprecipitating this receptor from pervanadate stimulated or unstimulated transduced A20 cells expressing full-length CLEC-2 (Fig. 4B). In addition, as we had done for other receptors (6, 29), we expressed the chimeric receptor at comparable levels in Syk-sufficient and Syk-deficient B cell lines (data not shown), and examined the production of IL-2 in response to zymosan stimulation. Although addition of zymosan to the Syk-sufficient cells expressing the chimera induced the production of IL-2, the Syk-deficient cells did not show any response to this particle (Fig. 4C). Finally, we also examined RAW264.7 macrophages expressing the chimeric receptor by confocal microscopy, following a short 2-min exposure to zymosan, and could clearly detect the activation of Syk around the phagosome, as measured by staining for phospho-Syk (Fig. 4D). Similar activation of Syk was absent in the cells expressing the Y7F mutant chimeric receptor (data not shown). Thus, these data demonstrate that CLEC-2 signals via Syk kinase in myeloid cells.

CLEC-2 does not induce the respiratory burst

Signaling via dectin-1 has been shown to activate the respiratory burst in macrophages in a Syk-dependent manner (39). The respiratory burst is an important antimicrobial mechanism in neutrophils, and as CLEC-2 signals via Syk, we wondered whether this receptor was also mediating this activity. We examined this response in the RAW264.7 macrophages transduced with the various chimeric receptors following stimulation with zymosan. Somewhat surprisingly however, macrophages expressing the chimeric receptors failed to induce a respiratory burst in response to these particles (Fig. 5A). In contrast, macrophages expressing dectin-1 induced a robust respiratory burst, as expected (39). Furthermore, this response was also absent in peripheral blood neutrophils stimulated with rhodocytin (Fig. 5B). Thus, these data show that despite signaling via Syk kinase, CLEC-2 does not induce the respiratory burst.

Discussion

The study of the dectin-1 cluster of NK-like C-type lectin receptors has provided important insights into mechanisms underlying homeostasis and immunity (4, 7). Arguably one of the most important discoveries has been the identification of receptors containing cytoplasmic ITAM-like motifs which can trigger cellular activation. These motifs possess only a single tyrosine, yet are able to recruit and signal via Syk kinase through a process which is not yet fully understood (29, 40). One receptor possessing this motif is CLEC-2, a molecule previously thought to be exclusively expressed on platelets and capable of triggering the activation of these cells (11). In this study, we show that CLEC-2 is also expressed on murine peripheral blood neutrophils. As in platelets (11, 15), we show that the cellular functions of CLEC-2 are mediated through its ITAM-like motif and that the receptor can induce intracellular signaling via Syk kinase.

The ITAM-like motif shows a striking similarity to that of dectin-1, suggesting that CLEC-2 may possess many of the functions of dectin-1 (15, 41). Indeed, we have shown that CLEC-2 can trigger phagocytosis and the induction of TNF- α , and it is possible that the receptor may also be able to induce the production of a number of other cytokines and chemokines in neutrophils (38). Furthermore, CLEC-2 mediated cytokine production may be amplified by costimulation through the TLR pathway, as has been shown for dectin-1 (36, 37, 41, 42). Thus, like dectin-1 (29, 41) and CLEC9A (6), CLEC-2 functions as an activation receptor on myeloid cells.

The ability of CLEC-2 to mediate phagocytosis is likely to involve the highly charged cytoplasmic triacidic cluster (DED), in addition to the ITAM-like motif. This cluster is conserved in dectin-1, but not CLEC9A, and mutation of these residues in dectin-1 has been shown to abolish particle uptake (39). Furthermore, although possessing an ITAM-like motif, CLEC9A does not mediate phagocytosis (6). However, it is still unknown if CLEC-2-mediated uptake requires Syk in neutrophils. For dectin-1, the requirement for Syk is cell-type specific; in dendritic cells dectin-1-mediated uptake involves Syk, but in macrophages this process is independent of Syk and occurs through uncharacterised, and possibly novel, pathways (26, 29). In neutrophils, phagocytosis mediated by the unrelated activation receptor, CEACAM3, which possesses a traditional ITAM motif, was shown to involve Syk, but the requirement for this kinase was shown to be dependent on the nature of the ligand (43). Although we have clearly demonstrated that CLEC-2 has the potential to mediate phagocytosis, the physiological relevance of this activity remains to be determined.

