Human CLEC18 gene cluster contains C-type lectins with differential glycan-binding specificity
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CAPSULE

Background: CLEC18 is a novel C-type lectin not being characterized.

Results: The amino acid residue in the CTLD of CLEC18 contribute to the differential glycan-binding ability.

Conclusion: CLEC18 members are expressed in immune and non-immune cells, and preferentially bind to fucoidan, beta-glucan, and galactan.

Significance: The biochemical features, tissue distribution, and the glycan-binding specificity suggest that CLEC18 may contribute to host immunity against pathogens.

ABSTRACT

The human C-type lectin 18 (clec18) gene cluster, which contains clec18a, clec18b and clec18c three loci, is located in human chromosome 16q22. Even though the amino acid sequences of CLEC18A, CLEC18B, and CLEC18C are almost identical, several amino acid residues located in the C-type lectin-like domain (CTLD) and the Sperm-Coating Protein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) domain, also known as the cysteine-rich secretory proteins/antigen 5/pathogenesis-related 1 proteins (CAP) domain, are distinct from each other. Genotyping by real-time PCR and sequencing further shows the presence of multiple alleles in clec18a/b/c loci. Flow cytometry analysis demonstrates that CLEC18 (CLEC18A, B and C) are expressed abundantly in human peripheral blood cells. Moreover, CLEC18 expression is further upregulated when monocytes differentiate into macrophages and dendritic cells (DCs). Immunofluorescence staining reveals that CLEC18 are localized in the endoplasmic reticulum (ER), Golgi apparatus, and endosome. Interestingly, CLEC18 are also detectable in human sera and culture supernatants from primary cells and 293T cells overexpressing CLEC18. Moreover, CLEC18 bind polysaccharide in Ca\(^{2+}\)-independent manner, and the amino acid residues S/R\(^{339}\) and D/N\(^{421}\) in CTLD domain contribute to their differential binding abilities to polysaccharides isolated from Ganoderma lucidum (GLPS-F3). The S\(^{339}\) (CLEC18A) → R\(^{339}\) (CLEC18A-1) mutation completely abolishes CLEC18A-1 binding to GLPS-F3, and sugar competition assay shows that CLEC18 preferentially bind to fucoidan, beta-glucans, and galactans. Since proteins with SCP/TAPS/CAP domain are able to bind sterol and acidic glycolipid, and are involved in sterol transport and beta-amyloid aggregation, it would be interesting to investigate whether CLEC18 modulate host immunity via binding to glycolipids, and are also involved in glycolipid transportation and protein aggregation in the future.

INTRODUCTION

The superfamily of C-type lectin (CLEC) comprises a large group of glycoproteins with divergent functions, including host-pathogen interaction and cell-cell interaction (1,2). The signature of CLEC is the presence of a stretch of 115-130 amino acid residues known as C-type lectin-like domain (CTLD), which is originally identified as carbohydrate-recognition domain (CRD) in a family of Ca\(^{2+}\)-dependent animal lectins, (3). The CTLD fold has a characteristic double-loop ('loop-in-a-loop') stabilized by two highly conserved disulfide bridges (4). The long loop region contains a conserved ‘WND’ or
‘ENC’ motif for Ca\(^{2+}\)-binding and dimerization in some C-type lectins (5). The CTLDs of CLEC members bind a variety of glycans, and it is now clear that even CTLDs with similar structures can bind ligands in distinct ways. Based on their ligand-binding properties, CTLD can be divided into two groups based on the presence of ‘Glutamic Acid-Proline-Asparagine (EPN)’ and ‘Glutamine-Proline-Aspartic acid’ (QPD) motifs in long loop region. CTLDs with ‘EPN’ motif usually bind mannose, N-acetylglucosamine, and fucose, while CTLDs with ‘QPD’ motif usually bind galactose and N-acetylgalactosamine (5). However, whether this is a general rule to all the members of C-type lectins need to be further confirmed.

Recently, more and more evidence shows that not all proteins with CTLD interact with Ca\(^{2+}\) in the long loop region (6). The classical CLEC (such as selectins, collectins, and mannose binding proteins) bind glycan in Ca\(^{2+}\)-dependent manner, while members of Syk-coupled CLEC receptors (such as Dectin-1/CLEC7A and MDL1/CLEC5A) bind glycan in Ca\(^{2+}\)-independent manner (7,8). Moreover, proteins with less closely related CTLDs (such as CD69, CD72, KLRF1 of NK receptor family) do not appear to have carbohydrate-binding activity.

In human genome, there are at least 57 CTLD-containing proteins divided into XVI groups (9). Among these proteins, we are especially interested in group XV, the CLEC18 family, for following reasons: 1) the clec18 gene cluster contains three genes (clec18a, clec18b, and clec18c) located in human chromosome 16q22.1 (clec18a, clec18c), and 16q22.3 (clec18b); 2) Translation of clec18 cDNA predicted an N-linked polypeptide with C-type lectin domain (CTLD) in the C-terminus, and the SCP/TAPS (Sperm-Coating Protein/Tpx-1/Ag5/PR-1/Sc7) domain [also known as CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1) domain], which is a conserved tertiary \(\alpha\)-\(\beta\)-\(\alpha\) structure stabilized by di-sulfide bonds. 3) Proteins of SCP/TAPS/CAP domain have been proposed to play significant roles in host-pathogen interactions, reproduction, development, and immune function (10,11); 4) several amino acid residues located in the CTLD and SCP/TAPS/CAP domains are distinct among the sequences retrieved from the NCBI database. However, the biochemical features of CLEC18 have not been tested yet.

In this study, we developed a real-time reverse-transcription PCR-based platform with fluorescent hybridization probe to detect the presence of CLEC18 in various cell lines and primary cells, followed by direct sequencing cDNAs to confirm the PCR-based typing method. We found that CLEC18 mRNAs and proteins were detectable in various cell lines and human peripheral blood cells. Furthermore, CLEC18 encode N-linked glycoproteins located in endoplasmic reticulum (ER), Golgi apparatus (GA), and endosomes. Moreover, amino acid residues S/R339 and D/N 421 located in the CTLD domain contribute to their differential glycan binding specificity. This observation suggests that the CLEC18 may bind to various glycoconjugates with distinct affinity, and contribute to differential immune responses to glycoconjugates expressed on foreign antigens or altered self-antigen in ER, GA, and endosomes.

