Clec12a inhibits MSU-induced immune activation through lipid raft expulsion

Ying Xu1,*, Dingka Song1,2,*, Wei Wang3, Shixin Li4, Tongtao Yue3, Tie Xia1, Yan Shi1,5

Monosodium uric acid (MSU) crystal, the etiological agent of gout, has been shown to trigger innate immune responses via multiple pathways. It is known that MSU-induced lipid sorting on plasma membrane promotes the phosphorylation of Syk and eventually leads to the activation of phagocytes. However, whether this membrane lipid-centric mechanism is regulated by other processes is unclear. Previous studies showed that Clec12a, a member of the C-type lectin receptor family, is reported to recognize MSU and suppresses this crystalline structure-induced immune activation. How this scenario is integrated into the lipid sorting-mediated inflammatory responses by MSU, and particularly, how Clec12a intercepts lipid raft-originated signaling cascade remains to be elucidated. Here, we found that the ITIM motif of Clec12a is dispensable for its inhibition of MSU-mediated signaling; instead, the transmembrane domain of Clec12a disrupts MSU-induced lipid raft recruitment and thus attenuates downstream signals. Single amino acid mutagenesis study showed the critical role of phenylalanine in the transmembrane region for the interactions between C-type lectin receptors and lipid rafts, which is critical for the regulation of MSU-mediated lipid sorting and phagocyte activation. Overall, our study provides new insights for the molecular mechanisms of solid particle-induced immune activation and may lead to new strategies in inflammation control.

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Introduction

Lipid raft composed of tightly packed lipid molecules is critical for membrane-initiated signaling in eukaryotic cells (Sezgin et al, 2017). Previous studies highlighted the importance of lipid rafts in regulating various biological processes (Simons & Ikonen, 1997; Varshney et al, 2016). Relocation of membrane receptors in and out of the lipid raft region upon ligand recognition is an essential mechanism in controlling activation and subsequent signal transduction. Recently, the concept of liquid-ordered (Lo) and liquid-disordered (Ld) domains have been raised by membrane biologists. The composition and dynamic behavior of the Lo domain mimic lipid rafts and make it rather interesting to investigating the biological role of phase separation on plasma membrane in receptor-mediated signal transduction (van der Goot & Harder, 2001; Quinn & Wolf, 2009). Of immunological interest, the translocation of TCR into cholesterol-enriched lipid raft region is a prerequisite for T cell activation (Janes et al, 2000). In innate immunity, it is also reported that the activation of pattern recognition receptors requires the involvement of lipid rafts (Ruysschaert & Lonez, 2015). Reciprocally, some transmembrane proteins are capable of affecting the dynamics and aggregation of lipid molecules on the plasma membrane as well (Devaux, 1992; McIntosh et al, 2003; Corradi et al, 2018). The plasma membrane has a highly hydrophobic core, and the presence of sphingolipids, such as sphingomyelin, along with omnipresent cholesterol, creates an even and high degree of horizontal packing (Diaz-Rohrer et al, 2014). This design leads to preferential selection of transmembrane domains (TMD) of cell surface proteins. The composition, arrangement, and chemical property of amino acid residues in TMDs are also critical in determining repulsion and attraction towards various lipid species, hence affecting the behaviors of surrounding lipid molecules (Lorent & Levental, 2015). How such interactions influence intracellular signals is an emerging field of investigation.

Lipid rafts can participate in transmembrane signaling in multiple ways. For instance, GPI-anchored cell surface molecules, without any intrinsic signaling moieties, can lead to intracellular kinase activation by virtue of its association with the membrane lipids (lipid rafts) (Štefanová et al, 1991). For Group I metabotropic glutamate receptors, their association with lipid rafts drives caveolin (a component of a subset of lipid rafts)-dependent intracellular Ca\(^{2+}\) signaling (Roh et al, 2014). Therefore, lipid rafts can be seen as a conduit or a platform for organizing cell surface–signaling events. Our previous work showed that “lipid sorting” on plasma membrane upon monosodium urate crystal (MSU) binding recruits cholesterol-enriched microdomains...
on the MSU-binding site (Ng et al, 2008), promoting the engagement of immune tyrosine activating motif (ITAM)-containing molecules, either in the form of nondiscriminatory gathering of ITAM-containing transmembrane proteins or the intracellular protein moesin that has a cryptic ITAM at its N-terminus, to the plasma membrane (Mu et al, 2018). In addition, Alum, the commonly used adjuvant in vaccines, was also shown to be active in regulating lipid domain composition on innate immune cells to exert its adjuvant effects (Flach et al, 2011).

As solid structure recognition is an ancient mechanism pre-dating immune receptors (Mu et al, 2018), our proposal provides a generic mechanism for phagocyte interaction with almost an infinite number of solid structures. Although no protein receptor is known to be responsible for MSU binding and immune activation, it was reported that the immune tyrosine-inhibiting motif (ITIM)-containing C-type lectin receptor Clec12a recognizes and inhibits MSU-induced inflammatory signals (Neumann et al, 2014). With the ITIM motif on the intracellular domain, it was reasonable to speculate an ITIM-mediated inhibitory effect of Clec12a would work through dephosphorylation that offsets the non-receptor kinase activities, such as those from Syk or common Fc γ chain (Lahoud et al, 2009). If this were true, it would represent one of the least studied signaling cascades, that a lipid sorting-based ITAM activation event was countered by a solid particle-specific receptor via its ITIM functions. However, whether this chain of events can take place has not been formally investigated.

Here, we report that Clec12a was indeed a negative regulator for MSU-mediated immune activation. However, the inhibitory activity of Clec12a was independent of its ITIM motif. In contrast with the prediction from previous studies (Lahoud et al, 2009), ITIM mutants exerted full suppressing capacity. Interestingly, we found that TMD of Clec12a was critically needed for the suppression. TMD domain swapping of Clec12a with several other Clec family members all showed a comparable level of suppressing efficacy, with the only exception of Clec4a. Imaging analysis indicated that whereas MSU crystals recruited lipid rafts domains upon contact, the presence of Clec12a TMD drove their dispersion, whereas Clec4a TMD failed to show this effect. As an additional piece of evidence, Clec12a appeared to reduce silica-induced lipid raft association on live cells if this molecule was pulled into the lipid rafts via an artificial link on the silica surface, leading to depressed immune responses. Collectively, these data revealed a critical role of Clec12a TMD in disrupting MSU-induced lipid domain aggregation and downstream immune activation, providing new insights for future studies on immune recognition of solid particles.

