

Endosomal TLR3 co-receptor CLEC18A enhances host immune response to viral infection

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Human C-type lectin member 18A (CLEC18A) is ubiquitously expressed in human, and highest expression levels are found in human myeloid cells and liver. In contrast, mouse CLEC18A (mCLEC18A) is only expressed in brain, kidney and heart. However, the biological functions of CLEC18A are still unclear. We have shown that a single amino acid change (S₃₃₉ → R₃₃₉) in CTLD domain has profound effect in their binding to polysaccharides and house dust mite allergens. In this study, we further demonstrate that CLEC18A and its mutant CLEC18A(S339R) associate with TLR3 in endosome and bind poly (I:C) specifically. Compared to TLR3 alone, binding affinity to poly (I:C) is further increased in TLR3-CLEC18A and TLR3-CLEC18A(S339R) complexes. Moreover, CLEC18A and CLEC18A(S339R) enhance the production of type I and type III interferons (IFNs), but not proinflammatory cytokines, in response to poly (I:C) or H5N1 influenza A virus (IAV) infection. Compared to wild type (WT) mice, ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice generate higher amounts of interferons and are more resistant to H5N1 IAV infection. Thus, CLEC18A is a TLR3 co-receptor, and may contribute to the differential immune responses to poly (I:C) and IAV infection between human and mouse.

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Human C-type lectin 18 (*clec18*) gene cluster contains three loci *clec18a*, *clec18b*, and *clec18c*, and each *clec18* family member encodes a polypeptide of 446 amino acids comprising a C-type lectin-like domain (CTLD) in the C terminus and a sperm-coating protein (SCP) domain (also known as CAP or TAPS domain) in the N terminus¹. The three human C-type lectin member 18A/B/C (CLEC18A/B/C) polypeptides are almost identical except polymorphic amino acid residues located in CTLD and SCP/CAPT/APS domains¹. Human CLEC18A is ubiquitously expressed in human tissues with higher expression levels in myeloid cells and liver. However, the biological function of CLEC18A is still unknown, nor do we understand the impact of polymorphic amino acid residues in CTLD.

It has been shown that endosomal Toll-like receptors (TLRs) recognize diverse danger signals and nucleic acid components released from uncoated virions, and activation of endosomal TLRs induces the production interferons (IFNs) and proinflammatory cytokines to initiate inflammatory responses². Endosomal TLR3 is regarded as a sentinel to RNA viruses, and activation of TLR3 leads to TRIF (TIR domain-containing adaptor-inducing IFN- β)-dependent IFN production and nuclear factor- κ B (NF- κ B)-dependent proinflammatory cytokine production³. Interestingly, the differential outcome of TLR3-mediated signaling between human and mouse have been noted^{4,5}. Compared to wild-type (WT) mice, TLR3^{-/-} mice produced lower levels of inflammatory mediators and displayed less cell infiltration after H3N2 influenza A virus (IAV) infection, despite higher virus titer being noted in the lung⁶. This observation suggests that TLR3-mediated signaling prefers to induce proinflammatory cytokine release and has a detrimental effect in IAV infection in mice. In contrast, human TLR3 is as important as RIG-I in the production of type I and type III IFNs after IAV infection⁷, and intranasal administration of TLR3 agonist reduced lung viral titers and mortality in mice⁸. These observations suggest that TLR3 activation is able to produce IFNs to protect the host from IAV infection. However, the molecular mechanism responsible for the differential TLR3-mediated signaling between human and mouse is still unknown.

Since polymorphic amino acid residues were found in the CTLD domain and SCP domain among members of CLEC18 family, and distinct glycan-binding affinity in vitro was found between the CTLD domain of CLEC18A(S339) and CLEC18A-1(R339)¹, we are interested to understand the differential functions of S339 versus R339 in host immunity in vivo. To address this question, we generate CLEC18A(S339R) mutant, which is identical to CLEC18A except amino acid 339 (S $\rightarrow$ R). Here we reported that CLEC18A and CLEC18A(S339R) associate with TLR3 and bind poly (I:C) directly. To understand their roles in TLR3-mediated signaling, we generated ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice to investigate their functions in poly (I:C) stimulation and IAV infection. We found that macrophages and alveolar epithelial cells (AECs) from ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice produce higher amounts of IFNs (type I and type III) and IFN-stimulated genes (ISGs) after incubation with poly (I:C) or H5N1 IAV. Furthermore, ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice are more resistant to H5N1 infection with higher survival rates than WT littermates (CLEC18A(S339R) > CLEC18A > WT). These observations suggest that CLEC18A and CLEC18A(S339R) act as TLR3 co-receptors in the endosome, and may contribute to the differential immune responses to TLR3-mediated signaling between human and mouse.

Results

Expression of mouse endogenous CLEC18A and generation of ROSA-CLEC18A/ CLEC18A(S339R) knock-in mice. While

human CLEC18A is ubiquitously expressed and highest expression levels were noted in immune cells and liver¹, mouse CLEC18A (mCLEC18A) messenger RNA (mRNA) is only expressed in brain, kidney, and heart (Fig. 1a). To confirm the expression of mCLEC18A, we generated human anti-mCLEC18A monoclonal antibody (mAb) to detect mCLEC18A in mouse tissues (lanes 1–8, Fig. 1b). We harvested cell lysates from 293T cells transfected with vector alone (lane 9, Fig. 1b) or Flag-tagged full-length mCLEC18A (lane 10, Fig. 1b), followed by fractionation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before blotting to polyvinylidene difluoride (PVDF) paper. The blots were incubated with anti-mCLEC18A mAb (lanes 9 and 10, Fig. 1b) and anti-Flag mAb (lanes 9 and 10, Fig. 1c), respectively, to determine the expression of mCLEC18A. We found that anti-mCLEC18A mAb is able to detect endogenous mCLEC18 in the brain (lane 1, Fig. 1b) and 293T cells transfected with full-length mCLEC18A (lane 10, Fig. 1b), while the expression levels of mCLEC18A in heart and kidney (lanes 3 and 7, Fig. 1b) were too low to be detected under the same condition. We further examined the expression of endogenous mCLEC18A by flow cytometry (Supplementary Fig. 1). Even we can detect 293T cells transfected with full-length mCLEC18A complementary DNA (cDNA) (Supplementary Fig. 1d), we cannot detect mCLEC18A in mouse peripheral blood, bone marrow, and splenocytes. These observations further confirmed that the tissue distribution of mCLEC18A is distinct from the human counterpart.

To understand the functions of CLEC18A-mediated immune responses in vivo, we generated ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice to express human CLEC18A and CLEC18A(S339R), respectively, to address this question. We found that CLEC18A and CLEC18A(S339R) were expressed ubiquitously, and the highest expression level was found in the brain, bone marrow, and splenocytes in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice (Fig. 1d). The expression of human CLEC18A and CLEC18A(S339R) in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice tissues is similar to what we observed in human tissues¹, although the expression levels are lower than that in human liver and kidney. Western blot analysis further confirmed the expression of CLEC18A and CLEC18A(S339R) in mouse bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) (Fig. 1e).

CLEC18A enhances IFN production and ISG expression after influenza virus infection. As mouse BMDMs do not express endogenous CLEC18A, we compared the responses of BMDMs isolated from WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice to various TLR ligands, respectively, followed by examining the expression of type I IFNs (IFN- α and IFN- β) and proinflammatory cytokines. Among the TLR ligands tested, high molecular weight (HMW) poly (I:C) enhanced the expression of IFN- α , IFN- β , IFN- γ -inducible protein 10 (IP-10), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α). Interestingly, the expression levels of IFN- α , IFN- β , and IP-10 were further upregulated in ROSA-CLEC18A and ROSA-CLEC18A(S339R) BMDMs (Fig. 2a). Similar to poly (I:C) stimulation, higher expression levels of IFN- α and IFN- β were found in ROSA-CLEC18A and ROSA-CLEC18A(S339R) BMDMs stimulated with RNA viruses, including H5N1 IAV and dengue virus (DV) (Fig. 2b). In contrast, other TLR ligand has no obvious effect to upregulate cytokine production (Supplementary Fig. 2). We further knocked down the endogenous CLEC18 in human macrophage to confirm its role in TLR3-mediated signaling. We found that knockdown of CLEC18 significantly reduced poly (I:C) and virus-, but not LPS-, induced IFN production (Supplementary