**EXPERIMENTAL PROCEDURES**

Reverse transcription and cloning of CLEC18 by
polymerase chain reaction—Total RNA was extracted from cells using Trizol according to the supplier's instructions, then subjected to reverse transcription using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington ON, Canada) as template for PCR amplification. The CLEC18 cDNAs were amplified by denaturing for 60 sec at 95°C, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 70°C for 45 sec, and extension at 72°C for 80 sec. The amplified cDNA fragment was ligated into yTA vector (RBC TA cloning vector kit) before subcloned into pCMV-Tag4A vector (Stratagene) for expression in mammalian cells. To sequence the CLEC18 cDNA fragment extending from SCP/TAPS domain to CTLD domain (nucleotide 211 to 1304), cDNA fragment was performed by a thermocycler (GeneAmp® PCR System 9700, Applied Biosystem®). The PCR program (denature/annealing/extension) for amplification is as following: 98°C, 10 sec/63°C, 30 sec/72°C, 70 sec for 30 cycles.

Determination of CLEC18 alleles by real-time PCR—The polymorphism of CLEC18 A/B/C was determined by real time PCR with hybridization probes (Roche Life Science). The position of primer are as shown in Figure 2, while the sequences of PCR primers and hybridization probe are listed in Table 1. PCR was performed by a thermocycler (LightCycler480® II, Roche), followed by heat denaturation at 95°C for 60 sec to determine CLEC18 polymorphism. The PCR program (denature/annealing/extension) for amplification of CTLD domain and is as following: 95°C, 10 sec/66°C, 10 sec/72°C, 10 sec. The amplification cycles were followed by a melting cycle, in which DNA was denatuated at 95°C for 60 s using a rate of 4.4°C/s, cooled to 40°C for 60 sec using a rate of 1.5°C/s and held for 30 s. Temperature was then raised to 75°C with a transition rate of 0.03°C/s. Fluorescence was continuously monitored during the melt.

Isolation of PBMC and preparation of human macrophages and dendritic cells—Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy human donors by standard density-gradient centrifugation with Ficoll-Paque (Amersham Biosciences). To prepare primary macrophage, CD14+ cells were purified from PBMCs by high-gradient magnetic sorting, using the VarioMACS technique with anti-CD14 microbeads (Miltenyi Biotec GmbH). Cells were then incubated in complete RPMI 1640 medium (Hyclone) supplemented with human M-CSF or GM-CSF/IL-4 for 7 days as described previously (12,13).

Western blotting and flow cytometry analysis—For western blotting analysis, cells (1 x 10⁶) were lysed by RIPA buffer, follow by fractionation on 12 % SDS-PAGE, before blotting onto PVDF membrane. Lysates were probed with anti-CLEC18 mAb (clone 3A9E6), followed by incubation with peroxidase-conjugated goat-anti-mouse polyclonal antibody (Millipore AP181P) and Immobilon™ Western Chemiluminescent HRP Substrate (Millipore™). For flow cytometry analysis, cells were permeabilized with 0.1% saponin and incubated with the Alexa 647–conjugated anti-CLEC18 mAb (clone 3A9E6), then were examined by flow cytometer (Verse™, BD Biosciences). Data were analyzed by the FlowJow™ software.

Immunofluorescence staining—Adherent cell were fixed with 4% paraformaldehyde for 1 hr, then were permeabilized with 0.5% Triton X-100 in
PBS for 10 min before incubation in blocking buffer [10%BSA in PBS] for 60 min. Cells were then incubated with anti-CLEC18 mAb (40µg/ml in 3%BSA/PBS, RT 1 hr), anti-calreticulin (dilution range 1:100 in 3% BSA/PBS, 4°C overnight), anti-GM130 (dilution range 1:150 in 3% BSA/PBS, 4°C overnight), and anti-EEA1 (dilution range 1:100 in 3% BSA/PBS, 4°C overnight), respectively. After washing, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Jackson Immuno), TRITC-conjugated goat anti-rabbit IgG (Jackson Immuno) for 1 hr and then Hoechst 33342 for 10 min. Samples were then mounted and observed under fluorescence Cofocal microscopy (Olympus FV10i). Alternatively, cells were incubated with Mitotracker red (250nM) or Lysotracker (100 nM) at 37°C for 1 hr to observe their colocalization with CLEC18.

Immunoprecipitation and mass spectrometry analysis—To understand whether CLEC18A is a secretory protein, pCMV-Tag4A or pCMV-Tag4A-CLEC18A (2 µg) was transfected into 293T cells (2×10^5), respectively, followed by incubation at 37°C for 48 hr before harvesting. Cell lysates or supernatant were incubated with Mitotracker red (250nM) or Lysotracker (100 nM) at 37°C for 1 hr to observe their colocalization with CLEC18.

Construction, expression, and purification of Fc-tagged Recombinant CLEC18.Fc Fusion Proteins—DNA fragments encoding CTLD domain of CLEC18A/B/C were amplified by reverse transcriptase-PCR and subcloned into pcDNA3.1-hIgG1 Fc (mut) vector to generate CLEC18A/A-1/B/C. Fc fusion proteins, respectively. PCR primers are listed in Table 1. The CLEC18B cDNA was generated by two steps PCR to insert exon B using CLEC18A cDNA as template. The CLEC18.Fc fusion proteins were overexpressed by using the FreeStyle 293 Expression System (Invitrogen). Briefly, 3 x10^7 293F cells in 28 ml of culture medium were transfected with a mixture of 30 µg of plasmid DNA and 60 µl of 293fectinTM in a total of 2 ml of Opti-MEM I (Invitrogen). The culture supernatants were harvested at day 3 and 5, and the recombinant CLEC18.Fc fusion proteins were purified by Protein A column (GE Healthcare).