Results

ITIM is not involved in Clec12a-mediated suppression of inflammatory response to MSU

As myeloid cells secrete interleukin 1beta (IL-1β) in response to MSU stimulation (Chen et al, 2006), the effects of Clec12a-mediated suppression can be assessed by the diminished IL-1β production. To establish a clean background, we used CRISPR/Cas9 to produce diploid deletion of clec12a gene in human monocyte THP-1 cells. The efficacy of Clec12a depletion was verified by Western blot (Fig S1A). The expression of Clec12a was then reestablished by lentiviral transfection (Fig S1A). As Clec12a has an intracellular ITIM domain responsible for SH2 domain-containing phosphatase 1/2 (SHP-1/2) recruitment and potentially transducing inhibitory effect, the ITIM mutant version of Clec12a (Y17F) was also generated (Fig S1A). Surface expression of Clec12a constructs were validated by anti-Clec12a Ab staining and flow analysis (Fig S1B). As expected, depletion of endogenous Clec12a-enhanced IL-1β and IL-18 production in response to stimulation of MSU, and overexpression of WT Clec12a significantly reduced the secretion of those cytokines. Unexpectedly, overexpression of the Clec12a ITIM mutant (Y17F) exhibited a comparable suppressive effect to WT (Fig 1A). To rule out that it was a peculiarity in THP-1 cells, we used similar overexpressing approaches on BMDCs from clec12a−/− mice. With those primary cells, ITIM mutation (Y7F) did not affect the inhibitory activity of Clec12a in response to MSU as well (Fig 1B). Accordingly, deletion of Clec12a led to increased production of mature Caspase-1 (p20), and the overexpression of either WT or Y7F mutant reinstated the suppressive effect (Fig 1C). This impact on inflammasome-associated p20 conversion likely reflected that lipid sorting-based signaling mechanism controls the intensity of phagocytosis, which is a prerequisite for intracellular NLRP3 inflammasome assembly. In line with this, the inhibitory efficacy on the intracellular apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) condensation (specks formation) after MSU stimulation was comparable between WT and Y7F mutant groups as well (Fig 1D). However, the absence or presence of any versions of Clec12a did not affect NF-κB activation (Fig S2A), which in the case of BMDCs, indicated the priming phase (signal 1) for inflammasome activation. Similarly, little impact on TNFα production in response to bacterial endotoxin LPS was observed in all versions of Clec12a transfectants (Fig S2B), suggesting that Clec12a is not involved in signal 1 activation, which is the prerequisite for inflammasome activation.

All these data implicated the possibility that the suppressive activity of Clec12a on MSU-induced inflammasome activation was independent of ITIM. To ascertain this point, HA-tagged WT or ITIM mutant Clec12a (Y7F) were overexpressed in murine DC line DC2.4 cells. The baseline expression of Clec12a on DC2.4 cells and the expression levels of various Clec12a constructs were assessed (Fig S1C). Notably, the baseline expression of Clec12a on DC2.4 cells is nearly undetectable and the expression levels of WT and ITIM mutant Clec12a were equivalent (Fig S1C). The surface expression of various Clec12a constructs on DC2.4 cells was further validated by flow cytometry (Fig S1D). Using EGFP-HA as the efficacy control, the interaction of Clec12a with SHP-1/2 was studied by co-immunoprecipitation (Co-IP). It showed that WT Clec12a was able to Co-IP both SHP-1 and SHP-2, whereas neither was pulled down with ITIM mutant Clec12a (Fig S2C and D). To further validate the ITIM-independent role of Clec12a inhibiting MSU-induced inflammasome activation, the full ITIM truncation version of Clec12a (Clec12a [ΔITIM]) was introduced and IL-1β secretion was assessed upon MSU stimulation. The overexpression of Clec12a (ΔITIM) significantly inhibits MSU-induced L-1β secretion in both mouse BMDC and THP-1 cells, which was comparable with the single amino acid mutation version of Clec12a ITIM domain (Fig S2E). These data
Lipid disruptional effects of Clec12a in response to MSU

CD80/86, another set of surface markers re-accessed. RT-PCR analysis revealed that the amounts of clec12a transcripts in clec12a occurs before the Syk recruitment (Fig 2A and B). Expression of CD80/86 under MSU stimulation, suggesting that the blockage either WT or Y17F mutant Clec12a in DC2.4 cells markedly reduced the p-Syk level under MSU stimulation, which helped reveal its point of interception with Zap70, which is the obligatory conduit for ITAM-based tyrosine protein kinase Syk phosphorylation (Ng et al, 2008), aggregating in response to solid particle binding including MSU led to stereotypic, non-phagocytic monocytes, both of which provide ITAMs required for Syk membrane recruitment (Mu et al, 2018). To investigate the involvement of Clec12a in “MSU-lipid raft-Syk” signaling axis, endogenous Clec12a-deficient Hela cells were transfected with either GFP-fused WT Clec12a, Y17F mutant Clec12a, or an empty vector, and cells were stimulated with MSU and stained with cholera toxin B subunit (CTB) that specifically recognizes ganglioside GM1 for lipid raft visualization and colocalization analysis. Hela cells were used because they are non-phagocytic, permitting a steady imaging status without any phagocytic progression, while at the same time retaining the autonomous membrane events. In the presence of MSU stimulation, strong colocalization between MSU and lipid rafts was observed in the vector transfectant, which was consistent with previous reports (Fig 3A) (Ng et al, 2008). However, overexpression of either the WT or Y17F mutant Clec12a largely disrupted the association of MSU and lipid rafts, with a reciprocal-increased association between MSU and lipid rafts, with a reciprocal-increased association (Fig 3A). Pearson’s coefficient in both WT and Y17F mutant Clec12a transfectant groups was significantly reduced compared with their unstimulated controls (Fig 3B). It has been reported that Clec12a is internalized upon MSU recognition (Gagné et al, 2013). To exclude the possibility of Clec12a internalization indicating that the intrinsic association between ITIM and SHP-1, which mediates the bulk of suppression activities in other scenarios involving CLR, was operational in our system. However, this dephosphorylation event was not used by Clec12a to reduce MSU-mediated inflammatory effects.

**ITIM-independent role of Clec12a in attenuating MSU induced DC activation**

MSU is an endogenous danger signal in inducing inflammatory response (Shi et al, 2003). MSU-mediated phagocyte activation is not limited to NLRP3 inflammasome, it was shown that lipid raft aggregation in response to solid particle binding including MSU led to tyrosine protein kinase Syk phosphorylation (Ng et al, 2008), which, along with Zap70, is the obligatory conduit for ITAM-based immune activation. We assessed whether Clec12a negatively impacted Syk activation, which helped reveal its point of interception in MSU signaling cascade. The results showed that expression of either WT or Y17F mutant Clec12a in DC2.4 cells markedly reduced the level of p-Syk under MSU stimulation, suggesting that the blockage occurs before the Syk recruitment (Fig 2A and B). Expression of CD80/86, another set of surface markers reflecting DC activation, was also accessed. RT–PCR analysis revealed that the amounts of CD80/86 transcripts in clec12a-/- BMDCs were substantially elevated upon MSU stimulation, yet overexpression of either WT or Y17F mutant Clec12a significantly reduced CD80/86 transcription (Fig 2C). Similar results were observed at the protein levels by surface CD80/86 staining and flow cytometry analysis (Fig 2D). Taken together, these results demonstrated that Clec12a can inhibit DC activation in response to MSU crystals in general.