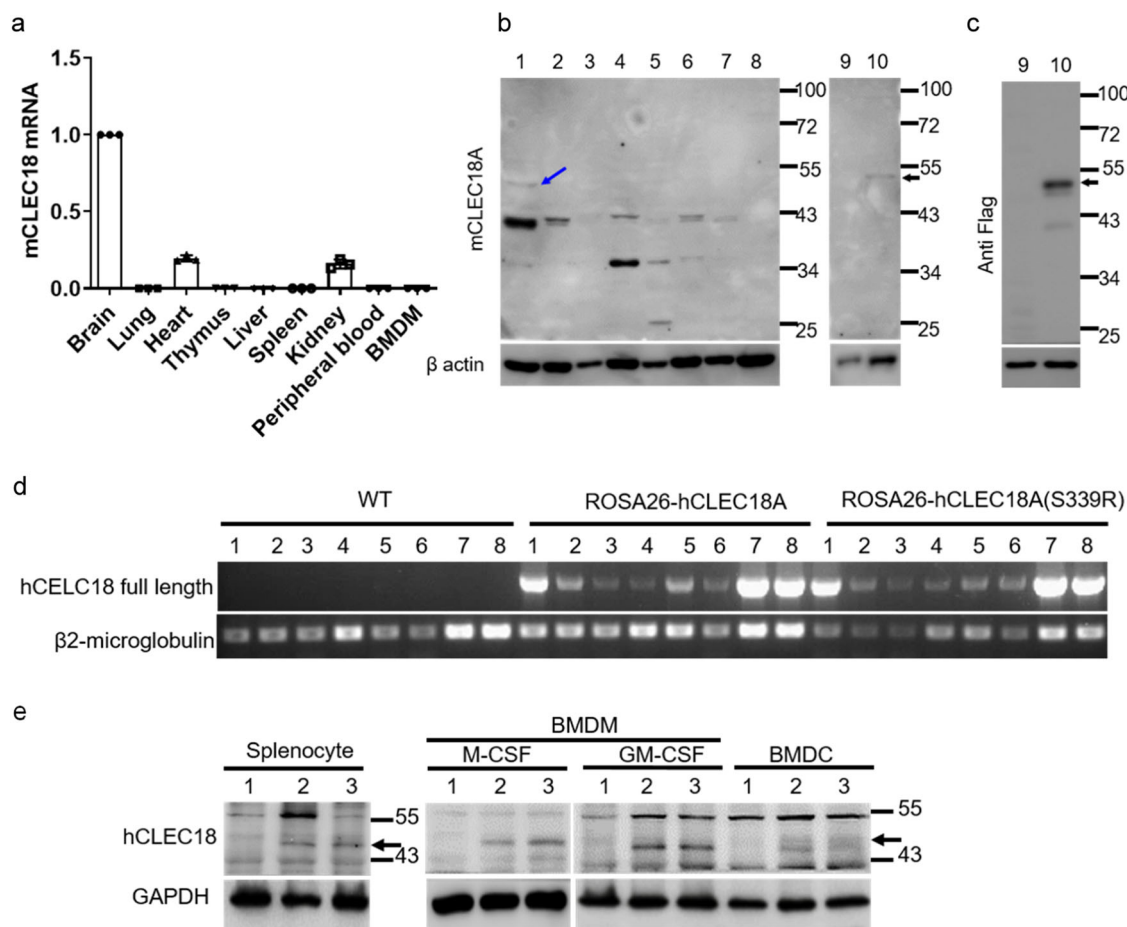


Fig. 1 Expression mouse endogenous CLEC18A and generation of ROSA- CLEC18A/CLEC18A(S339R) knock-in mice. a–c Expression of mouse endogenous CLEC18A (mCLEC18) mRNA and protein in B6 mice was determined by RT-PCR (**a**) and WB (**b**, **c**). 1: Brain, 2: Lung, 3: Heart, 4: Thymus; 5: Liver, 6: Spleen, 7: Kidney, 8: Bone marrow; 9: 293T cells transfected with pCMV-Tag4A (vector control); 10: 293T cells transfected with pCMV-Tag4A-mCLEC18 (50 kDa). Arrow indicates the position of mouse CLEC18 ($n = 3$). **d** Expression of human CLEC18A and CLEC18A (S339R) in ROSA-CLEC18A and CLEC18A(S339R) mice was determined by PCR. 1: Brain; 2: Lung; 3: Heart; 4: Liver; 5: Spleen; 6: Kidney; 7: Bone marrow; 8: Splenocyte ($n = 3$). **e** Detection of human CLEC18 protein (50 kDa) in mouse bone marrow-derived dendritic cells (BMDCs), as well as macrophages (BMDMs) cultured under M-CSF and GM-CSF, respectively. 1: Wild type mice (WT); 2: ROSA26-CLEC18A mice; 3: ROSA-CLEC18A(S339R) mice. Arrow indicates the molecular weight (50 kDa) of human CLEC18A/CLEC18A(S339R) (hCLEC18) ($n = 3$).

Fig. 3). To further examine the role of CLEC18A and CLEC18A (S339R) in TLR3-mediated signaling, human HT1080 cells were transfected by small interfering RNAs (siRNAs) to knock down endogenous CLEC18A. After recovery overnight, the transfected cells were transfected with pMACS.Kk-HA(C)-hCLEC18A and pMACS.Kk-HA(C)-hCLEC18A(S339R) to express CLEC18A and CLEC18A(S339R), respectively. We found that overexpression of CLEC18A and CLEC18A(S339R) enhanced the expression of IFN- α and IP-10 after poly (I:C) stimulation (Supplementary Fig. 4). These observations further confirm that CLEC18A is able to enhance TLR3-mediated signaling. Moreover, we examined the expression of ISGs critical in viral infection^{9,10}. While Ch25h and Mx1 were upregulated, the expression of ISG15 was similar in both ROSA-CLEC18A and ROSA-CLEC18A(S339R) BMDMs post H5N1 IAV and DV infections (Fig. 2c). In addition, virus titer and the expression of matrix protein (M) and nucleoprotein (NP) were downregulated in ROSA-CLEC18A and CLEC18A (S339R) BMDMs (Fig. 2d). It is interesting to note that CLEC18A (S339R) is more potent than CLEC18A to enhance IFN production and suppress virus replication in all the assays.

In addition to type I IFNs, we further investigated the expression of type III IFN, which plays a critical role in anti-influenza virus infection¹¹, in AECs after H5N1 IAV infection.

Compared to WT mice, both type I IFNs (IFN α 4 and IFN β) and type III IFN (IFN λ 2/3) were upregulated, while the expression of TNF- α and IL-6 was similar in AECs isolated from ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice (Fig. 3a). Moreover, the expression of M and NP genes was suppressed in AECs expressing human CLEC18A and CLEC18A(S339R) (Fig. 3b). Similar to what we observed in macrophages, CLEC18A(S339R) is more potent than CLEC18A to induce IFN expression and suppress virus replication in virus-infected AECs, suggesting that the polymorphic amino acid residue [S₃₃₉→R₃₃₉] in CTLD domain has a profound effect to upregulate IFN production and suppress H5N1 IAV replication.

CLEC18A and CLEC18A(S339R) associate with TLR3 and bind poly (I:C) directly. Because CLEC18A is located in the endosome, we asked whether CLEC18A associated with TLR3 in the presence of poly (I:C) or H5N1 IAV virus, respectively. We found that HMW poly (I:C) and H5N1 IAV increased the efficiency of fluorescence resonance energy transfer (FRET) between TLR3-CLEC18A, respectively (Fig. 4a, b). Because TLR3 is expressed in early endosome (pH 6.3)^{12,13}, we further asked whether CLEC18A.Fc and CLEC18A(S339R).Fc bind poly (I:C) directly by

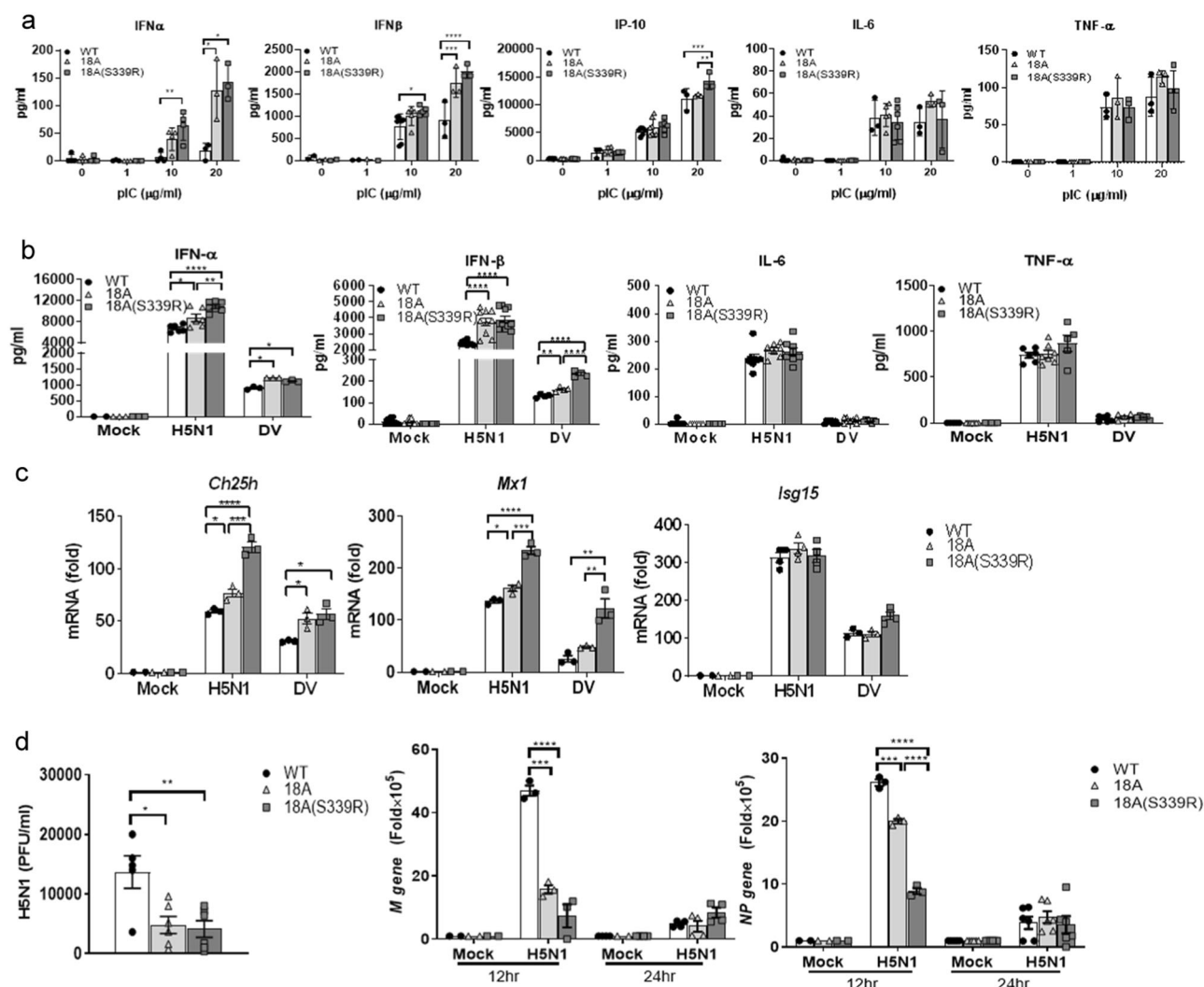


Fig. 2 CLEC18 enhances IFN production and ISG expression to suppress virus replication. **a** BMDMs (1×10^6 /well) from WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice were incubated with HMW poly(I:C) for 24 h, followed by harvesting cells to determine the expression of cytokines by ELISA ($n = 3-6$). **b, c** BMDMs (1×10^6 /well) from WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice were incubated with RNA viruses (H5N1 MOI = 2; DV MOI = 30) for 24 h, followed by harvesting cells to determine cytokines expression by ELISA ($n = 3-10$) (**b**) and IFN-stimulating genes (ISG) expression by quantitative RT-PCR ($n = 3$) (**c**). **d** Virus replication was determined by plaque assay (left), and quantitative RT-PCR to determine the expression of virus structure genes (M) and nucleoprotein (NP) (right) ($n = 3-10$). One-way ANOVA was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$, for WT group versus group of CLEC18A or CLEC18A(S339R). H5N1 type A influenza virus, DV dengue virus.

bio-layer interferometry at pH 6.3. We found that CLEC18A(S339R).Fc bound HMW poly(I:C) ($K_D = 8.17E-09$) with similar affinity as TLR3.Fc ($K_D = 5.59E-09$), whereas the binding affinity of CLEC18A.Fc to poly(I:C) ($K_D = 2.10E-08$) is slightly lower than CLEC18A(S339R).Fc under the same condition (Supplementary Table II). Interestingly, co-immobilization of TLR3.Fc with CLEC18A.Fc or CLEC18A(S339R).Fc, respectively, further increases binding affinity to HMW poly(I:C). The affinity between poly(I:C) and TLR3.Fc/ CLEC18A(S339R) ($K_D \leq 1.0E-12$) is much higher than TLR3.Fc alone ($K_D = 5.59E-09$), while TLR3.Fc/CLEC18A ($K_D = 4.46E-10$) is slightly higher than TLR3.Fc alone. Co-localization of human CLEC18A with human and mouse TLR3 in early endosome was also observed in human macrophage (Fig. 4c) and mouse BMDMs from ROSA-CLEC18A/ CLEC18A(S339R) mice (Fig. 4d). To further confirm the association between CLEC18 and TLR3 contributes to enhanced IFN production, we added the TLR3/dsRNA (double-stranded RNA) complex inhibitor, which inhibited IFN production via competing HMW poly(I:C) binding

to TLR3, to disrupt the TLR3/CLEC18/dsRNA complex. We found that IFN production was reduced by the TLR3/dsRNA complex inhibitor in a dose-dependent manner (from 30 to 100 μ M) in WT BMDM. In contrast, IFN production and the efficiency of FRET between TLR3-CLEC18A/ CLEC18A(S339R) was not reduced in lower doses (10 and 30 μ M) of TLR3/dsRNA complex inhibitor until it reaches 100 μ M in BMDMs isolated from ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice (Fig. 5a, b). These observations suggest that the binding affinity between HMW poly(I:C) and TLR3-CLEC18A/CLEC18A(S339R) complexes were increased, thereby requiring a higher concentration of inhibitor to suppress HMW poly(I:C)-induced cytokine production. Furthermore, enhanced phosphorylation of TBK1 and IRF3 was observed in BMDMs isolated from CLEC18A and CLEC18A(S339R) mice (Fig. 5c). Thus, we conclude that CLEC18A and CLEC18A(S339R) act as TLR3 co-receptor in the endosome, thereby enhancing TLR3-mediated signaling and IFN production after incubation with HMW poly(I:C).