Polysaccharide binding assay—Samples of GLPS-F3 were weighed, dissolved, and diluted with 100 mM Tris buffer (pH 9.5) to give 20µg/ml as described previously (14). Briefly, the GLPS-F3 was immobilized in the wells of 96-well plates (50µl/well, corning) and incubated overnight at 4°C, followed by incubation with 200µl of blocking buffer (2% BSA/TBST) for 1 h at room temperature before washing with TBST (0.05% Tween 20/TBS) twice. Each well were subjected to mass spectrometry analysis using the Thermo Finnigan LTQ FT Ultra High Performance Mass Spectrometer (Conquer scientific). The secretory CLEC18 in culture supernatants and sera was determined by ELISA kit (CUSABIO Life Science).
incubated with 100µl of CLEC.Fc fusion protein (2µg/ml in 2mM MgCl₂, 2 mM CaCl₂, 1% BSA/TBST) in the presence or absence of various glycans (Megazyme) as competitors for 1 h at room temperature. After washing with TBST, well was incubated with 100µl of peroxidase-conjugated goat anti-human IgG Ab (Jackson Immuno) in 1% BSA/TBST (1:5000) at room temperature for 30 min, followed by addition of 100 µl of tetramethylbenzidine substrate (BD) for 20 min, and reaction was stopped by 1N H₂SO₄ before subjecting to analysis by ELISA reader (TECAN).

Alternatively, fusion protein incubated with zymosans at 4°C for 1 hr and washed with PBS, followed by incubation with anti-hIgG1-FITC (1:200) at 4°C for 30 min and then were analyzed using a Verse™ flow cytometer (BD Biosciences) flow cytometer (BD Biosciences), and data were analyzed using FlowJow™ software.

RESULTS

Chromosomal location and structure of clec18 gene cluster— The clec18 gene cluster contains three loci: clec18a (16q22.1), clec18c (16q22.1) and clec18b (16q22.3). Both clec18a (Gene ID: 348174) and clec18c (Gene ID: 283971) comprise 12 exons, while a predicted extra exon (denoted as exon B) located between exon X and exon XI is found in clec18b (Gene ID: 497190) (Figure 1A & B). clec18a (NM_182619.3) and clec18c (NM_173619.3) encode a 446 amino acid (a.a.) polypeptide, respectively, while clec18b is predicted to encode a 455 amino acid polypeptide with the extra nine amino acids (LVWLSAAMG) within CTLD domain (Figure 1C).

Compared with CLEC18A, CLEC18C is almost identical with CLEC18A except two amino acids (T91 → I91, V118 → A118) located in SCP/TAPS/CAP domain (a.a. 45 – a.a. 190) and one amino acid (D421 → N421) in WND motif located in CTLD. In addition, four amino acid residues (T91 → I91, V118 → A118, A173 → T173, R185 → G185) located in SCP/TAPS domain (a.a. 45 – a.a. 190) and one amino acid residue (V196 → I196) located between SCP/TAPS/CAP domain and EGF region (a.a. 232 – a.a. 292) were noted among CLEC18A, CLEC18B, and CLEC18C. Moreover, CLEC18A-1 (AAI41809.1), which contains three distinct amino acid residues from CLEC18A (V118 → A118 and T151 → M151 in SCP/TAPS/CAP domain and S339 → R339 in CTLD) was found in human genome database (Figure 1C).

Alignment of human CLEC18 with CLEC18 of other species shows that CLEC18 is highly conserved in CTLD, but the predicted nine amino acid insertion is only found in human CLEC18B and chimpanzee CLEC18 (Figure 1D). The typical motifs of CTLDs, such as “WIGL” (a.a. 370-373), “QPD” (a.a. 399-401), and “WND” (a.a. 419-421), are found in both human and non-human CLEC18. It is interesting to note that the “D421/430” located in the “WND” motif of CLEC18A/B is replaced with “N 421” in human CLEC18C. Moreover, the basic amino acid residues R322 and R329 found in human and chimpanzee are replaced with G322 and G/E329 in non-primate. Furthermore, the aliphatic amino acid residues I360, A385 and H403 found in human and chimpanzee are replaced with T/M/S369, T/D385 and Q403 in non-primate CLEC18.

Detection of CLEC18 alleles by real time reverse-transcription PCR (RT-PCR) — Due to
the highly conserved cDNA sequences among CLEC18, nucleotide residues (C/A1017 and G/A1261) located in CTLD were used to discriminate each member of CLEC18 family by hybridization probes-based real time RT-PCR assay. In addition, a probe hybridizing the extra exon B was used to detect CLEC18B (Figure 2). We found that CLEC18A mRNA was expressed in almost all the cell lines tested, while CLEC18A-1 was detectable in CHME3, A549 and the human peripheral blood cells (PBCs) of three donors. CLEC18C mRNA was also detectable in some cell lines, but was less prevalent than CLEC18A. However, we cannot detect CLEC18B mRNA in all the cell lines by PCR using the probe hybridizing the exon B (Table 2A). We further investigated the expression of CLEC18 mRNAs in human peripheral blood cells (PBMCs) from healthy donors. While CLEC18A mRNAs was detectable in all the samples tested, the expression of CLEC18A-1 and CLEC18C was less prevalent than CLEC18A. However, CLEC18B mRNA is not detectable in all the samples tested (Table 2B).

We further examined the presence of other CLEC18A/B/C alleles in cell lines and human peripheral blood cells (PBCs) by sequencing the CLEC18 cDNAs amplified by RT-PCR. Interestingly, two discrepancies with the reference sequences were found: 1) The dominant amino acid residues located in the SCP/TAPS/CAP domain of CLEC18A are T91A118M151A173R185V196, not the T91V118T151A173R185V196 (reference sequence: NP_872425.2) (Table 3A); 2) cDNA clones with the I91A118T151I173G185I196 (characteristic of CLEC18B) in SCP/TAPS/CAP domain do not contain the extra nine amino acids (LVWLSAAMG) derived from exon B, among all the cell lines and human PBCs we sequenced (Table 3 B&C). This observation suggests that the putative exon B is absent in human CLEC18B, and multiple alleles are present in all the members of CLEC18 family.

**CLEC18 are N-linked glycoproteins detectable in cell lysates and culture supernatant**—We further investigated the expression of CLEC18 by Western blotting and flow cytometry. We found that anti-CLEC18 mAb (clone 3A9E6) detect a specific band migrating at 50 kDa in 293T cells transfected with CLEC18A and CLEC18C cDNAs, respectively (Figure 3A). The peptide was eluted for mass spectrometry analysis, and the sequences of peptides matched CLEC18 perfectly (Figure 3B). Because two potential glycosylation sites (N144 and N243) were predicted when analyzing CLEC18 by Vector NTI software (version 11), we asked whether CLEC18 are N-linked glycosylated proteins. To address this question, Flag-tagged CLEC18A-transfected 293T cells were incubated with tunicamycin to observe the change of M.W. of CLEC18. We found the CLEC18A polypeptide migrated from 50 kDa to 45 kDa in a time-dependent manner when probed with anti-Flag mAb (Figure 3C), and similar result was observed after PNGase F treatment (Figure 3D). This observation indicates that CLEC18A is a 50 kDa N-linked glycoprotein.