**Clec12a interrupts MSU-induced lipid raft aggregation**

Lipid raft clustering either condenses ITAM-containing membrane receptors or recruits intracellular moesin to the inner leaflet of the plasma membrane, both of which provide ITAMs required for Syk membrane recruitment (Mu et al, 2018). To investigate the involvement of Clec12a in “MSU-lipid raft-Syk” signaling axis, endogenous Clec12a-deficient Hela cells were transfected with either GFP-fused WT Clec12a, Y17F mutant Clec12a, or an empty vector, and cells were stimulated with MSU and stained with cholera toxin B subunit (CTB) that specifically recognizes ganglioside GM1 for lipid raft visualization and colocalization analysis. Hela cells were used because they are non-phagocytic, permitting a steady imaging status without any phagocytic progression, while at the same time retaining the autonomous membrane events. In the presence of MSU stimulation, strong colocalization between MSU and lipid rafts was observed in the vector transfectant, which was consistent with previous reports (Fig 3A) (Ng et al, 2008). However, overexpression of either the WT or Y17F mutant Clec12a largely disrupted the association of MSU and lipid rafts, with a reciprocal-increased association between MSU and lipid rafts (Fig 3A). Pearson’s coefficient in both WT and Y17F mutant Clec12a transfectant groups was significantly reduced compared with their unstimulated controls (Fig 3B). It has been reported that Clec12a is internalized upon MSU recognition (Gagné et al, 2013). To exclude the possibility of Clec12a internalization
that might affect our results, we performed a time-course assay of MSU-induced Clec12a internalization by flow cytometry. It revealed that MSU stimulation does not induce significant endocytosis of Clec12a in 1 h (Fig S3A), which in addition, lagged behind the time of MSU stimulation in our experiment settings (5–10 min) and would not affect the interpretation to our results.

Ganglioside GM1 staining of CTB reflects a proportion rather than the total amount of lipid rafts on the plasma membrane, we specifically calculated the colocalization rate between MSU and CTB staining lipid rafts on different cell types. It shows that the colocalization rate between MSU and CTB-positive areas in clec12a−/− cells is less than 1, suggesting the existence of CTB-negative lipid rafts. In addition, in the presence of WT or Y17F mutant Clec12a, the colocalization rate between MSU and CTB substantially reduced (Fig S3B). These results support our hypothesis that Clec12a suppresses MSU-induced lipid raft recruitment independent of its intracellular ITIM. In addition to CTB staining, other strategies were also developed for lipid raft probing (Carquin et al, 2014). To further verify lipid raft visualization by CTB staining in our study, we used another lipidic membrane fluorescent probe, di-4-ANEPPDHQ, for direct membrane staining and GP imaging analysis (Dinic et al, 2011). It revealed that the MSU-contacted region is associated with the Lo domain, indicating the recruitment of lipid rafts in the MSU-contacted place (Fig S3C).

To determine the role of Clec12a and lipid raft on MSU-induced downstream signals, Syk phosphorylation was studied on different cells upon MSU stimulation with or without MβCD treatment. It showed that MβCD efficiently depleted cholesterol on cells (Fig 3C). Furthermore, the enhanced Syk phosphorylation in the absence of Clec12a interference was abrogated by MβCD treatment, suggesting...
the Clec12a regulation was at the step of lipid organization (Fig 3D). On the other hand, the Syk phosphorylation was low in the presence of Clec12a, with or without MβCD. These data suggest that that interaction between Clec12a and MSU inhibited the lipid raft aggregation in response to MSU crystals (Fig 3D). In supporting this, MβCD treatment significantly suppressed IL-1β secretion in clect12a−/− BMDCs in response to LPS plus MSU stimulation, demonstrating the dominant role of lipid rafts in transducing MSU-initiated pro-inflammatory signals (Fig S3D and E).

It has been also reported that MβCD treatment can affect the overall organization of the plasma membrane (Zidovetzki & Levitan, 2007), or even lead to the formation of solid ordered

Figure 3. Clec12a disrupts monosodium uric acid (MSU)-induced “lipid sorting.”

(A) Confocal images of the distribution of lipid raft (red) and Clec12a-eGFP (or empty vector) (green) on the plasma membrane. Endogenous Clec12a-deficient Hela cells overexpressing with the indicated vectors were stained with Alexa-Fluor 647-conjugated CTB, stimulated with MSU (or left untreated), and monitored under a confocal microscope. Wavelengths of Ex were set at 488 nm (eGFP) and 633 nm (AF647), respectively; wavelengths of Em were set at 493–561 nm (eGFP) and 688–756 nm (AF647), respectively. Bright field images were also captured for MSU visualization. Magnification of oiled-immersed objective lens: 63x. Images were processed by Image J, the BF images (first row), lipid raft (red, second row), eGFP (green, third), and merged images (fourth row) were displayed. Arrows indicate MSU-contacted regions on the plasma membrane. Scale bar: 10 μm.

(A, B) Statistical analysis of the colocalization rate between lipid raft and Clec12a-eGFP in (A).

(C) Efficiency of cholesterol depletion by MβCD. DC2.4 cells overexpressing with the indicated vectors were stimulated with MSU, MSU + MβCD or left untreated. Cells were washed with PBS for three times and the amount of retained cholesterol was detected.

(D) DC2.4 cells overexpressing with the indicated vectors were stimulated with MSU, MSU + MβCD or left untreated, Syk phosphorylation was detected by Western blot. Data are represented from of three independent experiments. CTB, chloro toxin subunit B; MβCD, methyl-beta-cyclodextrin; Ctrl, control; BF, bright field.
or gel domains (Giocondi et al., 2004). To exclude the cytotoxic effect of MβCD on cells, cell death events were analyzed after MβCD treatment. It shows that MβCD stimulation in our setting does not induce significant cell death (Fig S3F). Nevertheless, as MβCD treatment may cause reorganization of the plasma membrane beyond lipid raft disruption, our results of MβCD treatment can be used only as supporting but not definitive evidence for the involvement of lipid rafts. Collectively, these results highlight the role of lipid rafts in mediating Clec12a’s inhibiting role in MSU-induced DC activation.

Figure 4. The transmembrane region of Clec12a is critical in the inhibition of monosodium uric acid (MSU)-induced lipid sorting. (A) Transmembrane sequence comparison of Clec12a and other family members. (B) As in Fig 3A, endogenous Clec12a-deficient HeLa cells were overexpressed with different Clec12a transmembrane mutants and monitored under a confocal microscope. All parameters were set as same as Fig 3A. Scale bar: 10 μm. (B, C) Statistical analysis of the colocalization rate in (B). (D) As in Fig 3D, cells were transfected with different Clec12a mutants: the transmembrane domain (TM) of Clec12a swapped with the same region from Clec4a (TM_Clec4a) or Clec7a (TM_Clec7a) or Clec9a (TM_Clec12a) or kept unchanged (TM_Clec12a). Later, DC2.4 cells overexpressing different Clec12a transmembrane mutants were stimulated with MSU or left untreated. Syk phosphorylation was analyzed by Western blot. (B, E) Confocal imaging of HeLa cells overexpressing WT or L46F, L57F mutation of Clec12a and stimulated with MSU as in (B). Scale bar: 10 μm. (E, F) Statistical analysis of the colocalization rate in (E). Data are represented from of three independent experiments. TM, transmembrane; L, leucine; F, phenylalanine.
Clec12a TMD interferes with lipid raft sorting