One of the most surprising discoveries was the inability of CLEC-2 to induce the respiratory burst, despite signaling via Syk kinase. The activation of Syk by the Fc γ R, dectin-1, and CEACAM3 has been shown to induce the respiratory burst in a variety of cell types, including neutrophils (35, 39, 43, 44).

In fact, this response is completely absent following stimulation of these receptors in Syk-deficient cells (39, 44). In macrophages stimulated with zymosan, however, the activation of Syk by dectin-1 and the subsequent respiratory burst occurred only in a subset of cells, yet the assembly of the NADPH oxidase on the zymosan phagosomes occurred in all cells (39). Taken together, these data therefore suggest that there is another component/pathway stimulated by these receptors, in addition to Syk, that is required for the induction of the respiratory burst.

Our analysis of CLEC-2 expression indicates that high levels of expression of the receptor occurs only on circulating neutrophils, and that the receptor is only weakly expressed on bone marrow or elicited inflammatory cells. This suggests that its expression is tightly regulated and implies a specific function for the receptor on circulating cells. The expression of CLEC-2 on neutrophils, however, may only occur in mice, as we did not detect expression on human peripheral polymorphonuclear leukocytes using commercially available Abs (data not shown). Although not analyzed in detail, we also observed that expression of CLEC-2 could be up-regulated on murine monocytes, following stimulation with Pam₃CSK₄, but not other TLR agonists tested, suggesting some specificity in this response. It is possible that CLEC-2 may be similarly regulated on human polymorphonuclear leukocytes or other leukocytes, which were not examined in this study. Why the expression of this receptor appears to be primarily restricted to circulating cells is unclear, and despite the identification of both endogenous and exogenous ligands, the physiological role of CLEC-2 is still unknown.

Like other receptors in the dectin-1 cluster (4), CLEC-2 may function as a pattern recognition receptor. The expression of this receptor on neutrophils certainly implies a role in innate immunity, as these short-lived cells provide a first-line of defense against infection and are essentially required for the control of bacterial and fungal infections (38). A role in immunity is also suggested by the ability of specific TLR agonists to induce CLEC-2 expression on monocytes, and the phagocytic capacity of this receptor may be important for the clearance of blood-borne pathogens, although such interactions have yet to be documented. However, other than rhodocytin, only HIV has been identified to possess an exogenous CLEC-2 ligand and

rather than being protective, the interaction with CLEC-2 may promote transfer of infectious HIV-1 particles (17).

The primary function of CLEC-2, however, may be in the regulation of homeostasis through the recognition of endogenous ligands. One endogenous ligand that has been identified is podoplanin, a mucin-like protein that is expressed on a variety of cell types including osteoblasts, keratinocytes, fibroblasts, airway epithelia, renal tubular epithelial cells, lymphatic endothelial cells, and certain tumor cells, although podoplanin is not expressed on blood vessel endothelium (45). Thus, under normal circumstances, circulating neutrophils and platelets would not come into contact with this ligand. However, the interaction of CLEC-2 with podoplanin following tumor invasion has been proposed to promote platelet activation and aggregation, which may be associated with tumor metastasis (11, 13). Metastasis is also promoted by inflammation, induced in part by neutrophils (46, 47), and it is tempting to speculate that the interaction of podoplanin with neutrophil-expressed CLEC-2 may contribute to this process.

Finally, the simultaneous recognition of CLEC-2 ligand(s) by both platelets and neutrophils may contribute to the interactions between these two cell types and to the activation and cross-talk of the inflammatory and coagulation pathways. These interactions are known to be important for the control of infection, and for limiting inflammatory pathology, but are also involved in the development of disease (48, 49). We are currently determining whether CLEC-2 is involved in these processes, and the possibility that this receptor recognizes other endogenous and exogenous ligand(s).

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Disclosures

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