We further examine its expression in human PBCs and CD14+-derived macrophages and dendritic cells by flow cytometry and Western blotting. To address this question, CD14+-derived dendritic cells were incubated with GM-CSF+IL-4 (25 ng/ml+20 ng/ml), GM-CSF (10 ng/ml), and M-CSF (10 ng/ml) for 7 days,
respectively, to differentiate into dendritic cells (DCs), GM-macrophages (GM-Mϕ) and M-macrophages (M-Mϕ). We found that CLEC18 are abundantly expressed in human PBCs, including T cells (CD3⁺), B cells (CD19⁺), granulocytes (CD66b⁺), and monocytes (CD14⁺) (Figure 4A). Compared with monocytes, CLEC18 were further upregulated in monocyte-derived DCs, GM-Mϕ, and M-Mϕ (upper, Figure 4B), and protein expression level in accord with CLEC18 mRNA levels determined by real time PCR (lower, Figure 4B). This observation suggested the CLEC18 expression is upregulated by cytokines. However, the expression of CLEC18 was low and barely detectable in several hematopoietic cell lines (Figure 4C) and non-hematopoietic cell lines (data not shown).

It has been shown that members of protein with SCP/CAP domain are soluble proteins (11,15), thus we asked whether CLEC18 are secretory proteins. To address this question, culture supernatant of pCMV-Flag-CLEC18A-transfected 293T cells was incubated with anti-Flag mAb to pull down soluble CLEC18A, followed by western blot analysis using anti-CLEC18 mAb (clone 3A9E6). We found that CLEC18A is not only detectable in culture supernatant of pCMV-Flag-CLEC18A-transfected 293T cells (Figure 4D), but also in the culture supernatant of DCs, GM-Mϕ and M-Mϕ (upper, Figure 4E), as well as in human sera (lower, Figure 4E) as determined by ELISA. This observation suggests that CLEC18A is a secretory protein.

Subcellular localization of CLEC18—Because CLEC18 were detectable in cell lysates, we further investigated its subcellular localization. To address this question, human CD14⁻-derived M-Mϕ was incubated with anti-CLEC18A mAb, followed by incubation with Alex488-conjugated goat anti-mouse mAb. To determine its subcellular localization, M-Mϕ was also co-incubated with both anti-GM 130 mAb (rabbit), anti-calreticulin mAb (rabbit), and anti-EEA1 mAb (rabbit), respectively, followed by incubation with TRITC-conjugated goat-anti-rabbit mAb. We found that CLEC18 were co-localized with GM 130 (marker for Golgi apparatus), calreticulin (marker for endoplasmic reticulum), and EEA1 (marker for early endosome), but not with mitotracker (mitochondria dye) or lysotracker (lysosome dye) under the same condition (Figure 5). This observation suggests that CLEC18 are located in ER, Golgi apparatus, and early endosomes.

Tissue distribution of CLEC18—We further used human tissue array to detect the expression of CLEC18 by immunohistochemistry staining. We found that CLEC18 were expressed in human spleen (leukocytes), brain (microglia), liver (hepatocytes), gall bladder (epithelial cells), fallopian tube (epithelial cells), and testis (epithelial cells) (Figure 6). This observation suggests that CLEC18 are not only expressed in hematopoietic lineages, but also expressed in and epithelial and parenchymal cells.

Differential glycan binding ability of CLEC18A, CLEC18A-1 and CLEC18C—We further investigated the role of amino acid residues S/R339 and D/N421 in CTLD of CLEC18 family in binding ability to glycans. To address this question, S339 and D421 located in CTLD of CLEC18A and mutants were ligated
with pcDNA3.1-hIgG Fc (mut) to get pCLEC18A.Fc, pCLEC18A-1.Fc, and pCLEC18C.Fc, respectively, followed by transfection to 293F cells to get recombinant fusion proteins as described previously (8,14,16). To understand whether the presence of exon B (encoding ‘LVWLSAAMG’) affects glycan-binding ability, we inserted the nine amino acids into CLEC18A CTLD to generate pCLEC18B.Fc. The recombinant CLEC18A.Fc, CLEC18A-1.Fc, and CLEC18C.Fc fusion proteins were then used to hybridize with the synthetic oligosaccharide glycan arrays from CFG (Consortium for Functional Glycomics, slides #14508, #14506, #14507; PMT70 v5.0 Alexa48810,20,11 HJ). However, all the fusion proteins did not bind any glycans spotted on the glass slides, because the signals are weak (RFU < 10000) and coefficient variation is high (CV > 50%) (Supplementary Data 1A~1C). Thus, we further test their binding ability to F3 polysaccharides isolated from medicinal fungi *Ganoderma lucidum* (GLPS-F3), followed by glycan competition assay to determine binding specificity as described previously (14,16).

It has been shown that GLPS-F3 contains abundant polysaccharides comprising glucose, mannose, fucose, galactose, xylose, GlcNAc, and rhamnose (17), and GLPS-F3 is able to interact with several C-type lectins and Toll-like receptors (14). Thus, we examined the interaction between CLEC18 and GLPS-F3 using Dectin1.Fc and DC-SIGN.Fc as control. Similar to Dectin1.Fc, DC-SIGN.Fc, we found that CLEC18A.Fc and CLEC18C.Fc also bound to F3 polysaccharides, while $S_{339} \rightarrow R_{339}$ mutation (CLEC18A-1.Fc) and insertion of nine amino acid ‘LVWLSAAMG’ in CTLD domain (CLEC18B.Fc) abolished their binding ability to GLPS-F3 (Figure 7A). Interestingly, EDTA was unable to abolish binding of Dectin1.Fc, CLEC18A.Fc, and CLEC18C.Fc to F3 polysaccharide, though EDTA inhibited DC-SIGN.Fc binding to F3 efficiently under the same condition (Figure 7A). Similar observation was observed when incubating fusion proteins with zymosans (Figure 7B) and house dust mite (Figure 7C). Since Dectin-1.Fc binding to beta-glucans is independent of Ca$^{++}$ (7), this observation suggests that CLEC18 binding to F3 polysaccharides and zymosans is also Ca$^{++}$-independent, and amino acid residue $S_{339}$ is critical for binding to polysaccharides.