C-type lectin family members are single-span type II transmembrane proteins (Dambuzo & Brown, 2015). As their TMD are exposed to a high hydrophobic lipid core (Janes et al, 2000; Varshney et al, 2016), a potential mechanism for Clec12a’s inhibitory activity was to use its extracellular C-terminal domain as the specificity determinant for MSU (Neumann et al, 2014), whereas interrupting lipid raft aggregation via its TMD. To address this, the TMD of Clec12 was swapped with three other Clec family members, Clec 4a, 7a, and 9a (Fig 4A). The expression levels were determined to be similar using Clec12 C-terminal-specific antibody (Fig S4A). Flow cytometry analysis further revealed that the mutation of the TMD of CLEC12A does not affect its surface expression or binding affinity to the anti-Clec12a antibody (Fig S4B). Colocalization of Clec12a mutants, lipid rafts, and MSU were imaged as above. Interestingly, swapping with TMDs from Clec7a (TM_Clec7a) or 9a (TM_Clec9a) had results similar to WT Clec12a, yet replacing with Clec4a TMD (TM_Clec4a) significantly diminished the expulsion effect of Clec12 in MSU-induced lipid raft accumulation (Fig 4B and C). Consistently, Western blot results showed that Syk phosphorylation only took place when TMD was replaced by that of Clec4a (Fig 4D). Specifically, TMD switching from Clec12a to Clec4a dampened the inhibitory effect of Clec12a on MSU-induced Syk phosphorylation, indicating the opposite functions of TMDs from Clec4a and Clec12a in transducing MSU-related signals (Fig 4D). To determine the physiological role of the TMD in transducing Clec12a’s inhibitory signal, IL-1β secretion under stimulation was analyzed in various Clec12a TMD mutation transfecants (Fig S4C). Overexpression of WT Clec12a suppressed IL-1β secretion, whereas TMD swapping with Clec4a (but not others) restored the level of secreted IL-1β. To verify this, more precise mutagenesis of TMD in Clec12a was conducted (based on both WT and TM_Clec4a versions of Clec12a) and IL-1β secretion was analyzed. It showed that although elongation or truncation of TMD did not affect the inhibitory effect of WT Clec12a, similar modifications on the TM_Clec4a version of Clec12a, especially four amino acids (aa) deletion in TMD, significantly reduced the secretion of IL-1β, indicating that a mechanism intrinsic to this particular TMD makes it unable to suppress MSU-mediated activation (Fig S4D). Sequencing of TMDs from those Clec family members revealed that 12a, 7a, and 9a are formed mostly by small hydrophobic amino acids which might make the TMD loosely packed as a result of having so many bulky side-chains (Diaz-Rohrer et al, 2014; Lorent et al, 2017). Clec4a, on the other hand, contains several phenylalanines (Phe or F) which might make the TMD loosely packed as a result of having so many bulky side-chains (Diaz-Rohrer et al, 2014; Lorent et al, 2017). To ascertain this, we separately introduced four F amino acids into Clec12a TMD by mutagenesis of Leucine (L) to F (Fig S4F). Surface expressions of various mutants were also ascertained to be equivalent on both THP-1 and Hela cells (Fig S4F). Mutations of leucine to phenylalanine at position 53 and 54 largely impaired membrane targeting of Clec12a (data not shown). For the rest, Clec12a mutants L46F and L57F were less likely to be recruited into and disrupted lipid rafts (Fig 4E), Clec12a-mediated inhibition of MSU induced IL-1β secretion was also removed by F mutations (Fig S4G). As TMD mutations tend to impact surface expression, this work cannot be systematically carried out for all positions; our preliminary data nevertheless indicated that Clec12a may interfere with MSU activation by virtue of a tightly packed alpha helical hydrophobic core of its TMD.

Clec12a-mediated inhibitory effect is autonomous

Our data suggested that Clec12a mediated the inhibitory effect via its TMD, although it remained unknown whether such an effect was applicable in the phagocytosis of other solid crystals. Silica crystals similarly activate phagocytes, whereas no negative receptor has been identified yet (Hornung et al, 2008). To test the inhibitory activity of Clec12a TMD in silica-induced immune activation, streptavidin-coated silica crystals were co-cultured with THP-1 with biotin-labeled Clec12a antibody, which artificially trapped Clec12a underneath the silica crystals on the plasma membrane, creating an artificial colocalization of Clec12a with aggregated lipid rafts formed in response to silica crystal binding (Fig 5A, scheme). IL-1β production was measured accordingly, and it revealed that the TMDs of Clec12a, Clec7a, and Clec12a were all effective in suppressing silica-induced IL-1β secretion. In contrast, TMD replacement with Clec4a essentially removed the inhibitory activity of Clec12a (Fig 5B). Colocalization of different Clec12a mutants with lipid rafts was also visualized and it showed a trend similar to that of MSU with inhibitory TMD-expelling lipid rafts (Fig 5C and D). Taken together, these results revealed that Clec12a TMD operated as a strong interference for crystal-induced lipid raft aggregation, suggesting a new molecular basis for its suppression of MSU-induced immune activation.

In vivo activity of Clec12a in inhibiting MSU-induced acute inflammation via lipid raft aggregation

To better understand the physiological role of Clec12a in MSU-induced inflammation in vivo, a mouse model of air pouch gout was established (Pessler et al, 2008). Briefly, C57BL/6 or clec12a−/− mice were injected with sterile-filtered air underneath the back skin, which provided a suitable environment for MSU inoculation and fluid accumulation mimicking the pathogenesis of gout (Fig 6A, scheme). The results showed that MSU injection led to the secretion of proinflammatory cytokines including IL-1β and IL-6 in both C57BL/6 and clec12a−/− mice, whereas the amount of secreted cytokine in the clec12a−/− group was higher compared with WT control. Meanwhile, dual administration of MSU and MβCD led to a significant abrogation of cytokine secretion in both groups, and the fold difference was no longer found between C57BL/6 and clec12a−/− mice. Sole administration of MβCD did not induce any inflammation as expected (Fig 6B and C). These results suggest that the inhibitory effect of Clec12a was no longer applicable in the absence of lipid rafts. Flow cytometry was performed for neutrophil infiltration analysis (Fig 5S for gating strategy). It revealed that the infiltration of neutrophils upon MSU administration and the number of infiltrated cells in clec12a−/− mice were significantly higher than the one in WT control. However, MβCD co-treatment suppressed the infiltration of neutrophils in responses to MSU and no difference was observed between C57BL/6 and clec12a−/− mice (Fig 6D and E). To verify this, we established another mouse model via intra-plantar injection of MSU crystals (Lin et al, 2020; Shin et al, 2020).
2020). 18 h later, foot swelling of mice under different treatments was compared and inflammatory responses were evaluated. It showed that MSU administration induced more severe foot swelling in clec12a−/− mice than WT control, which was significantly relieved when treated in combination with MβCD (Fig 6F). The amount of retained cholesterol on infiltrated cells of each group was quantified, it showed that MβCD injection led to the reduction of cholesterol on cells (Fig 6G). Histopathologic analysis also revealed the differential infiltration of lymphocytes and was attenuated by co-administration of MβCD (Fig 6H). Collectively, these results demonstrated the physiological role of Clec12 in MSU-induced immune activation in vivo, which highlight the importance of lipid raft in transducing Clec12a-initiated signals in response to MSU stimulation.