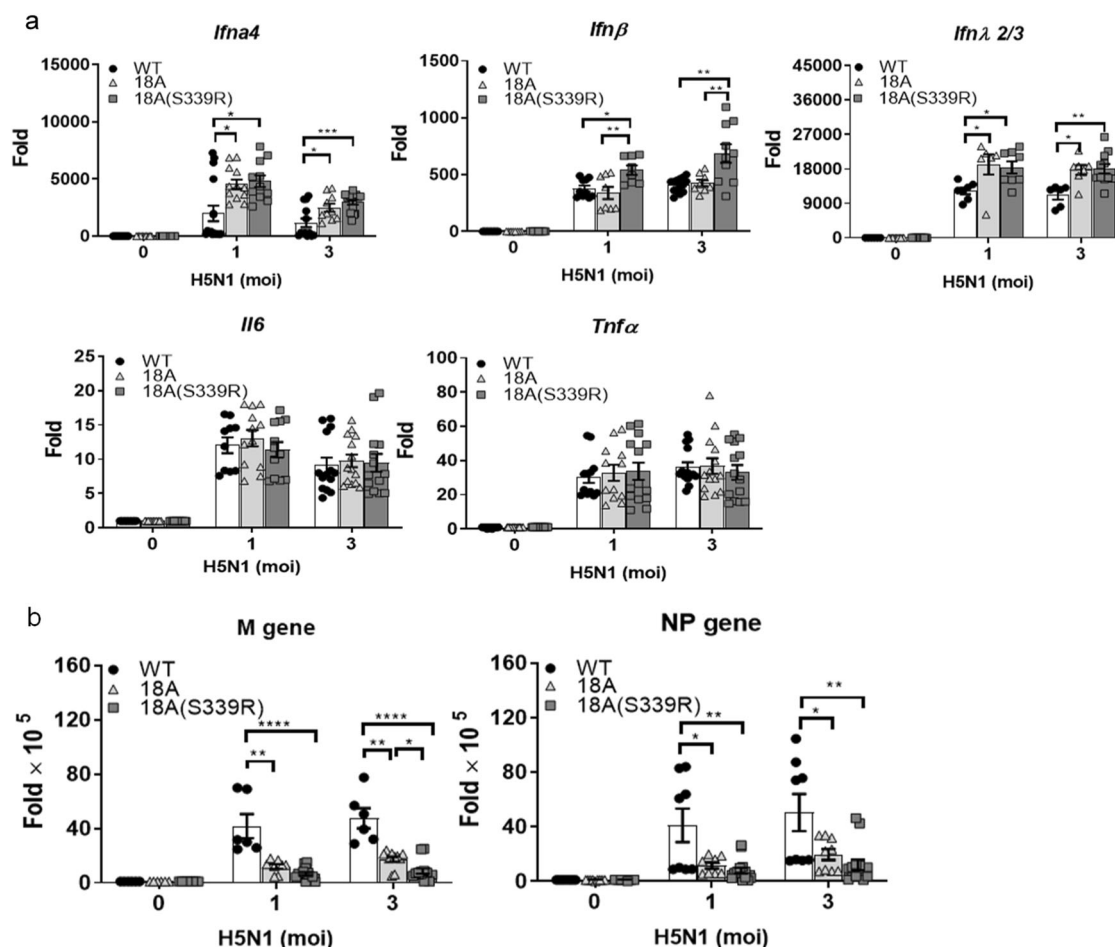


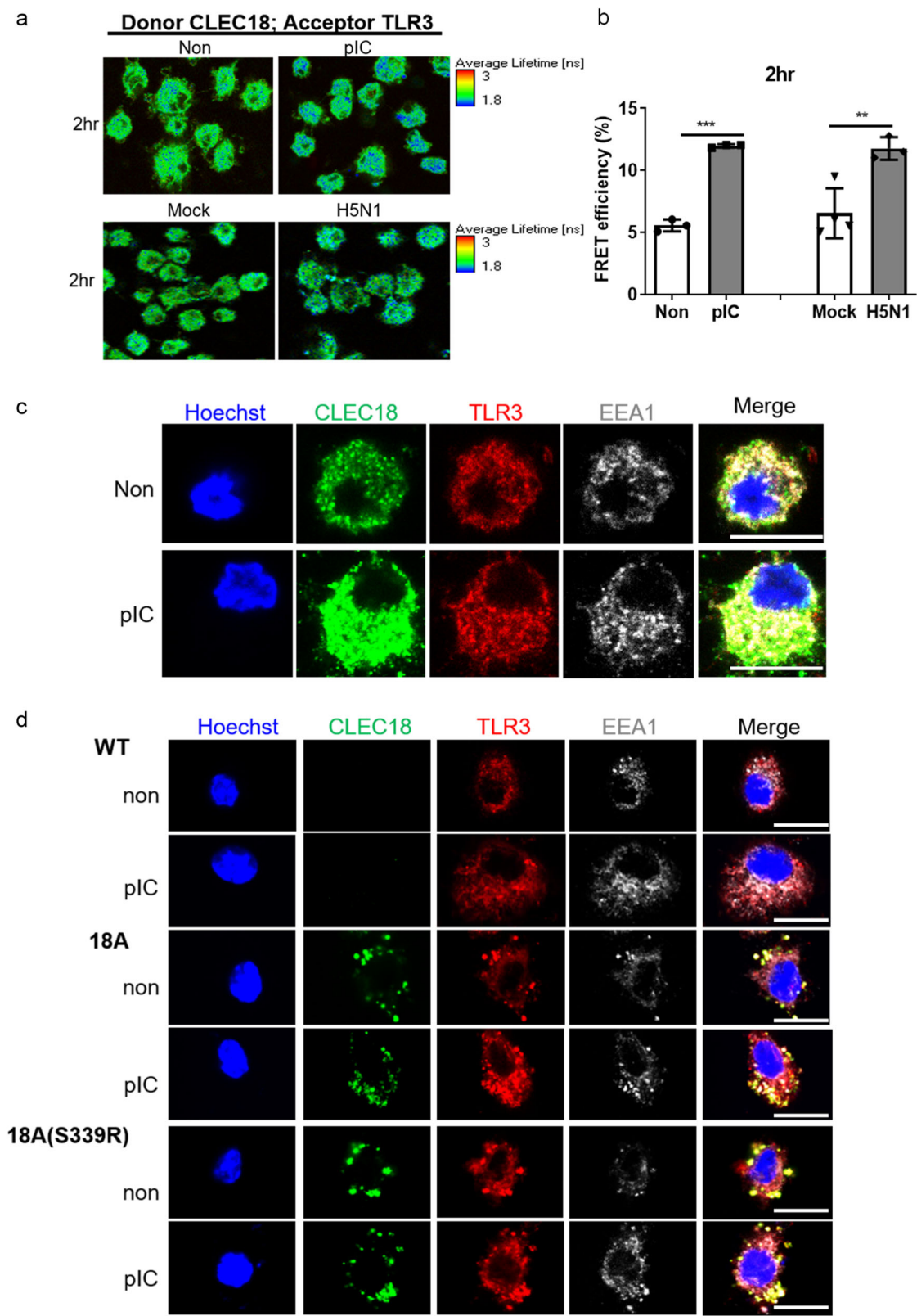
Fig. 3 CLEC18A and CLEC18A(S339R) modulate type I/III IFN expression and virus replication in lung alveolar epithelial cells. Pulmonary alveolar epithelial cells (2×10^5 /well) from WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice were incubated with H5N1 virus (MOI = 2) for 12 h, followed by harvesting cells to determine cytokine expression (a) and virus replication (b) by quantitative RT-PCR ($n = 7-12$). One-way ANOVA was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$, for WT group versus group of CLEC18A or CLEC18A(S339R).

Mapping of TLR3 and CLEC18A/ CLEC18A(S339R) interaction domain. We further identified the interaction domains between TLR3 and CLEC18A/CLEC18A(S339R) by immunoprecipitation using TLR3 deletion mutants (Fig. 6a). We found that CLEC18A and CLEC18A(S339R) associated with TLR3 deletion mutant without Toll/IL-1 receptor homology domain (TLR3 Δ TIR), but they were unable to associate with TLR3 deletion mutant without leucine-rich repeat (LRR) domain (TLR3 Δ LRR) (Fig. 6a–c). It has been reported that poly (I:C) interacts with TLR3 via C-terminal LRR20 and N-terminal-binding site LRR1^{14,15}. Therefore, we generated TLR3 Δ LRR₁₋₃ (TLR3 without 1–3 LRR repeats) and TLR3 Δ LRR₁₉₋₂₂ (TLR3 without 19–22 LRR repeats) to map the minimal region of TLR3 responsible for interacting with CLEC18A and CLEC18A(S339R). As shown in Fig. 6d–g, TLR3 mutants (TLR3 Δ LRR₁₋₃ and TLR3 Δ LRR₁₉₋₂₂) still interacted with CLEC18A and CLEC18A(S339R), respectively. This observation suggests that TLR3 interacted with CLEC18A and CLEC18A(S339R) via LRR₄₋₁₈, which is not overlapping with poly (I:C) interacting LRR1 and LRR20 of TLR3.