We further determined the glycan-binding specificity of CLEC18A and CLEC18C by sugar competition assay (14) using monosaccharides (Figure 8A) and polysaccharides (Figure 8B) as competitors. Even though mannose and fucose competed DC-SIGN.Fc binding to GLPS-F3 in a dose-dependent manner (left, Figure 8A), none of the monosaccharides (GlcNAc, galactose, mannose, fucose) were able to inhibit CLEC18A.Fc (middle, Figure 8A) and CLEC18C.Fc (right most, Figure 8A) binding to GLPS-F3 under the same condition. We further used polysaccharides to replace monosaccharides for competition assay. Among the fifteen polysaccharides tested (Table 4 & Figure 7D), fucoidan (sulfated fucose), laminarin ($\beta$-1,3-linked glucan with $\beta$-1,6-linked side chain), and galactan ($\beta$-4-GlcNac) could inhibit CLEC18A.Fc and CLEC18C.Fc binding to GLPS-F3 (lower, Figure 8B). Interestingly, pachyman ($\beta$-1,3-linked glucan) were less efficient than laminarin ($\beta$-1,3-linked glucan with $\beta$-1,6-linked side chain) to inhibit CLEC18A/C binding to GLPS-F3.
though pachyman was more efficient than laminarin to inhibit Dectin-1 binding to GLPS-F3 (upper right, Figure 8B). In contrast, mannan was unable to inhibit CLEC18A/C binding to GLPS-F3, even though mannan efficiently inhibited DC-SIGN binding to GLPS-F3 (upper left, Figure 8B). Therefore, the CTLD domain of CLEC18A/C preferred binding to sulfated fucose, β-glucan, and galactan.

**DISCUSSION**

Several unusual features of CLEC18 are also noted in this study: 1) It is surprising to find that CLEC18A/C bind GLPS-F3 in Ca++-independent manner, even though the typical WND domain are present in CLEC18A; 2) The presence of “Q399P400D401” tripeptide motif in CLEC18 CTLD predicts binding specificity to galactose and GalNAc. However, sugar competition assay showed that CLEC18A and CLEC18C displayed diverse binding specificity to various glycans, including galactan (β-GlcNac), fucoidan, and β-glucan. In addition, amino acid residue D421/N421 has mild effect on F3 binding, because laminarin and galactan have better inhibitory effect to CLEC18A.Fc (N421) than CLEC18C.Fc (D421) in higher concentration (100 μg/ml). Thus, amino acid residue S/R339 has decisive role to determine CLEC18 binding to GLPS-F3, while D/N421 only has mild effect to influence their binding to laminarin and galactan, but fucoidan (Figure 8B). In addition, the stronger inhibitory effects of laminarin (β-1,3-linked glucan with β-1,6-linked side chain) than pachyman (β-1,3-linked glucan) suggests that CLEC18A.Fc and CLEC18C.Fc seem preferentially binding to β-1,6-linked side chain of β-glucans. 3) CLEC18 are not only located in ER, Golgi apparatus, and endosome of primary macrophage (Fig. 5), but also detectable in culture supernatant of 293T cells overexpressing CLEC18 (Fig. 4D). However, overexpression often results in non-physiological cell response, thus the subcellular distribution of CLEC18 and the presence of soluble CLEC18 under physiological condition need to be further verified in the future.

In contrast to L-type lectins calreticulin/calnexins, which are ER membrane proteins acting as chaperons to bind GlcM3 (Glc-α1→3-Man-α1→2-Man-α1→2-Man) of Glc1Man9GlcNAc2-attached to glycoproteins in ER for N-linked glycosylation (18), no significant binding to GlcM3 or other glycans spotted on CFG glycan arrays was noted by recombinant CLEC18A/A-1/C fusion proteins (Supplementary Date 1A~1C). Furthermore, CLEC18A.Fc and CLEC18C.Fc bind GLPS-F3 (Figure 7A) and zymosans (Figure 7B) in Ca++-independent manner, and display diverse binding specificity to various glycans (Figure 7D and Table 4). Thus, CLEC18 seem not involved in N-linked glycosylation in ER and Golgi apparatus. This argument is further supported by the histochemical staining that CLEC18 are not ubiquitously expressed in all the cell types (Figure 6).

Because CLEC18 do not contain ‘H/KDEL’ tetrapeptide motif critical for ER-retention, we asked whether CLEC18 was retained in ER via association with other proteins. To address this question, Flag-tagged CLEC18A was precipitated from CLEC18A-transfected 293T cells by anti-Flag mAb, and the immunoprecipitates were subjected to mass spectrometry analysis. We found that CLEC18A was coimmunoprecipitated with ER protein
Grp78/Bip (data not shown). However, we were unable to co-precipitate CLEC18A and Grp78/Bip from cells with or without stress (data not shown), thus how CLEC18 were retained in ER and Golgi apparatus needs to be further investigated in the future.

It has been shown that proteins with the SCP/TAPS/CAP domain, such as the Pathogen-Related Yeast (PRY) proteins and human CAP family member CRISP2, are necessary and sufficient for lipid export and sterol binding (15,19). Since CLEC18 contains both CTLD and SCP/TAPS/CAP domains, it is reasonable to speculate that CLEC18 may bind glycolipids and are involved in the transport of glycolipids. The distinct amino acid residues in the SCP/TAPS/CAP domain may also contribute to the binding specificity and affinity of CLEC18 to glycolipids and other glycoconjugates. Furthermore, it has been speculated that the plant pathogenesis-related 1 protein, a host-defense protein with SCP/TAPS/CAP domain, may serve to inhibit pathogen proliferation by extracting sterols from the pathogen membrane (15). Therefore, CLEC18 may extract the sterol component of pathogens assembly in ER and Golgi apparatus (such as members of flaviviruses) and attenuate their infectivity. This speculation is supported by the fact that CLEC18 are upregulated in human macrophages and dendritic cells (Figure 4B), which are the major targets of flaviviruses. Furthermore, the Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1), a mammalian proteins with SCP/TAPS/CAP domain, is shown to interact with acidic phospholipids and inhibits Aβ aggregation (20).