Computational analysis of Clec12a TMD in interrupting MSU-induced lipid reorganization

In general membrane biology, biophysics analysis tends to suggest that receptor–ligand ligation leads to the demobilization of receptors, indicating a state of activation. In the case of Clec12a, we wondered if the high aggregation of Clec12a TMD can also block lipid domain formation. To address this, coarse-grained (CG) molecular dynamics (MD) simulations were performed on TMD of WT Clec12a in a multi-component membrane experiencing phase separation (Monticelli et al, 2008; Wu et al, 2014). Simulation results showed that most of the TMD of WT Clec12a were partitioned to the boundaries between Lo domain and Ld domain (Fig 7A), and showed higher affinity to unsaturated 1,2-diarachidonoyl-sn-glycero-3-phosphocholine (DAPC) in the disordered domain (Fig 7B), which might contribute to the ability of Clec12a to pull MSU out of lipid rafts because Clec12a can switch between Lo and Ld domains. To understand how different mutations influence Clec12a partitioning in the phase-separated membrane, all-atom (AA) MD simulations were performed. Compared with TMD of WT Clec12a, L46F and L57F mutants were more likely to associate with the Lo domain (lipid raft, Fig 7C), which is consistent with confocal imaging results and the limited inhibitory effect of swapped Clec4a TMD in response to MSU. To elucidate why such mutation favors lipid rafts, AA MD simulations were performed on L46F and L57F mutants of Clec12a in the Lo domain. Interestingly, the side chain of benzene in F46 and F57 formed π-π stacking with surrounding cholesterol (Fig 7D and E). We calculated change of the interaction energy between specific residues and cholesterol (Fig 7F). The positive values for L46 and L57 manifested unfavorable
Figure 6. In vivo role of Clec12a in inhibiting monosodium uric acid-induced acute inflammation via lipid raft aggregation.
(A) Establishment of mouse air pouch gout model via back skin air injection and MSU administration. (B, C, D) Fluid in the pouch of mouse back was extracted and the amount of pro-inflammatory cytokines IL-6 (B), and IL-1β (C) in supernatants were detected via ELISA. (D, E) Fluid in the pouch of mouse back was extracted and the infiltration of total cells (D) and neutrophils (E) were detected via flow cytometry. (F) Thickness of mice footpads under different treatments. (G) Efficiency of cholesterol depletion by MβCD in vivo. Infiltrated cells of each group were counted and diluted to 1 million per sample; the concentration of cholesterol was detected as described above. (H) H&E staining images of mouse footpads under different treatments as indicated. Magnification: 20x. Scale bar: 100 μm. Five mice per group was setup in mouse experiments and data are represented from of three independent experiments.
association between WT Clec12a and lipid raft. After mutation, the energy changes became negative, suggesting favorable interactions of mutants with lipid raft. By contrast, L46F induced lower energy than L57F because the residue of F46 locates shallower in the membrane to form more stacking contacts with cholesterol. Collectively, these results suggested a potential molecular mechanism of Clec12a TMD in inhibiting MSU-induced inflammatory signals via interference of lipid sorting.

Discussion

Immune sensing of solid particle is of interest in the field of innate immunity because of their adjuvanticity and their ability to drive crystallopathy. However, the underlying mechanism of solid particle recognition by immune cells has not been clarified yet, possibly because of the diversity and complexity of their molecular structures (Shi, 2012). As one of the most well-established DAMPs, MSU crystal was identified as the causative agent of gout (Shi et al., 2003; Rock et al., 2013) and has been extensively studied for its role in inflammation and innate immune response. It was shown that MSU has a strong ability to drive inflammation via NLRP3 inflammasome activation (Martinson et al., 2006) and Syk-dependent inflammatory phagocytosis (Ng et al., 2008). Whether there is a cell-surface receptor for MSU has remained a mystery. Neumann et al identified Clec12a as the receptor for MSU on the plasma membrane by constructing the fusing protein with the extracellular domain of Clec12a and Fc part of IgG, the recognition of MSU by Clec12a was also revealed by the cell reporter system (Neumann et al., 2014).

Although the interaction between Clec12a and MSU was established, it is still unclear about the mechanism for Clec12a’s inhibitory function. ITIM motif in Clec12a per current dogma appears to be sufficient in explaining the attenuation. This proposal had not been vigorously tested. Our current study shows that Clec12a interrupts MSU-induced lipid raft aggregation to produce the inhibitory effect. This finding illustrates how this protein receptor Clec12a can negatively impact a lipid sorting-based innate activation. We, for the moment, do not know why the ITIM signaling, which is intact in our system by co-precipitation assay, did not contribute to the inhibition. One possibility is that the membrane lipid ligation-mediated phagocytosis is exceedingly strong and rapid, and the dephosphorylation event may be of limited potency in reversing the process. This possibility remains to be tested in the future.

Regarding the molecular mechanism of Clec12a interfering with MSU-induced lipid sorting, our molecular simulation data suggested that the TMD of WT Clec12a has more favorable interactions with the Rd domain. Given this fact, upon Clec12a binding with MSU, it inhibits the recruitment of Lo domain in the MSU-contacted region. L to F mutations on position 46 or 57 promotes the interaction of Clec12a to the Lo domain, which reduced the threshold for MSU-induced lipid sorting in the presence of Clec12a. Previous studies proposed key determinants of the transmembrane sequence in interacting with lipid domains, which include the length, amino acid composition, modifications, etc. (Lorent et al., 2017). Our simulated data suggested that differential behaviors of Clec12a and its mutants in interacting with the cell membrane are subjected to enthalpy-driven partitioning of Clec12a in different lipid domains.
Because the Lo phase is relatively tight, and interactions between surrounding lipids are strong, introduction of external proteins may cause a huge loss of enthalpy, which may not be offset by the increase in entropy during the process. The Ld phase, on the other hand, is less dense and more favorable for Clec12a, and because lipids in the Ld phase are already poorly organized, introduction of protein does not lead to significant increases of entropy. Therefore, Clec12a repels the Lo phase whereas having stronger affinity to the Ld phase. The fact that L-F mutation drives Clec12a toward the Lo phase can be explained by an additional finding: the π–π interaction of F residues in the TMD with cholesterol in Lo. It is likely that both mechanisms are at work to promote better acceptance of non-inhibiting, Clec4a-like TMD into lipid rafts. In contrast, forced entry of an inhibiting TMD, such as under the pulling force of extracellular Clec12a binding to MSU surface, disrupts lipid sorting and the ensuing phagocytic signaling. In addition, it was reported that phosphorylation of the Clec12a ITIM domain depends on receptor clustering in flotillin-rich membrane domains and this could be disrupted by MjCD (Paré et al., 2021). Therefore, it would be intriguing to compare the phosphorylation of SHP-1/2 of two Clec12a TMD mutants, which would be insightful for future studies on the relationship between lipid membrane partitioning of receptors and intracellular signal transduction.