We further generated CLEC18A deletion mutants to identify minimal domain responsible for interaction with TLR3. We found that all the CLEC18 deletion mutants interact TLR3 efficiently except CLEC18 Δ 28–292 a.a. (Fig. 6h–k). Therefore, we conclude that CTLD domain is dispensable for CLEC18A–TLR3 interaction, and TLR3 interacts with all the rest parts of CLEC18A, including CAP-, EGF- and EGK-like domains.

CLEC18 enhances poly (I:C)-induced inflammatory reaction in vivo. We further asked whether CLEC18A and CLEC18A (S339R) enhanced HMW poly (I:C)-induced inflammatory reaction in vivo. To address this question, mice were inoculated with HMW poly (I:C) via intranasal route, and then were sacrificed at days 1 and 4 post inoculation to examine lung inflammation by histochemical staining. Compared to WT littermates, severe cell infiltration was noted in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice (CLEC18A(S339R) > CLEC18A > WT) at days 1 and 4 post inoculation (Fig. 7a). We found that production of type I IFNs (IFN- α , IFN- β), type III IFN (IFN λ 2/3), and IP-10 was upregulated in ROSA-CLEC18A(S339R) and ROSA-CLEC18A mice (Fig. 7b). This observation further supports the notion that CLEC18A(S339R) is more potent than CLEC18A to enhance poly (I:C)-induced inflammation.

CLEC18A and CLEC18A(S339R) protect host from H5N1 IAV-induced inflammation and lethality. We further examined the effect of CLEC18A and CLEC18A(S339R) in host defense against H5N1 IAV infection. To address this question, mice were inoculated with H5N1 IAV (1500 PFU/mouse) via intranasal route to observe body weight change and survival rate for 21 days. We found that CLEC18A(S339R) is more potent than CLEC18A to protect mice from H5N1 IAV-induced body weight loss (Fig. 8a) and lethality (Fig. 8b). Compared to WT mice, less cell infiltration and pulmonary inflammation were noted in ROSA-



CLEC18A and ROSA- CLEC18A(S339R) mice after H5N1 IAV infection (Fig. 8c). Moreover, virus titer was suppressed dramatically in CLEC18A(S339R) mice (Fig. 8d), and the expression of viral M and NP mRNAs is downregulated in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice at day 7 post infection (Fig. 8e). We further investigate the cytokine amount in bronchoalveolar lavage fluid (BALF) by enzyme-linked

immunosorbent assay (ELISA) (Fig. 8f). At days 4 and 7 post infection, higher levels of IFN- α , IFN- β , and IP-10 were noted in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice. Moreover, a higher level of IFN λ 2/3 was observed on day 4, but not on day 7 post infection, while the expression of proinflammatory cytokines (TNF- α , CCL2, and CCL5) was similar in WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice (Fig. 8f). These observations

Fig. 4 CLEC18A and CLEC18A(S339R) associate with TLR3 and bind poly (I:C) directly. **a, b** Human macrophages (2×10^5) were incubated with HMW poly (I:C) or H5N1 virus (MOI = 2) for 2 h, followed by incubation with mouse anti-human CLEC18 mAb and rabbit anti-TLR3 mAb. After washing, samples were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG mAb (donor) and Alexa Fluor 546-conjugated goat anti-rabbit IgG mAb (acceptor). Cells were examined under a confocal microscope (TCS-SP5-MP-SMD, Leica), and images were analyzed by the “SymPhoTime” software. The image of the fluorescence lifetime is shown by false colors. Green color represents a long lifetime of CLEC18A, whereas blue color represents the reduced lifetime attributed to fluorescence resonance emission time (FRET) between Alexa Fluor 488 and Alexa Fluor 546 ($n = 3-4$). **c, d** Human macrophages (6×10^4 /well) (**c**) and BMDMs (6×10^4 /well) from WT and CLEC18 KI mice (**d**) were incubated with HMW poly (I:C) for 2 h, followed by immunostaining using mAb against CLEC18A/CLEC18A(S339R), TLR3, and EEA1, respectively. Cells were examined by a confocal microscope (TCS-SP5-MP-SMD, Leica). Green color: human CLEC18A and CLEC18A(S339R); red color: TLR3; white color: EEA1. pIC poly (I:C). Scale bar: 10 μ m ($n = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

indicated that CLEC18A and CLEC18A(S339R) are able to upregulate the expression of type I and type III IFNs to suppress H5N1 IAV replication in vivo.

Discussion

It has been demonstrated that human macrophages and DCs only produce type I IFNs, but not TNF and IL-6, after TLR3 ligand stimulation. In contrast, mouse macrophages produce both type I IFNs and proinflammatory cytokine after poly (I:C) stimulation⁴. However, the underlying mechanism of the differential responses between human and mouse cell after poly (I:C) stimulation is still unknown. In this work, we demonstrated that human CLEC18A and CLEC18A(S339R) associate with TLR3, and act as co-receptor to bind poly (I:C). Interestingly, enhanced expression of type I and type III IFNs was noted in macrophages and epithelial cells isolated from ROSA-CLEC18A and CLEC18A(S339R) mice, respectively.

It is surprising to find that poly (I:C) binds to CLEC18A ($K_D = 2.10E - 08$) and CLEC18A(S339R) ($K_D = 5.59E - 09$) with high affinity. Both CLEC18A and CLEC18A(S339R) are typical C-type lectins with “QPD,” “WND,” and “WIGL” motifs. The presence of QPD motif is in accord with its preferential binding to galactan¹, while the “WND” motif is shown to promote binding to galactose and *N*-acetylgalactosamine¹⁶. Furthermore, the characteristic “WIGL” motif is highly conserved in canonical CTLD sequences¹⁶. Thus, CLEC18A is the first canonical C-type lectin that can bind poly (I:C) and acts as TLR3 co-receptor to enhance anti-viral immunity.

It is interesting to note that a single amino acid change [$S_{339} \rightarrow R_{339}$] in CTLD domain has a potent effect to enhance type I and type III IFN productions and anti-viral immunity. Compared to BMDM-CLEC18A, BMDM-CLEC18A(S339R) expressed higher levels of IFNs (Fig. 2b) and ISGs (Fig. 2c) with lower level of viral NP gene (Fig. 2d) expression after H5N1 IAV infection. Moreover, higher expression of IFN- β with a lower level of viral M gene was found in lung epithelial cells isolated from ROSA-CLEC18A(S339R) mice (Fig. 3b). This observation is in accord with a stronger induction of p-TBK1 and p-IRF3 in CLEC18A(S339R) (Fig. 5c). Furthermore, higher levels of type III IFN (Fig. 8f) with lower levels of virus titer and less lung inflammation were found in ROSA-CLEC18A(S339R) mice (Fig. 8c, d). The differential responses between CLEC18A and CLEC18A(S339R) alleles are in accord with a higher survival rate of ROSA-CLEC18A(S339R) mice after H5N1 IAV infection (Fig. 8b). Thus, the polymorphic amino acid residue S339R in the CTLD of CLEC18A seems contributing significantly in host immunity to H5N1 infection. Further study is needed to confirm the role of CLEC18A(S339R) allele in host defense against various microbial infections in the future.

In this work, we demonstrated that both CLEC18A and CLEC18A(S339R) bind to poly (I:C) and enhances poly (I:C)-induced IFNs production via forming a heterocomplex with TLR3. The higher affinity of CLEC18A(S339R) than CLEC18A may be attributed to the positive charge of R_{339} located in the

CLEC18A(S339R) CTLD, thereby increasing its binding to negatively charged poly (I:C). Moreover, ROSA-CLEC18A (S339R) mice produce higher amount of type I IFNs than ROSA-CLEC18A mice, and are more resistant to H5N1 IAV infection. These observations suggest that ROSA-CLEC18A(S339R) allele may be beneficial to host upon RNA virus infection. This speculation is supported by our observation that DV induced higher amount of IFNs in ROSA-CLEC18A(S339R) than ROSA-CLEC18A and WT mice. Among the species we examined¹, *Takifugu rubripes* CLEC18A contains N corresponding to S/R_{339} in CTLD, while mice also contain “S” in CTLD domain. In addition to amino acid residue 339, another polymorphic amino acid residue was found at 421 in CTLD domain [$(D_{421} \text{ (CLEC18A)} \rightarrow N_{421} \text{ (CLEC18C)})$]. The $D_{421} \rightarrow N_{421}$ mutation converts the “WND” motif into WNN, and thus may attenuate their binding to galactose and *N*-acetylgalactosamine¹⁶, and even other glycan and non-glycan ligands. In addition to CTLD domain, polymorphic amino acid residues located in N-terminal SCP/CAP domain was also noted¹. Thus, CLEC18 is a highly polymorphic C-type lectin, and large-scale human population is necessary to understand whether another polymorphic amino acid residue exists in positions 339 and 421, and investigate the association of CLEC18A polymorphic amino acid residues in host defense against virus, bacteria, and other microbes in the future.

In previous studies, we found that human CLEC18 is detectable in culture supernatant¹ and human serum^{17,18}. However, we cannot detect human CLEC18A/CLEC18A(S339R) in the ROSA-CLEC18 knock-in mouse serum. It may be attributed to lower expression of human CLEC18A/CLEC18A(S339R) in the knock-in mice, because the expression levels of CLEC18 in human immune cells and liver¹ are much higher than what we observe in ROSA-CLEC18A/CLEC18A(S339R) mice (Fig. 1a–c). Even though the functions of soluble CLEC18 are still unknown, CLEC18 serum level correlates with the stage of hepatitis B virus (HBV) infection and is a potential biomarker to predict HBeAg loss and seroconversion in CHB patients under nucleos(t)ide analog therapy¹⁷. Moreover, higher CLEC18 serum level positively correlated with viral loads¹⁸ in HCV patients. These observations suggest that CLEC18 may also participate in the pathogenesis of liver inflammation caused by hepatitis B virus and HCV. Our observation that CLEC18A acts as a TLR3 co-receptor is the first step to explore its potential role in innate immunity against viral infection, and more studies are necessary to understand how CLEC18A contributes to host innate immunity against viral infections in the future.