All the above evidence suggest that CLEC18 may be involved in the recognition and transportation of glycoconjugates, and it would be interesting to investigate how the polymorphic amino acid residues in SCP/TAPS/CAP and CTLD domains determine CLEC18 binding specificity and affinity to various glycoconjugates in the future.

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Conflict of interest:
The authors declare no competing financial interests.

Author contributions
Contribution: Y.-L.H., F.-S.P., Y.-T. T. and. H.-C. M. designed, performed, and analyzed experiments; T.-L. H., C.-Y. W., T.-Y. C., W.-B. Y., C.-H. C. and C.-H. W. provided reagent and technique support; and S.-L.H. designed and analyzed experiments and wrote the manuscript.
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**FOOTNOTES**

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FIGURE LEGENDS

FIGURE 1. Chromosomal location, structures, protein sequence and alignment. (A) The clec18 gene family are located in human chromosome 16q22.1 (clec18a and clec18c) and 16q22.3 (clec18b).
(B) The clec18a and clec18c comprise 12 exons, respectively, while an extra exon (exon B) is predicted in clec18b. (C) The clec18a and clec18c encodes a polypeptide of 446 a.a., respectively, while clec18b is predicted to encodes a polypeptide of 455 a.a. due to the extra 9 amino acid residues translated from the exon B in clec18b. The polymorphic amino acid residues among members of CLEC18 family are indicated. (D) Multiple sequence alignment of CTLD of human CLEC18 and other species. Only human genome contains three genes (clec18a, clec18b and clec18c), while other species only contain one gene (clec18a) in the genome. Only human and chimpanzee CLEC18 are predicted to contain exon B with nine-inserted amino acid residues (LVWLSAAMG) in the CTLD. *: the polymorphic amino acid residues (339 and 421) found in human CLEC18 family.

FIGURE 2. Typing of CLEC18 alleles by hybridization probes. Detection of CLEC18 alleles by real-time RT-PCR using hybridization probes. CLEC18 cDNAs from cell lines or primary cells are used as template, and amplified by primer pairs and hybridization probes to detect polymorphism in nucleotide 1017 (primers A and A’ with probes H1 and H2), nucleotide 1261 (primers C and C’ with probes H5 and H6). Exon B is detected by primers B and B’ with probes H3 and H4.

FIGURE 3. CLEC18 are an N-linked glycoprotein. (A) The 293T cells were transfected with pCMV-Tag4A vector (V), pCMV-Tag4A-CLEC18A (18A), or pCMV-Tag4A-CLEC18C (18C), followed by western blot analysis using anti-CLEC18 mAb (clone 3A9E6). (B) Mass spectrometry analysis of peptides recognized by anti-CLEC18 mAb (clone 3A9E6). The 50 kDa peptides were eluted for mass spectrometry analysis. The peptides (underlined) matched perfectly to CLEC18. (C) CLEC18A-transfected 293T cells were incubated with tunicamycin for various time points, followed by western blot analysis to determine the presence of N-linked glycosylation using anti-Flag mAb. D: DMSO. (D) Alternatively, 293T cells were transfected with vector (V) or CLEC18A (18A) for 48hr, and then collected lysate (20μg) untreated (lane 1) or treated with 500 units of PNGase F (lane 2) for 37℃ 2hr, followed by western blot analysis using anti-Flag mAb.

FIGURE 4. CLEC18 are a secretory protein. (A) Detection of CLEC18 by flow cytometry and real-time PCR in human peripheral blood cells. Shadow: isotype control; Dash line: anti-CLEC18 mAb. (B&C) Detection of CLEC18 by western blot and real-time PCR in DC/Mφ (B) and cell lines (C). Arrow: CLEC18. (D) The 293T cells were transfected with vector or pCMV-Tag4A-CLEC18A for 48 hours, and the supernatant or lysates incubated with anti-Flag mAb to pull down CLEC18, followed by Western blot analysis. (E) Detection of secretory CLEC18 in culture media and human sera. Culture supernatants (upper) of monocyte, dendritic cells, and macrophages and human sera (lower) were determined by ELISA (CUSABIO Life Science).
FIGURE 5. CLEC18 are localized in ER, Golgi apparatus, and early endosomes. CD14+ monocyte-derived macrophage (M-MΦ) were incubated with Alexa488-conjugated anti-CLEC18 mAb (green) in the presence TRITC-conjugated anti-GM130 mAb (red), TRITC-conjugated anti-calreticulin (red), TRITC-conjugated anti-EEA1 mAb (red), Mitotracker (red), or Lysotracker (red), respectively. Cells were examined under a confocal microscopy (Olympus FV10i). Scale bars: 10µm.

FIGURE 6. Histochemical staining of human tissue array. The formalin-fixed human tissue array (prepared from normal human tissues in Taipei Veterans General Hospital) was incubated with anti-CLEC18 mAb (3A9E6) followed by HISTOMOUSETM-MAX kit (Zymed) according to the vendor’s instructions. Arrow heads: leukocyte (spleen), microglia (brain), hepatocyte (liver), epithelial cells (gall bladder, testis, fallopian tube).

FIGURE 7. Determination of sugar binding ability of CLEC18. (A) Polysaccharides isolated from *G. lucidum* F3 fraction (GLPS-F3) were immobilized on ELISA plate (20µg/ml), followed by incubation with Dectin-1.Fc, DC-SIGN.Fc, and CLEC18.Fc family proteins (2µg/ml), in the presence or absence of EDTA (5mM). (B) Incubation of Dectin-1.Fc or CLEC18.Fc family proteins (10 µg/ml) with zymosan (10 mg/ml, sigma), in the presence or absence of EDTA. The number represents the ‘mean fluorescence intensity’. (C) House dust mite (5µg/ml) were immobilized on ELISA plate, followed by incubation with Dectin-1.Fc, DC-SIGN.Fc, and CLEC18.Fc family proteins (2µg/ml), in the presence or absence of EDTA (5mM). (D) Interaction of CLEC18A/C with various polysaccharides (as shown in Table 4) by ELISA. N: non competitor.