Despite continuous debates for decades, the concept of lipid raft, which refers to microdomains enriched in saturated lipids, sphingolipids, and cholesterol, on the plasma membrane, has been well accepted by communities as the “supporting platform” where most membrane receptors can be activated (Hancock, 2006). Biophysical studies revealed that, in contrast to Ld phases containing loose-packed unsaturated lipid species, the physical chemistry of lipid raft (or Lo domain) is more rigid because of tight interactions among different components, which supports the conformational and oligomerizing state alteration of membrane proteins required for signal transduction (Dietrich et al., 2001; Kaiser et al., 2009). The molecular basis for lipid raft formation is the evident affinity between cholesterol and saturated lipids, whereas both of them are repulsive to unsaturated lipids (Levental et al., 2020), which ultimately lead to liquid–liquid phase separations (Dietrich et al., 2001). In living cells, lipid rafts are more dynamic because they are regulated by variable factors, of which, transmembrane proteins are in general detrimental for the formation and dissociation of lipid rafts through peptide–lipid interactions (Simons & Sampaio, 2011; Sezgin et al., 2017). It has been proposed that several transmembrane protein motifs are responsible for specific interactions with different lipid species. For instance, Pleckstrin homology (PH) domains and Phox homology (PX) domains are predominantly responsible for recognizing phospholipids; the cholesterol consensus motifs (characterized by L/V(X)(1–5)Y(X)(1–5)R/K) are responsible for cholesterol recognition; whereas V(X)(XX)L(X)Y is one of the best characterized sphingolipid-binding motifs (Björklöf et al., 2014). Nevertheless, a universal rule for lipid–peptide interaction is still lacking because of insufficient information by far. Our data suggested that, in the context of Clec12a, single amino acid mutation (L→F) in the TMD is sufficient to permit lipid domain formation, which appears to be less stringent than other lipid recognition motifs. One unexpected result is that three out of four Clec family members tested show a similar ability to block MSU-mediated activation, with the only exception of Clec4a with a total of six F in its TMD. Whether type II TMDs with packed transmembrane helix all have similar ability to interfere with lipid raft organization when co-localized is unknown. If so, whether addition of large side chain aa reverses the inhibition is also intriguing. The inhibitory effects of Clec12a exerts by its TMD are conceptually novel. Although how often this mechanism in used in type II transmembrane proteins remains a new territory, our work may help pave the way for developing membrane–lipid-targeted drugs/therapies, such as in the clinical treatment of gout.

There are several limitations of our study. First, it remains elusive for the involvement of membrane lipids in the recognition of Clec12a and MSU crystals. A previous study has shown that MSU specifically binds to Clec12a-Fc fusion protein but not Clec7a-Fc or Clec9a-Fc, indicating the direct interaction between Clec12a and MSU (Neumann et al., 2014). However, it was performed in a cell-free system that the effect of lipid membrane, particularly lipid rafts, in MSU recognition by Clec12a was not addressed. The binding activity of Clec4a with MSU and the role of lipid raft in such a process have not been determined as well. Regarding the imaging analysis, it was reported that the CTB pantemeric structure can bind five molecules of GM1 and induce coalescence of lipid rafts (Day & Kenworthy, 2015). Although we have performed GP analysis to verify the formation of lipid raft under the MSU-contacted region, additional visualizing approaches are still required to further validate the involvement of Clec12a in the dynamics of membrane lipids in the formation of lipid rafts. Another limitation is the lack of strategies for investigating the biological functions and underlying mechanism of lipid rafts in vivo. Unlike proteins that can be precisely manipulated with genetical and chemical interventions, there are very few approaches for lipid raft manipulation. MjCD has been widely used for lipid raft disruption based on its capability of cholesterol extraction, although it could lead to other unexpected consequences. These questions would need to be investigated by follow-up studies in the future.

Materials and Methods

Mice

C57BL/6 mice were obtained from Jackson Laboratories. clec12a<sup>−/−</sup> mice were kindly provided by Dr. Xin Lin’s laboratory from School of Medicine, Tsinghua University. All mice were bred and housed per approved protocols at Tsinghua University Animal Facilities (ICUAC).

Cell culture

Hela cells were kindly provided by Dr. Xiaohong Fang’s laboratory from the Institute of Chemistry, China Academy of Science, and 293FT cells were kindly provided by Dr. Yeguang Chen’s laboratory from the School of Medicine, Tsinghua University, DC2.4 and THP-1 were kindly provided by Dr. Kenneth Rock’s laboratory from the University of Massachusetts Medical School, THP-1 and Raw264.7 cells were obtained from ATCC. Cell culture follows ATCC instructions that all immune cell types like DC2.4, THP-1, and Raw264.7 were
Plasmids

pLVX-IRE-zsGreen plasmid was kindly provided by Dr. Wei Guo’s laboratory from School of Medicine, Tsinghua University. pcAGin and pCAGin-N-EGFP plasmids were customized by our laboratory, lentICRISPR v2 (Plasmid #52961) was obtained from Addgene. All plasmids were verified by sequencing before use.

Reagents

Murine GM-CSF (315-03-1000) and murine IL-4 (214-14-000) were obtained from Peprotech; protein A/G agarose was obtained from Abmart; sodium orthovanada was obtained from Yeason Biotech; phorbol 12-myristate 13-acetate (TPA) mixture solution (S1819) and PMSF were obtained from Beyotime biotech; Lipofectamine 2000 (11668-019) was obtained from Thermo Fisher Scientific; β-mercaptoethanol (07604) and DMSO (V900090) were obtained from Sigma-Aldrich. Biotin Labeling Kit (E-LK-B002) was obtained from Elabscience. Di-4-ANEPPDHQ (D36802) was from Thermo Fisher Scientific. Total cholesterol (TC) content assay kit (BC1985) was obtained from Solarbio. Alexa Flour 647-conjugated Annexin-5/PI kit was purchased from 4A biotech.

Antibodies

Anti-human IL-1β functional grade purified antibody (16-7018-85), anti-IL-1β monoclonal antibody (13-7016-85), anti-mouse IL-1β capture antibody were obtained from ebioscience; anti-Clec12a (C-terminal) (ab193941) was obtained from Abcam; HRP-conjugated anti-mouse IgG (7076), β-actin (13 × 10^5) rabbit mAb (4970) and anti-human Caspase-1 were obtained from CST; anti-mouse Caspase-1 p20 (AG-20B-0042) was obtained from Adipogen; human MCL/ Clec12a antibody (MAB29461-100) and mouse MCL/Clec12a antibody (AF2950) were obtained from R&D systems; PE-conjugated rat anti-mouse CD371 (Clec12a) antibody (562773) was obtained from BD biosciences; PE-conjugated anti-human CD371 (Clec12a) monoclonal antibody (H83) (12-9878-42), allophycocyanin-conjugated p-Syk (Tyrs41) monoclonal antibody (monotc) (17-9014-42), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG F(ab′)2 secondary antibody (31573), and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG F(ab′)2 secondary antibody (31553) were obtained from Invitrogen; HA-tag (26D11) mouse monoclonal antibody (M20003L) was obtained from Abmart; mouse IgG1 isotype control antibody (130-113-762) was obtained from MACS.