Methods

Reagents. Culture media and supplements were purchased from Gibco. Chemical reagents and chemical inhibitors were purchased from Sigma and Calbiochem, respectively. TLR ligands, including poly (I:C) (tlr-pic), cl097 (tlr-c97-5), ODN2395 (tlr-2395), Pam3CSK4 (tlr-pms), and LPS (tlr-3pelps) were purchased from InvivoGen. Murine or human macrophage colony-stimulating factor (M-CSF) and ELISA Kits (TNF, IL-6, IFN- λ 2/3, CCL2, CCL3, CCL5, and IP-10) was obtained from R&D Systems. Anti-phospho-TBK1 (#5483), anti-TBK1 (#3504), anti-phospho-IRF3 (#4947), anti-IRF3 (#4302), anti-phospho-I κ B α (#9246), and

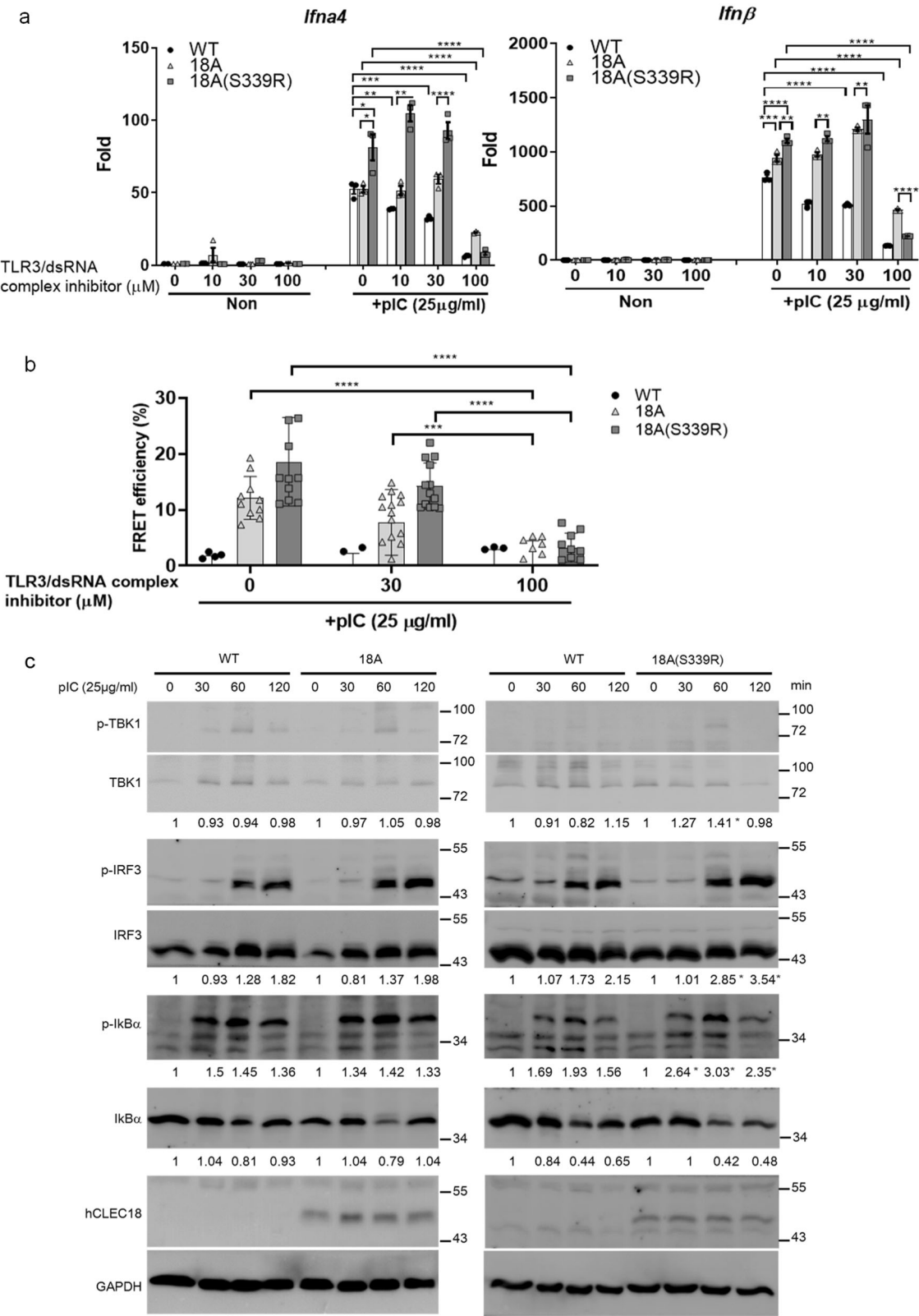
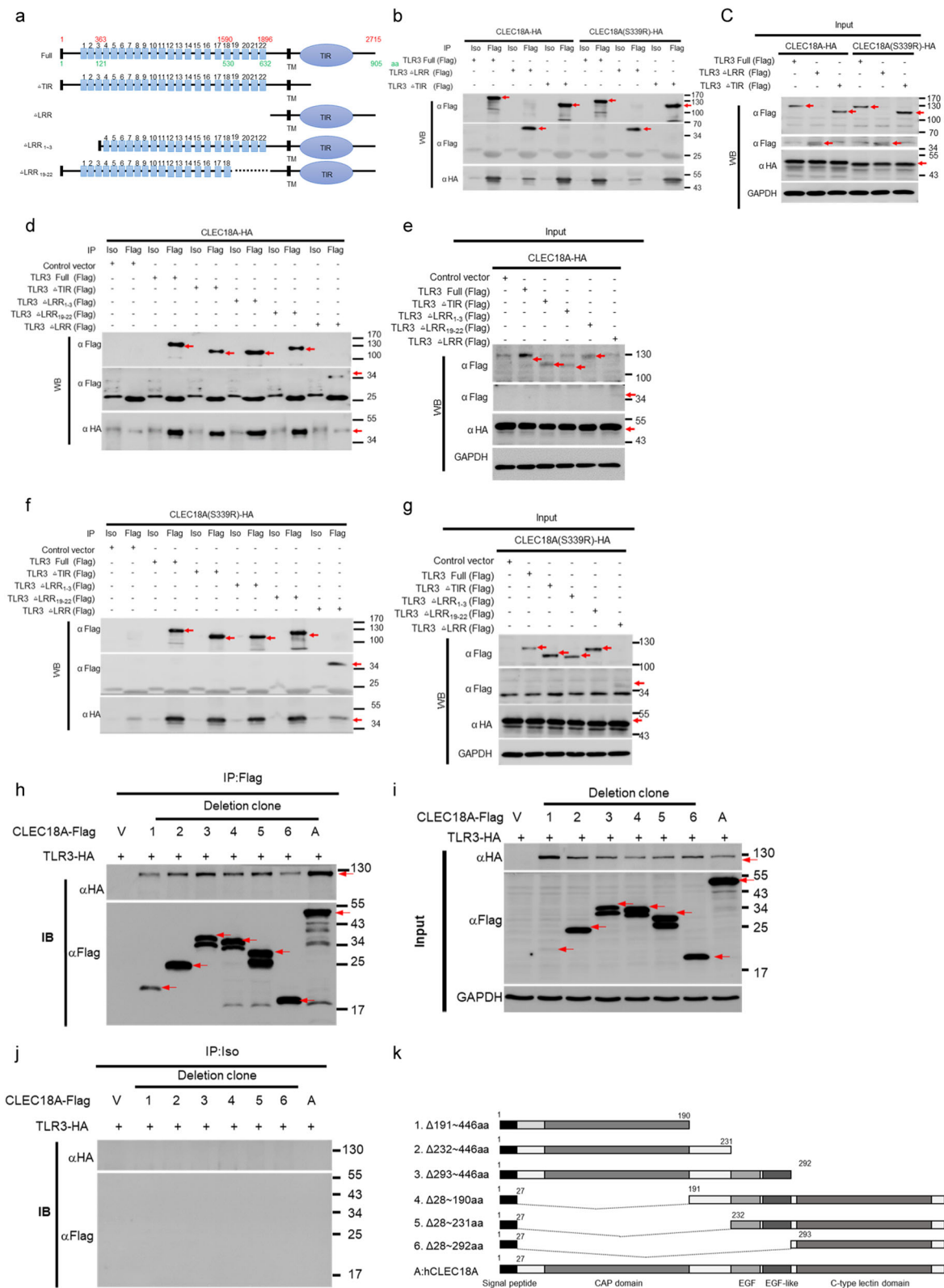


Fig. 5 CLEC18A and CLEC18A(S339R) enhance TLR3-mediated signaling and IFN production. **a** BMDMs (1×10^6 /well) from WT and CLEC18 KI mice were preincubated with various concentrations of TLR3/dsRNA complex inhibitor for 1 h, followed by the addition of HMW poly (I:C) (25 μ g/ml) for 12 h before harvesting samples to determine cytokine expression by RT-PCR ($n = 3$). **b** Alternatively, BMDMs (3×10^4 /well) were preincubated with various concentrations of TLR3/dsRNA complex inhibitor for 1 h, followed by the addition of HMW poly (I:C) (25 μ g/ml) for 2 h for FRET assay ($n = 3-8$). **c** BMDMs (2.5×10^6 /well) were incubated with HMW poly (I:C) and then lysates were harvested at indicated time points, followed by harvesting samples to analyze protein phosphorylation by WB. pIC poly (I:C), min minute ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.



anti-IkBa (#4814) Abs were purchased from Cell Signaling Technology. TLR3 antibody (#GTx20260) and TLR7 antibody (#NBP2-24906) were purchased from Genetex and Novus Biologicals. TLR3/dsRNA complex inhibitor (#614310) was purchased from Calbiochem Merck Millipore. The transfection reagents used were as follows: ON-TARGETplus Non-targeting Control Pool (Dharmacon, D-001810-10-50), ON-TARGETplus Human TLR3 siRNA-SMARTpool (Dharmacon, L-007745-00-0020), HiPerFect Transfection Reagent (Qiagen, 301707), human CLEC18 siRNA#1 (Qiagen, SI03231088), human CLEC18 siRNA#2 (Qiagen,

SI04149502), AllStars Negative Control siRNA (Qiagen, 1027280), and AllStars Neg.siRNA AF 488 (Qiagen, 1027289).

Mice. Eight to twelve-week-old WT C57BL/6 and ROSA-CLEC18 mice were used for all the experiments. The ROSA-CLEC18A and ROSA-CLEC18A(S339R) knock-in mice were generated by the Transgenic Mouse Models Core. All animals were bred and housed at the Academia Sinica SPF animal facility and the

Fig. 6 Mapping of TLR3 and CLEC18A/CLEC18A(S339R) interaction domain. **a** Scheme of human TLR3 deletion mutants. **b–g** The 293T cells (3×10^6) were transfected with pMACS-Kk-HA-hCLEC18A/CLEC18A(S339R) (7.5 μ g) and pFlag-CMV1-hTLR3 (full-length and deletion mutants) (7.5 μ g) for 48 h, followed by immunoprecipitation using anti-Flag mAb. Immunoprecipitates were fractionated on SDS-PAGE and blotted to PVDF membranes before probing with anti-HA mAb and anti-Flag mAb, respectively. Blots were further incubated with peroxidase-conjugated anti-mouse IgG antibody and developed by ECL Kit. **h–k** The 293T cells (3×10^6) were transfected with pCMV-Tag4A-hCLEC18A (full-length and deletion mutants) and pUNO1-TLR3-HA for 48 h, followed by immunoprecipitation using anti-Flag mAb. Immunoprecipitates were fractionated on SDS-PAGE before blotting to PVDF membranes. Blots were probed with anti-HA mAb and anti-Flag mAb, respectively, followed by incubation with peroxidase-conjugate anti-mouse IgG antibody and developed by ECL Kit. Iso isotype control antibody, IP immunoprecipitation, WB western blot. Red arrows: CLEC18 or TLR3; V: vector only; [+]: Flag-tagged CLEC18A and deletion mutants were co-transfected with HA-tagged TLR3 expression vector (TLR3-HA). All WB experiments have been repeated at least three times.