FIGURE 8. Determination of sugar binding specify of CLEC18. Various monosaccharide (A) or polysaccharide (B) were used to compete DC-SIGN, Dectin-1, CLEC18A.Fc and CLEC18C.Fc (2µg/ml) binding to GLPS-F3 in the sugar competition assay.
TABLE LEGENDS:

TABLE 1. Primer lists. F indicated forward primer, R indicated reverse primer. Underlined nucleotides indicated complementary sequences of exon B.

TABLE 2. Typing of CLEC18 family in cell lines and human peripheral blood cell. Detection of CLEC18 family in cell lines (A) and human peripheral blood cells (B) by real-time RT-PCR using a LightCycler® 480 (Roche). +: detectable, -: undetectable. “*” were confirmed by cDNA clone sequencing.

TABLE 3. Sequencing of CLEC18 family in cell lines and human peripheral blood cell. Determination of CLEC18 polymorphism by cDNA sequencing. The polymorphic amino acid residues detected by sequencing more than 150 cDNA clones amplified from human cell lines and human primary cells. (A) The presence of CLEC18B was based on the presence of signature structure in CLEC18B (I91A118T151T173G185I196). However, we cannot detect exon B among all the cDNA clones we ever sequenced. Determination of CLEC18 alleles by cDNA sequencing in cell lines (B) and human peripheral blood cells (C). +: detectable, -: undetectable. “*” were confirmed by cDNA clone sequencing. “Φ”: real time PCR is negative, but sequencing is positive (but without the extra exon B).

CLEC18A*: minor allele; CLEC18B**: without exon B

TABLE 4. Polysaccharides used for sugar competition assay.
| Primer lists                                | Sequence                                      |
|--------------------------------------------|-----------------------------------------------|
| Primer for real time PCR                   |                                               |
| A                                          | 5’-AGCCAGGATGAAATGTCAGAGGAA-3’                |
| A’                                         | 5’-GGTGAGCCCGATCCAGAAGTTCC-3’                 |
| B                                          | 5’-GGGCTGGTGTGGCTGAGTGAGTG-3’                 |
| B’                                         | 5’-CGAGGGAGCCATGTGGTCA-3’                     |
| C                                          | 5’-CCGCCCAAGGACTCTCTCTCCG-3’                  |
| C’                                         | 5’-CGAGGGAGCCATGTGGTCA-3’                     |
| Hybridization probes                       |                                               |
| H1                                         | 5’-GATCAAGAGCCAGAAATGTCAGAGGAA-3’-FL          |
| H2                                         | LC610-5’-GACATCCGTGCTCGCTCTTATCTGGGCC-3’      |
| H3                                         | 5’-GCAAAACCCGCAACCCGTTACATCTGCC-3’-FL         |
| H4                                         | LC640-5’-GTTTGGCCAGAGCAGACTCTCCCCGG-3’        |
| H5                                         | 5’-TCAACTGGAAACAACCAGGCAGCGC-3’-FL            |
| H6                                         | LC670-5’-GCAAAACCCGAAACCAGGTTACATCTGCC-3’    |

| Primer pair for CTLD domain                | Sequence                                      |
|--------------------------------------------|-----------------------------------------------|
| CLEC18A F                                  | 5’-GGATCCAAAGGTGCAATTCTTCCTCCATAC-3’          |
| CLEC18A R                                  | 5’-GAATTCGTGGTGTCAGGCCTCCAGGACC-3’           |
| CLEC18A-1 F                                | 5’-CAGATCAAGAGACAAAGGTGAC-3’                 |
| CLEC18A-1 R                                | 5’-GCACTTTCTGCTCTTTGATCG-3’                  |
| CLEC18C F                                  | 5’-GCCCTTCAACTGGAACAAACGGCGCAGC-3’           |
| CLEC18C R                                  | 5’-GTTTGGATCAAGGTGACATTTTCCTCCCATCC-3’       |
| CLEC18B Part I F                           | 5’-GGCTGAGTCATGCTGACATGGGTGTGTCAGAGC-3’      |
| CLEC18B Part I R                           | 5’-GCACTGAGCCACCAGCAGGCCTGTTGTCAGAGCTGC-3’  |
| CLEC18B Part II F                          | 5’-GGCTGAGTCATGCTGACATGGGTGTGTCAGAGC-3’      |
| CLEC18B Part II R                          | 5’-GTTGAGTGAATTCGTGGTGTCAGGCCTCAGGACC-3’     |

F indicated forward primer, R indicated reverser primer. Underlined nucleotides indicated complementary sequences of exon B.
| Cell line   | CLEC18 mRNA |       |       |       |       |
|-------------|-------------|-------|-------|-------|-------|
|             | A  | A-1 | B  | C  |       |
| THP1        | +  | -   | -   | +  |       |
| U937*       | +  | -   | -   | -   |       |
| HL-60       | +  | -   | -   | -   | +    |
| K562*       | +  | -   | -   | -   |       |
| KU812       | +  | -   | -   | -   |       |
| Jurkat      | +  | -   | -   | -   | +    |
| CHME3       | +  | +   | -   | +  |       |
| A549        | -  | +   | -   | +  |       |
| BEAS-2B     | +  | -   | -   | -   |       |
| MCF-7       | +  | -   | -   | -   |       |
| HepG2       | +  | -   | -   | +  |       |
| 293T*       | +  | -   | -   | -   |       |
| SW480       | +  | -   | -   | +  |       |
| HT29        | +  | -   | -   | +  |       |

| Donor 1*    | +  | -   | -   | +  |       |
| Donor 2     | +  | -   | -   | +  |       |
| Donor 3     | +  | -   | -   | +  |       |
| Donor 4*    | +  | +   | -   | -   |       |
| Donor 5     | +  | -   | -   | -   |       |
| Donor 6     | +  | -   | -   | -   |       |
| Donor 7     | +  | -   | -   | -   |       |
| Donor 8*    | +  | +   | -   | -   |       |
| Donor 9*    | +  | +   | -   | -   |       |
| Donor 10    | +  | -   | -   | +  |       |

*: detectable, -: undetectable. "*" were confirmed by cDNA clone sequencing.
TABLE 3. Sequencing of CLEC18 family in cell lines and human peripheral blood cell