MSU crystal preparation

The preparation of crude MSU crystals (20–50 μm long in needle shape) was done following the published report (Luo et al, 2020). In brief, 1.68 g uric acid (Sigma-Aldrich) was dissolved in 400 ml of 25 mM NaOH solution and then left for overnight at RT. Crystals were harvested the next day after decanting off the supernatant. After washing three times with cold sterile PBS and filtered on a filter paper, crystals were left to dry completely at RT in a hood with fan for 3 d. Crude MSU was further placed into a 50 ml tube with appropriate amount of steel beads and homogenized for 6-h to make fine MSU (1–3 μm in diameter).

Molecular cloning

Human clec12a gene was cloned from the cDNA library of THP-1 and mouse clec12a gene was cloned from the cDNA library of bone marrow cells from C57BL/6 mice. The primer sequences are as follows: (5′–3′): murine clec12a forward, ATGCTGAGAAATTGTGTTA; murine clec12a reverse, TCACTCTATCTCCTGAGCCCA; human clec12a forward, ATGCTGATAGATTCTTTAC; human clec12a reverse, TCACTCTATCTCCTTATATG. All mutageneses of WT human or mouse Clec12a are detailed in the text. PCR-amplified fragments were cloned into pCAGIN-N-EGFP or pLVX-zsGreen, respectively, and all constructs were validated by sequencing.

Flow cytometry

For cell surface staining and FACS analysis, cells were stained with the indicated fluorophore-conjugated antibodies for 15 min under dark at RT, and then samples were washed with PBS buffer (with 2 mM EDTA) three times before flow analysis. For p-Syk flow analysis, cells were fixed, permeabilized, and stained with fluorophore-conjugated anti-p-Syk antibody for 1 h under dark at RT. Then, the samples were washed three times before FACS analysis. Fluorescent intensity of 1 × 10^4 was set as the cut-off value for p-Syk phosphorylation as previously described (Ng et al, 2008). For cell death detection, cells were treated with 10 mM MβCD or left untreated for 1 h, then the cells were washed with pre-warmed PBS for three times and incubated for an additional 5 h. The samples were stained with Annexin-5/PI according to manufacturer’s protocol and cell death events were recorded by flow cytometry.

Cell transfection

For transient transfection, cells were cultured in six-well plates until 70% confluent. 4 μg of endotoxin-free plasmids and 10 μl Lipofectamine 2000 were dissolved in 250 μl Opti-MEM for 5 min, respectively, then mixed and stayed for another 20 min before dropping into each well. 6 h later, cell supernatants were discarded and replaced with a fresh medium. 36–48 h later, transfection efficiency was monitored by fluorescent imaging (for vectors with fluorescent tag) or drug screening (for vectors with drug-resistant...
tag). To obtain chromosome-integrated transfectants, cells were treated with 250 ng/ml G418 screening and single clones were picked up by serial dilution. Overexpression of the inserted genes was verified by FACS or Western blot analysis. For lentiviral-mediated transfection, 293FT cells were transfected with lentiviral vectors along with the packaging plasmids PMD2.G and psPAX2 following the weight ratio of 4:1:3. To transfect one well in a six-well plate, the total amount of 9 µg plasmids and 9 µl of Neofect was used for making the mixture and the procedure is similar to transient transfections. 48–72 h later, cell supernatants were collected and filtered through a 0.45-µm syringe filter. For lentiviral infection of THP-1 cells, cells were incubated with a lentiviral supernatant and 8 µg/ml polybrene and centrifuged at 3,000g for 30 min; later, the cell pellets were resuspended in CCM with 1 µg/ml puromycin for selection. Positive clones were picked up, amplified, and verified by Western blot analysis.

Cell assay

For MSU stimulation, cells were treated with 100 µg/ml fine MSU crystals for indicated time or otherwise indicated; for MøjCD treatment, cells were treated with 10 mM MøjCD 1 h before additional stimulations. To detect Syk phosphorylation, 1 mM Na3VO4 was added to the manufacturer’s protocol. Briefly, cells were broken by an ultrasonic wave on ice (power 300 W, ultrasonic 2 s, interval 3 s, total time 3 min), centrifuged at 10,000 × g for 10 min, and then placed for test. For cholesterol quantification of cells collected from air-pouch fluid, cells from each sample were first counted and diluted to equal number (one million per sample) before detection to preclude the influence cell number on final readouts.

Real-time PCR

For real-time PCR analysis, total amount of RNA was extracted by Trizol and retro-transcribed into cDNA. The following primers were used to detect the expression of human or mouse CD80/86: human cd80 Forward: AAACCTGACATCTAGCAAA; human cd80 Reverse: GGTTTTGAATCTCGGGCATA; human CD86 Forward: GGAACGTCGTACAGTTCTTG; mouse cd80 Forward: CTGCAAAGGACTTCAGAAACCT; mouse cd86 Forward: GGTGGCCTTTTTGACACTCTC; mouse cd86 Reverse: TGAGGTAGAGGTTACC; human CD80 Forward: CTGCTCAAGGACTTCAGAAACCT; human CD80 Reverse: GGTTCTTGTACTCGGGCCATA; human CD86 Forward: CTGTTTCATCCTCGGGCATA; human CD86 Reverse: GGTTTTGAATCTCGGGCATA; human CD80 Forward: GGAACGTCGTACAGTTCTTG; mouse cd80 Forward: CTGCAAAGGACTTCAGAAACCT; mouse cd86 Forward: GGTGGCCTTTTTGACACTCTC; mouse cd86 Reverse: TGAGGTAGAGGTTACC; GAPDH Forward: ATCAAGAGGTGGTAAGCA; GAPDH Reverse: AGACAACCTGGTCTCAGTGT.

Co-IP

For SHP-1/2 immunoprecipitation, 4 µg anti-α-HA antibody were incubated with 100 µl protein A/G agarose for 2 h at RT. Cells (3 million/ml) were collected and lysed with immunoprecipitation lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM NaVO3, and protease inhibitor cocktails). The cell lysates were added into antibody–bead mixtures for 3 h at 4°C. Beads were then collected by low-speed centrifugation (1,000g for 5 min) and washed four times with immunoprecipitation lysis buffer (300 mM NaCl added). After the last wash, pellets were suspended with 70 µl 2× SDS loading buffer and boiled for 5 min before Western blot analysis.

Confocal microscopy

For endocytic efficiency detection, latex beads were mixed with 0.1 M borate buffer (pH 8.4), centrifuged at 1,500g for 5 min, and washed with 0.1 M borate buffer for an additional two times. Later, the latex beads were mixed with biotin-BSA at the final concentration of 350 µg/ml and incubated overnight, then washed with 1x PBS two times, and centrifuged at 1,500g for 5 min. The coated beads were stored at 4°C before use.