Laboratory Animal Center of National Defense Medical Center. Animal experiments were approved by the Institutional Animal Care and Use Committee at AS core (protocol ID 18-12-1243), and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Taiwanese Council of Agriculture.

Preparation of macrophages. To prepare mouse macrophages, bone marrow cells were isolated from femurs and tibias and incubated in RPMI-1640 complete medium supplemented with 10% (v/v) fetal bovine serum and 10 ng/ml of recombinant mouse M-CSF (R&D Systems) for 6 days. To prepare human macrophages, CD14⁺ monocytes isolated from peripheral blood mononuclear cells were incubated in RPMI-1640 medium supplemented with 10 ng/ml human M-CSF (R&D Systems) for 6 days to differentiate into macrophages¹⁹. The protocol of peripheral blood mononuclear cell isolation was approved by the Human Subject Research Ethics, Academia Sinica (Taiwan, number AS-IRB02-108175).

Isolation of primary mouse alveolar epithelial type II cells. C57BL/6 mice were sacrificed by an overdose of isoflurane. The inferior vena cava was cut and lungs were perfused with PBS via the right ventricle until it turned white in color. A small nick was made into the exposed trachea to insert a shortened 20 G angiocatheter that was fixed and a total volume of 3 ml of sterile dispase was injected (BD Biosciences), followed by injection of 500 μ l of sterile 1% low-melting agarose in PBS (Lonza). After a few minutes of incubation, the lungs were removed and placed into a tube containing 1 ml of dispase for 45 min (25 °C). Lungs were then transferred into a culture dish containing Dulbecco's modified Eagle's medium (DMEM)/10 mM HEPES/100 U/ml DNaseI (Sigma)/antibiotics for 5 min, and the tissue was carefully dissected from the airways and large vessels. The single cell was successively suspended through a 100 and 40 μ m strainers. The cell was centrifuged at $300 \times g$ for 10 min at 4 °C, then the cell was resuspended with RBC lysis buffer for 5 min to lyse the presence of RBCs, and finally, it was directly added to 10 ml DMEM supplemented with 10% FCS/HEPES and antibiotics. The cell was centrifuged and the resuspended cell was placed on prewashed 100-mm tissue culture plates that had been coated for 24–48 h at 4 °C with 42 μ g CD45 and 16 μ g CD32 (BD Pharmingen). After incubation for 2 h at 37 °C, type II cells were gently collected from the plate and filtered by 40 μ m strainers to suspend the single cell. AECs were plated into 6-well cell culture plates at 1.5×10^6 /well, followed by detecting prosurfactant protein C (Abcam, ab40879), the specific marker for alveolar epithelial type II cells, by flow cytometry to determine the purity.

Transfection of macrophages and HT1080 cells by CLEC18 siRNA and CLEC18 expression vectors. Human macrophages were seeded at 7×10^4 cells/well in 96-well microtiteration plates. After incubation at 37 °C overnight, cells were transfected with 100 nM siRNA in 2.5 μ l HiPerFect, and further incubated at 37 °C for 48 h before harvesting for the functional assay. Alternatively, HT1080 cells were seeded in 12-well microplate (1.2×10^5 cells/well) and incubated at 37 °C overnight, followed by incubation with 10 nM siRNA in 6 μ l HiPerFect at 37 °C for 48 h to knock down endogenous CLEC18. The siRNA-transfected HT1080 cell cells were then seeded in 12-well microplate (1.2×10^5 cells/well) and incubated at 37 °C overnight, followed by transfection with 1 μ g plasmids in 4 μ l Turbofect transfection reagent (Thermo Scientific, catalog number R0532), and further incubated at 37 °C for 24 h before functional assay.

Detection of human and mice CLEC18 by Western blot or flow cytometry. For Western blotting analysis, cells (1×10^6) were lysed by RIPA buffer, followed by fractionation on 12% SDS-PAGE before blotting onto PVDF membrane. Membranes were probed with anti-hCLEC18 mAb (clone 3A9E6 (1)) or anti-mCLEC18 mAb (home-made antibody C8G), followed by incubation with peroxidase-conjugated goat anti-mouse polyclonal antibody (Millipore, AP181P) or donkey anti-human polyclonal antibody (Jackson ImmunoResearch, 709-035-149). Blots were developed by the ImmobilonTM Western Chemiluminescent using horseradish peroxidase as the substrate (MilliporeTM). For flow cytometry analysis, primary cells were incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 antibody (BioLegend, 100408), PerCP-Cy5.5-conjugated rat anti-mouse CD8a (BD,

561109), PE/cyanine7-conjugated anti-mouse CD11b antibody (BioLegend, 101216), Alexa Fluor 488-conjugated anti-mouse Ly6G/Ly6C (Gr-1) antibody (BioLegend, 108417), Brilliant Violet 510-conjugated anti-mouse IA/IE antibody (BioLegend, 107636), Brilliant Violet 421-conjugated anti-mouse/human CD45R/B220 antibody (BioLegend, 103251), and then intracellular stain with Alexa Fluor 647-conjugated anti-mCLEC18 mAb (home-made antibody clone C5F). After staining, cells were analyzed by a FACSVerse[™] flow cytometer (BD Biosciences) and data were analyzed using the FlowJo software.

Virus. Original stocks of recombinant PR8 expressing HA and NA derived from either VN1203 (VNHA,NA) or 127 HK5498 (HKHA,NA) were obtained from HKU-Pasteur Research Pole^{20,21}. Both IAV strains were grown in Madin-Darby canine kidney cells (MDCK, ATCC[®]CCL-34[™]) according to a standard procedure and virus titer was determined by a plaque assay on confluent MDCK cells²². DVs (DV2-PL046) were propagated in C6/36 mosquito cell line, and viral titers were determined by plaque-forming assays using BHK-21 cells.

Inoculation of virus. Murine and human macrophages were infected with IAVs at a multiplicity of infection (MOI) = 2. After 1 h adsorption, cells were washed once with PBS, followed by incubation with culture medium (RPMI supplemented with 10% fetal bovine serum) for 12 h or 24 h. WT or ROSA-CLEC18 knock-in mice (9–12 weeks old) were anesthetized by intraperitoneal injection of ketamine and xylazine, followed by intranasal inoculation with H5N1^(HA,NA) virus²⁰ (1.5×10^3 PFU) in 20 μ l sterile PBS. Survival rate and body weight loss were monitored until day 21 post infection.

Immunofluorescence staining and fluorescence lifetime imaging microscopy-FRET. Murine and human macrophages were fixed and permeabilized, followed by incubation with anti-CLEC18 (60 μ g/ml), anti-TLR3 (20 μ g/ml), or anti-TLR7 (20 μ g/ml) Abs at 4 °C for 16–18 h, followed by incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 715-545-150) or Alexa Fluor 546-conjugated goat anti-rabbit IgG (Life Technologies, #A-11035). Samples were mounted with coverslips and observed under a confocal microscope (TCS-SP5-MP-SMD, Leica) and analyzed by the SymPhoTime software.

Immunoprecipitation and Western blotting. To understand whether hCLEC18A or hCLEC18A(S339R) associates with hTLR3, pMACS.Kk-HA(C), pMACS.Kk-HA(C)-hCLEC18A, and pMACS.Kk-HA(C)-hCLEC18A(S339R) (7.5 μ g) were co-transfected with pFlag-CMV1-hTLR3 (7.5 μ g), respectively, into 293T cells (3×10^6), followed by incubation at 37 °C for 48 h before harvesting. Cell lysates were preincubated with Sepharose beads to remove non-specific associated protein, followed by incubation with anti-Flag mAb-conjugated Sepharose beads (Sigma-Aldrich, A2220) or isotype mAb-conjugated Sepharose beads to pull down TLR3-associated proteins. The immunoprecipitates were fractionated on 12% SDS-PAGE and blotted onto PVDF membranes, which were probed with anti-Flag mAb and anti-HA mAb before incubation with peroxidase-conjugated goat anti-mouse polyclonal antibody (Millipore, AP181P or AP200P). Blots were visualized by adding ImmobilonTM western chemiluminescent horseradish peroxidase substrate (MilliporeTM). To determine CLEC18 domains associated with TLR3, pUNO1-hTLR3 (7.5 μ g) and pCMV-Tag4A-hCLEC18A (7.5 μ g) deletion mutants were transfected into 293T cells (3×10^6), followed by incubation at 37 °C for 48 h before immunoprecipitation.

RNA extraction and real-time PCR. Total RNA was extracted by TRIzol reagent (Invitrogen[™]) according to the vendor's instruction. First-strand cDNA was synthesized using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific). PCR reaction was performed by LightCycler[®] 480 System (Roche Applied Science). All primers are listed in Supplementary Table I.

Quantification of viral M and NP gene. To determine M and NP expression in infected mice, samples were harvested from infected mice for RNA extraction