(A)

| NCBI Database | SCP hotspots(91, 118, 151, 173, 185, 196) | CTLD hotspots (339, 421, 430) |
|---------------|------------------------------------------|---------------------------------|
| CLEC18A NP_872425.2 | T V T A R V | S D* |
| CLEC18A-1 AAH41809.1 | T A M A R V | R D* |
| CLEC18B NP_001011880.2 | I A T T G I | S D' |
| CLEC18C NP_775890.2 | I A T A R V | S N* |

| cDNA sequencing | SCP hotspots(91, 118, 151, 173, 185, 196) | CTLD hotspots (339, 421, 430) |
|-----------------|------------------------------------------|---------------------------------|
| CLEC18A         | T A M A R/G V | S D* |
| CLEC18A*        | T A/V T A R V | S D* |
| CLEC18A-1       | T A M A R V | R D* |
| CLEC18B**       | I/T A T T G I | S D' |
| CLEC18C         | I/T A T/M A R V | S N* |

(B)

| Cell line | CLEC18 mRNA |
|-----------|-------------|
|           | A | A-1 | B | C |
| U937*     | + | - | - | - |
| K562*     | + | - | ☐ | + |
| 293T*     | + | - | ☐ | + |
| THP1      | + | - | - | + |
| CHME3     | + | + | ☐ | + |
| HepG2     | + | - | - | + |
| A549      | + | + | ☐ | + |

(C)

| CLEC18 mRNA |
|-------------|
| A | A-1 | B | C |
| Donor 1* | + | - | - | + |
| Donor 4* | + | - | ☐ | - |
| Donor 8* | + | + | - | - |
| Donor 9* | + | + | - | - |

+: detectable, -: undetectable. “**” were confirmed by cDNA clone sequencing; 
“☐”: real time PCR is negative, but sequencing is positive (but without the extra exon B). 
CLEC18A*: minor allele; CLEC18B**: without exon B
| Number | Name                   | Structure                                                                 |
|--------|------------------------|---------------------------------------------------------------------------|
| 1      | Alpha-eylodelontrin    | six alpha-1,4-linked Glc ring molecule                                    |
| 2      | D- (+)-Cellobiose      | beta-1,4-linked Glc disaccharides                                        |
| 3      | Dextran                | alpha-1,6-linked glucan with 1-3 linked Glc side chains                   |
| 4      | Dextran                | alpha-1,6-linked glucan with 1-3 linked Glc side chains                   |
| 5      | CM-cellulose 4M        | beta-1,4-Glc-linked glucan                                               |
| 6      | Amylose                | alpha-1,4-Glc-linked glucan                                               |
| 7      | Laminarin             | beta-1,3-linked glucan with beta-1,6-linked side chains                   |
| 8      | Pachyman              | beta-1,3-linked glucan                                                    |
| 9      | Fucoidan               | sulfated fucose polymer                                                   |
| 10     | Galactan               | beta-1,4-linked galactan                                                  |
| 11     | Larch Arabinogalactan  | beta-1,4-linked galactan                                                  |
| 12     | Beta-gentiobiose       | beta-1,6-linked Glc disaccharides                                        |
| 13     | Galactosylmannotriose  | beta-1,4-linked Mannan with alpha-1,6-Gal side chain                      |
| 14     | Di-galactosylmannopentaose | beta-1,4-linked Mannan with alpha-1,6-Gal side chain                   |
| 15     | F3                     | polysaccharide from *Ganoderma lucidum*                                  |
Figure 2
Figure 3

(A) Tunicamycin (5 μg/ml)

(B) MLHPETSHPRLAILAVVLLALLGCMAFVVPDKOKMAGALNEKSFLLLFLSNE
LESWOPPAANMRSLEGSASLAQLGQARAALCTPETSLASGWLWTLQVGNMQLLLP
AGLVSFLEVWLSLFAGQFQSHAASECMANNATCCTHYTYIQWAVATSSQLGCGHLCAG
QAlAEAFVCAYSPRGNWEVNGTIVPYPKGMCSLCTASVSCCFKADFAGGLCEVP
RNFRMCGQNHRLN1STCRKCPFGYTGRCQVRCSLQCVHRFRERECSVCRI6G
YGGACATKXHFTTCGSLHGDTCNVSSEALETTYBRMKQREGGVLAOIKSOLV
ODLAFYLGELATTNYLIDSHKETERHPWGLTYTAKOSFRATGERGKTSFARGG
PHNHFHCNSVQGLQASAFNMDQOBCTKRBYYTFCAFEGHSNEGCS.

(C) 18A

(D) - V 18A
Figure 4

(A) CD3 16.7  CD19 15.5  
CD66b 97.8  CD14 7.76  

(B) CLEC18 55  GAPDH 43  

(C) HL-60  U937  Jurkat  Raji  
CLEC18 55  GAPDH 40  

(D) Supernatant  Lysate  
- V  18A  - V  18A  

(E) Human sera  
Donor1  Donor2  Donor3  

CD3 CD19
Mo          DC      GM-Mφ      M-Mφ

Count

LysateSupernatant
15.5 16.7
CD3 CD19

CD66b 97.8  CD14 7.76

HL-60  U937  Jurkat  Raji

Supernatant  Lysate
- V  18A  - V  18A

Culture supernatant

ng/ml

Mo  DC  GM-Mφ  M-Mφ

Human sera

ng/ml

Donor1  Donor2  Donor3

nd
Figure 5

![Image of fluorescence microscopy images showing the localization of various markers: Hoechst, CLEC18, GM130, Calreticulin, EEA1, Mitotracker, and Lysotracker. The images are arranged in rows and columns, with each row showing different combinations of markers.]
Figure 6

- Spleen
- Liver
- Fallopian tube
- Brain
- Gall bladder
- Testis
Figure 7

(A) GLPS-F2

(B) CaCl₂/MgCl₂  EDTA

Dectin-1  298  535
CLEC18A  73.6  39.7
CLEC18A-1  15.8  16.9
CLEC18B  22.4  23.3
CLEC18C  15.7  15.0
Human CLEC18 gene cluster contains C-type lectins with differential glycan-binding specificity
Ya-Lang Huang, Feng-Shuo Pai, Yun-Ting Tsou, Hsien-Chen Mon, Tsui-Ling Hsu, Chung-Yi Wu, Teh-Ying Chou, Wen-Bin Yang, Chung-Hsuan Chen, Chi-Huey Wong and Shie-Liang Hsieh

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