For lipid raft and Clec12a visualization under MSU stimulation, endogenous Clec12a-deficient Hela cells overexpressing indicated Clec12a-EGFP mutants were stained with CTB-647 at 37°C for 15 min, then monitored under ZEISS LSM 880 confocal laser scanning microscope. Images were captured and processed with Image J. For colocalization analysis between lipid raft and Clec12a, MSU-contacted regions (Regions of Interest) were picked up and the colocalization rate (Pearson’s correlation coefficient) between green signal (Clec12a) and red signal (lipid raft) were calculated basing on the fluorescent intensities via Image J plugin JACoP_jar (2.1.1) (Bolte and Cordelières, 2006). For colocalization analysis between MSU and lipid raft, the number of “CTB positive, MSU contacted” regions on cell membranes of each image was counted and divided by the total MSU-contacted regions in the same image. A total of 15 images of each group were processed for statistical analysis.

For silica stimulation and confocal imaging analysis, anti-Clec12a antibody was conjugated with biotin molecule following manufacturer’s instructions. Later, endogenous Clec12a-deficient Hela cells overexpressing indicated Clec12a-EGFP mutants were incubated with biotin-conjugated anti-Clec12a Ab and streptavidin-conjugated silica crystals for confocal imaging. Image J was used to analysis the rate of colocalization under different conditions as above.

GP imaging

Di-4-ANEPPDHQ is a potentiometric styryl dye that carries a dipole sensitive to lateral lipid packing density. Different densities of lipid domains are associated with “relaxed” or “rigid” states. Di-4-ANEPPDHQ is excited at 488 nm and the peak of its emission wavelength is subjected to the polarizing state of the surrounding membrane. In the Lo phase, the emission wavelength of di-4-ANEPPDHQ peaked at 550 nm, whereas it shifted to around 650 nm in the Ld phase (Jin et al, 2006). The generalized polarization (GP) analysis is a digitizing process to convert the fluorescent signal within a given range to a numeric value between –1 to 1. This derivatization produces a new image indicating intensity change between the original set boundaries. As previously described (Song et al, 2019), cells were stained with 5 µM Di-4-ANEPPDHQ in a serum-free medium for 30 min and washed three times with PBS. Samples were monitored under ZEISS LSM 880 confocal laser scanning microscope excited at 488 nm. Fluorescent signals of channel 1
(Ch1: 550–570 nm) and channel 2 (Ch2: 660–560 nm) were recorded. For generalized polarization (GP) analysis, the GP value is calculated basing on fluorescent intensity from each channel according to the following formula: GP=(Ch1-Ch2)/(Ch1+Ch2). Pseudo-color images were generated via Image J plugin (Calculate_GP_2). All images were processed via Image J.

**Air pouch and MSU injection-induced gout model**

The mouse model of air pouch-induced gout was established as described previously. Briefly, mice were injected twice with 3 ml of sterilized air under the back skin in 3-d interval. Later, 400 µl of MSU crystals (5 mg/ml in PBS) or equal volume of PBS were injected into the air pouch to induce acute inflammation; for certain groups, MβCD (0.35 M, 4 µl/mice) was injected 20 min later. 18 h later, MSU intra-plantar injection induced gout model

**MSU intra-plantar injection induced gout model**

40 µl MSU crystals (50 mg/ml in PBS) or PBS were intra-plantar-injected into mice; for certain groups, MβCD (0.35 M, 4 µl/mice) was injected 20 min later. 18 h later, mice were euthanized and foot swelling was compared. The inflammation of mice feet under different treatments was analyzed by H&E staining and histopathological analysis.

**Hematoxylin and eosin staining**

Footpad samples obtained 18 h after MSU crystal injection were immediately fixed in 4% buffered formalin for 48 h at RT. Formalin-fixed paraffin sections (4-µm thickness) were stained using hematoxylin and eosin. The tissue sections were deparaffinized and treated using 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity before H&E staining. The resulting images were examined using light microscopy (Olympus).

**MD simulation**

To extend time and length scales to be appropriate for simulating membrane phase separation and Clec12a TMD portioning, the widely used Martini CG force field was adopted (Monticelli et al, 2008). The multi-component membrane of 37 × 37 nm in lateral size was prepared using the CHARMM-GUI tool (Wu et al, 2014). Based on the ternary model consisting of saturated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), unsaturated 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPE), and cholesterol (CHOL), asymmetric lipid distributions across two leaflets were considered by introducing sphingomyelin (DPSM) to the outer leaflet. The lipid composition ratio in the outer leaflet was DPPC:CHOL:DOPE = 2:2:3:3, and the ratio in the inner leaflet was DPPC:CHOL:DOPE = 3:3:4. AA models of the WT Clec12a were derived from the AlphaFold Data Bank, with the TMD truncated for use in simulations. The AA protein model was transferred to the corresponding CG models using the martiniize.py script provided by the Martini force field. CG MD simulations were performed with the NPT ensemble using the semi-isotropic Berendsen barostat (P = 1 bar) with a coupling constant of 4 ps and compressibility of 5 × 10⁻⁵ bar⁻¹ in both lateral and membrane normal directions. The temperature was kept constant at T = 295 K using the V-rescale thermostat with a coupling constant of 4 ps. A cutoff of 1.2 nm was used for van der Waals interactions, and the Lennard–Jones potential was smoothly shifted to zero between 0.9 and 1.2 nm to reduce the cutoff noise. The coulombic potential, with a cutoff of 1.2 nm, was smoothly shifted to zero from 0 to 1.2 nm. The particle-mesh Ewald summation method was used to treat the long-range electrostatic interactions. The time step of simulations was 10 fs, with the neighbor list updated every 10 steps.

To acquire atomic-level information about how specific mutations influence Clec12a TMD interactions with lipid raft, a phase-separated membrane of 115 × 11.5 nm of the same composition ratio was constructed. Besides, a pure liquid-ordered (Lo) membrane consisting of DPPC, DPSM, and CHOL was also constructed to investigate interactions of Clec12a mutants with lipid raft. Periodic boundary conditions were considered in all three directions. Both CG and AA MD simulations were performed using the GROMACS 2019 software package (Rakhshani et al, 2019). Simulated snapshots were rendered by VMD (Humphrey et al, 1996).

**Statistics**

All experiments were repeated independently for at least three times. For mouse experiments, five mice per group were included. All plot graphs show the mean ± SEM. Statistical analysis for each independent experiment was performed with an unpaired t test. P-value of <0.05 was considered significant: *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

**Data Availability**

The data that support the findings of this study are available from the corresponding author upon request.

**Supplementary Information**

Supplementary information is available at https://doi.org/10.26508/lsa.202301938.

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Author Contributions

Y Xu: conceptualization, software, investigation, methodology, and writing—original draft and project administration.
D Song: supervision, validation, investigation, methodology, and writing—original draft and project administration.
W Wang: software, investigation, methodology, and writing—original draft.
S Li: supervision, investigation, and methodology.
T Yue: conceptualization, data curation, supervision, validation, and methodology.
Y Xu: conceptualization, software, investigation, validation, and writing—original draft.
Y Shi: conceptualization, supervision, funding acquisition, validation, and writing—review and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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