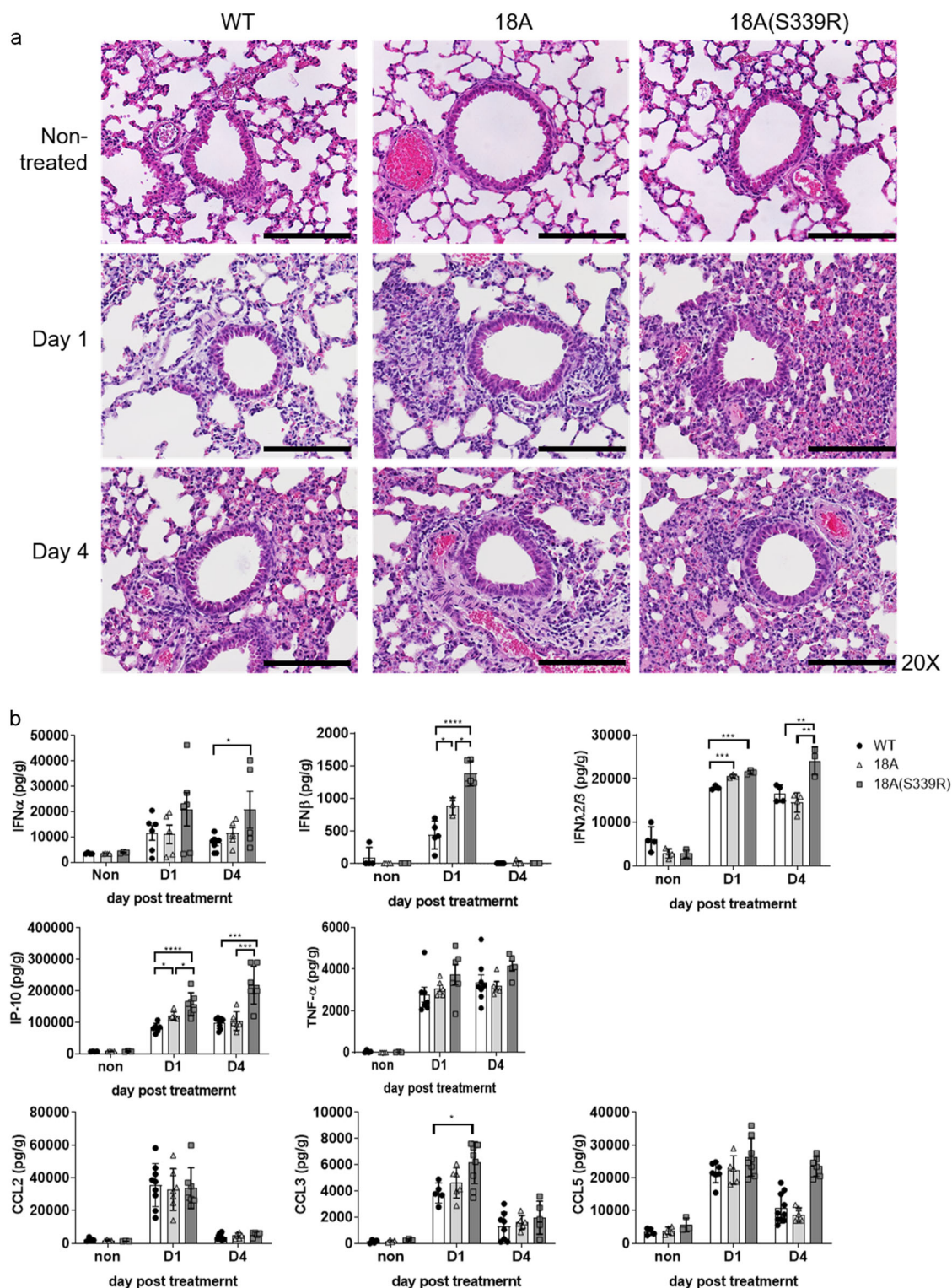


Fig. 7 CLEC18 enhances poly (I:C)-induced inflammatory reaction in vivo. WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice were inoculated with HMW poly (I:C) (30 μ g per mouse) via intranasal route, and then were sacrificed at day 1 (D1) and 4 (D4) post treatment. Lung sections were subjected to hematoxylin and eosin (H&E) staining ($n = 3-6$) (**a**), and cytokine in bronchoalveolar lavage was determined by ELISA ($n = 3-8$) (**b**). pIC: poly (I:C). One-way ANOVA was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ for WT group versus groups of CLEC18A or CLEC18A(S339R). Scale bar: 200 μ m.

using the TriRNA Pure Kit (Geneaid, Catalog No. TRP100), and first-strand cDNA was synthesized using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific) as per the vendor's instruction. Quantitative real-time PCR analysis was performed using the LightCycler[®] 480 System (Roche Applied Science).

Determination of virus titers in lung tissue. Lungs isolated from IAV-infected mice were homogenized with a homogenizer (MagNA Lyser Instrument, Roche Applied Science) in 0.7 ml PBS on ice, followed by centrifugation at 11,000 r.p.m. for 10 min at 4 °C. Supernatants were diluted serially in DMEM, and virus titers were determined by plaque assay on confluent MDCK cells.

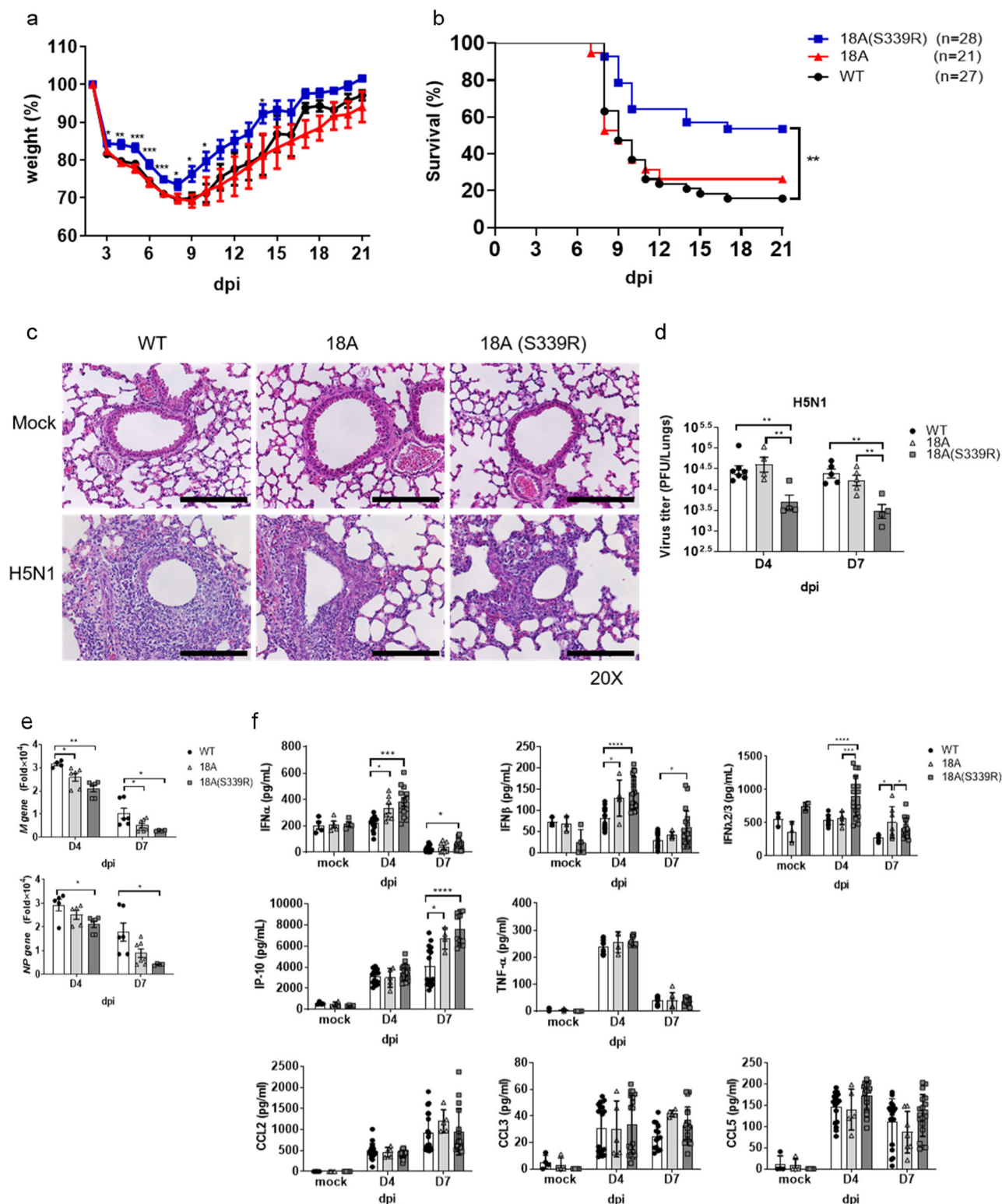


Fig. 8 CLEC18A and CLEC18A(S339R) protect host from IAV-induced inflammation and lethality. **a, b** WT, ROSA-CLEC18A, and ROSA-CLEC18A(S339R) mice were inoculated with H5N1 virus (1.5×10^3 PFU) by intranasal route to observe body weight changes (**a**) and survival rate (**b**) ($n = 21-28$). **c** Lung tissues harvested at day 4 post infection were subjected to hematoxylin and eosin staining ($n = 3$). **d, e** Virus titer in lung tissues was determined by plaque assay ($n = 4-6$) (**d**), while viral structure gene expression (M and NP) was determined by RT-PCR ($n = 4-6$) (**e**). **f** Cytokines contained in the bronchial alveolar lavage fluid (BALF) from WT and ROSA-CLEC18A/CLEC18A(S339R) mice at days 4 and 7 post infection were determined by ELISA ($n = 3-17$). d.p.i. days post infection. One-way ANOVA was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ for WT group versus group of CLEC18A or CLEC18A(S339R). Scale bar: 200 μ m.

Determination of cytokine levels by ELISA. The levels of cytokines in culture supernatant and BALF were determined by ELISA Kits according to the vendor's instruction. All samples were stored at -80°C before assay.

Administration of poly I:C to mice by intranasal route. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, followed by intranasal administration of saline or 30 μg HMW poly I:C (InvivoGen) in 20 μl sterile saline. Poly I:C was freshly prepared for each experiment according to the vendor's instruction.

Bronchoalveolar lavage. All the cell infiltrates and contents in lung tissues were harvested by infusing 3 ml PBS to lung six times. All the samples were centrifuged at 1600 r.p.m. for 10 min at 4°C and the supernatant was stored at -80°C before use.

Statistics and reproducibility. Data were analysed using GraphPad Prism 8 or FlowJo, and the data were shown as mean $\pm$ SEM. Statistical significance was determined using unpaired one- or two-way analysis of variance with the Tukey's multiple comparison test. The statistical significance of survival rate was determined using log-rank test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary information files). The source data underlying graphs shown in the main figures are presented in Supplementary Data 1. Full blots are shown in Supplementary Fig. 5.

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Acknowledgements

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Author contributions

Y.-L.H.: designed, performed experiments, data analysis, and wrote the manuscript; M.-T.H., and P.-S.S.: provided technical support; T.-Y.C.: provided technical support; R.-B.Y.: provided reagents; A.-S.Y. and C.-M.Y.: generation of anti-mouse CLEC18A mAb; W.-C.C. and Y.-W.H.: provided technical support; S.-L.H.: designed, performed experiments, data analysis, and wrote the manuscript

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s42003-021-01745-7>.

Correspondence and requests for materials should be addressed to S.-L.H.

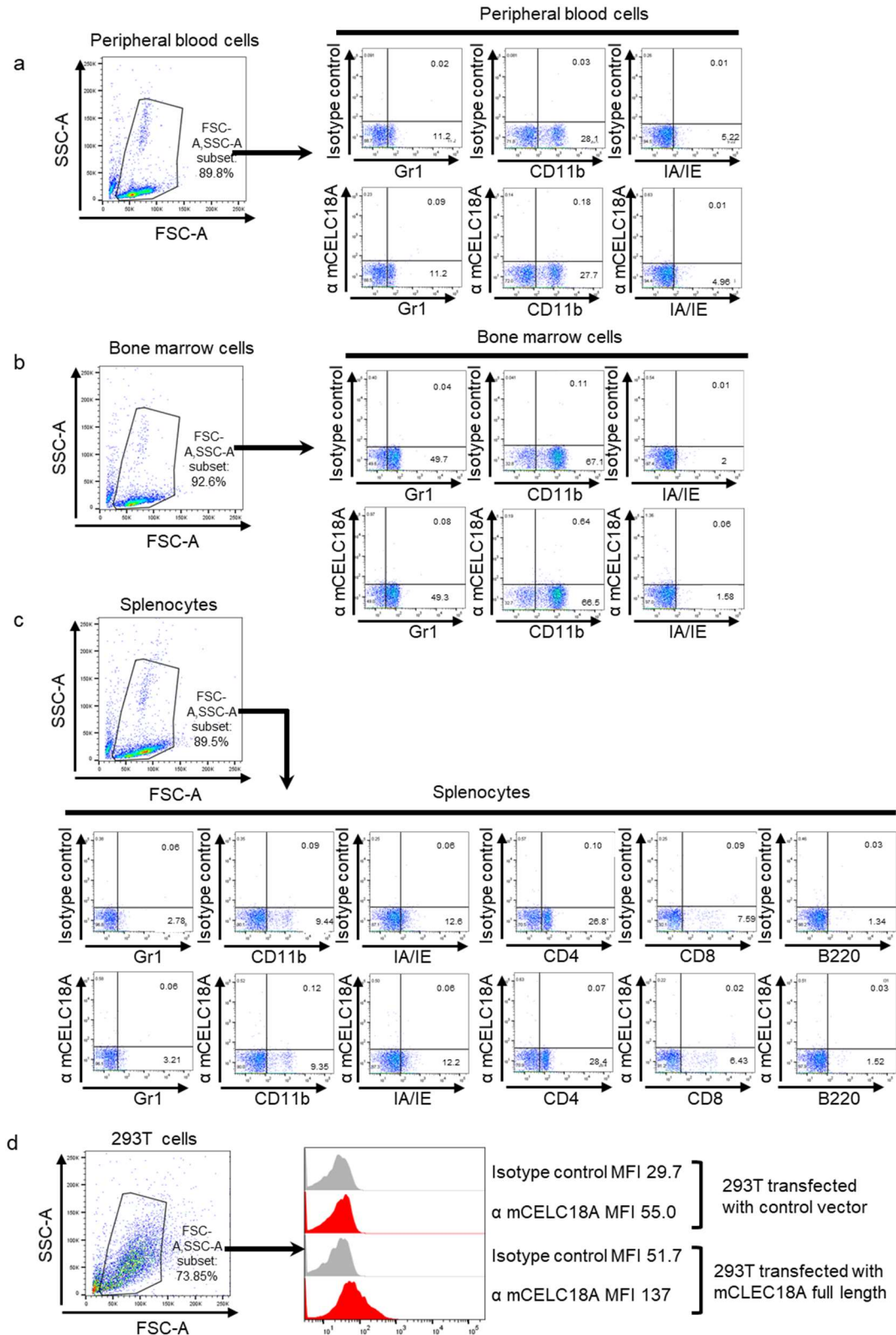
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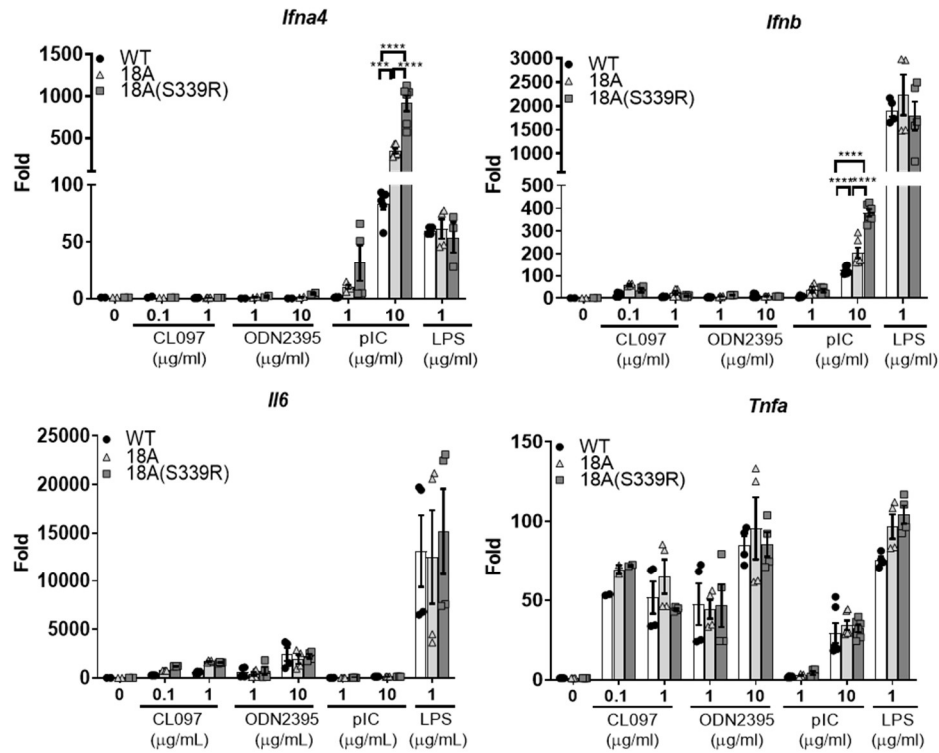


Supplementary Figure 1. Determination of mCLEC18A expression by flow cytometry

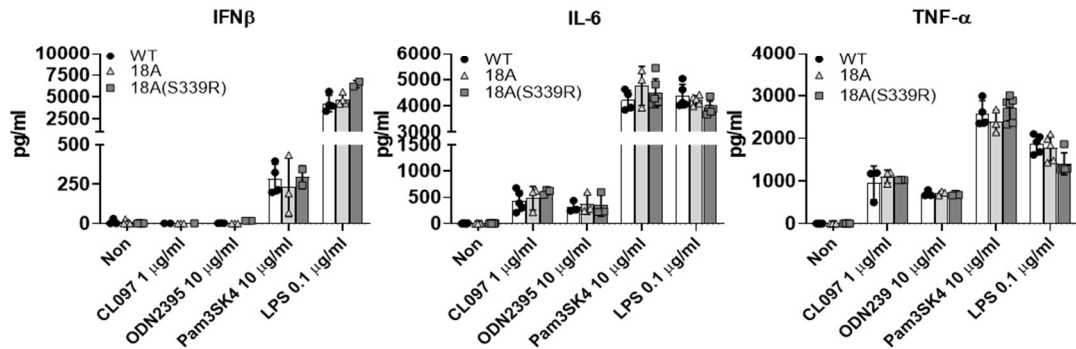
Mouse peripheral blood cells (a), bone marrow cells (b), and splenocytes (c) were

isolated from WT mice, followed by incubation with fluorochrome-conjugated mAb to detect various cell surface markers and endogenous intracellular CLEC18A by flow cytometry. (d) Detection of endogenous mCLEC18A by flow cytometry. The 293T cells were transfected with pCMV-Tag4A-mCLEC18A to express mCLEC18A as positive control for intracellular staining.

a

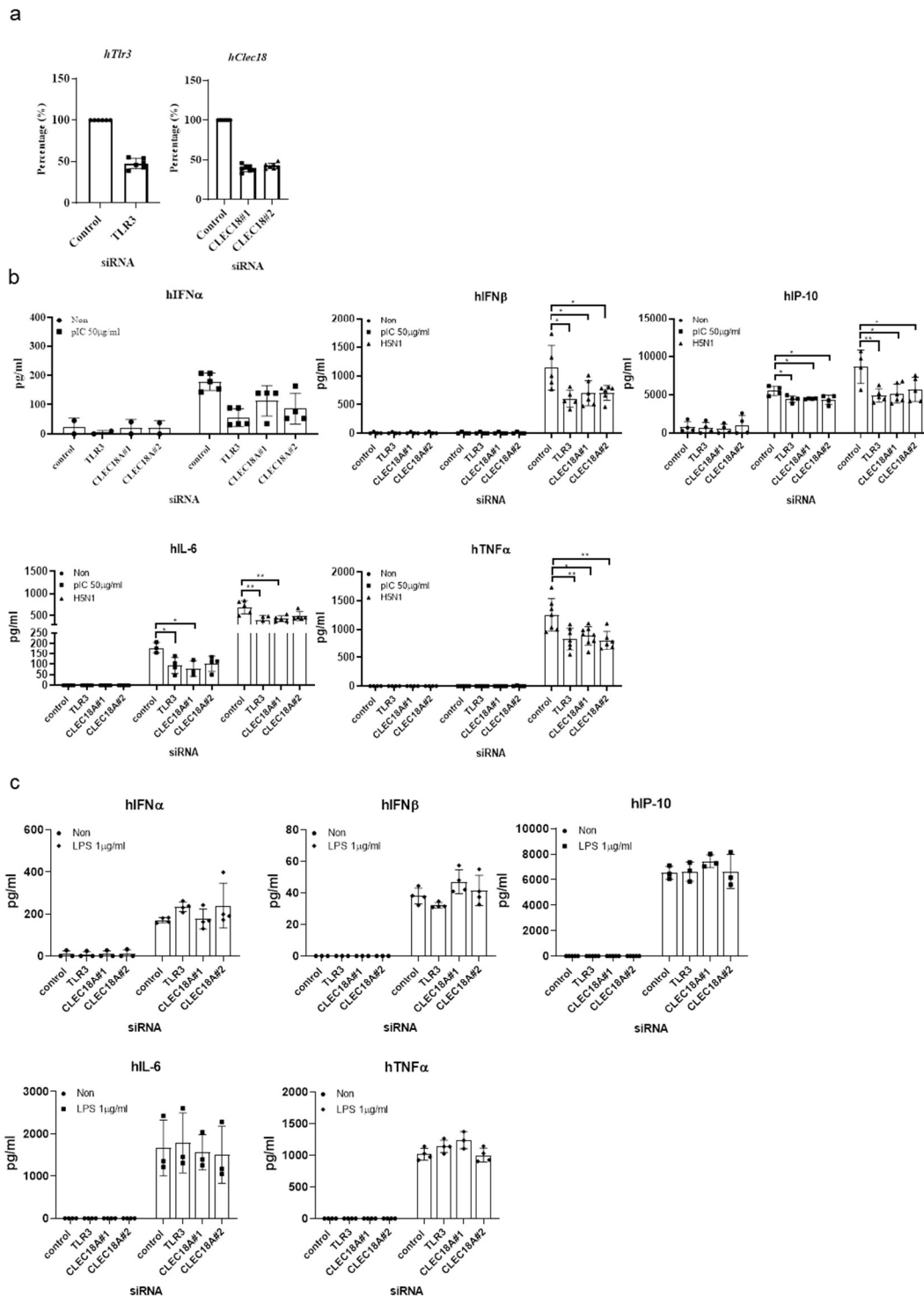


b



Supplementary Figure 2. Effect of TLR ligands to induce cytokine expression in BMDMs

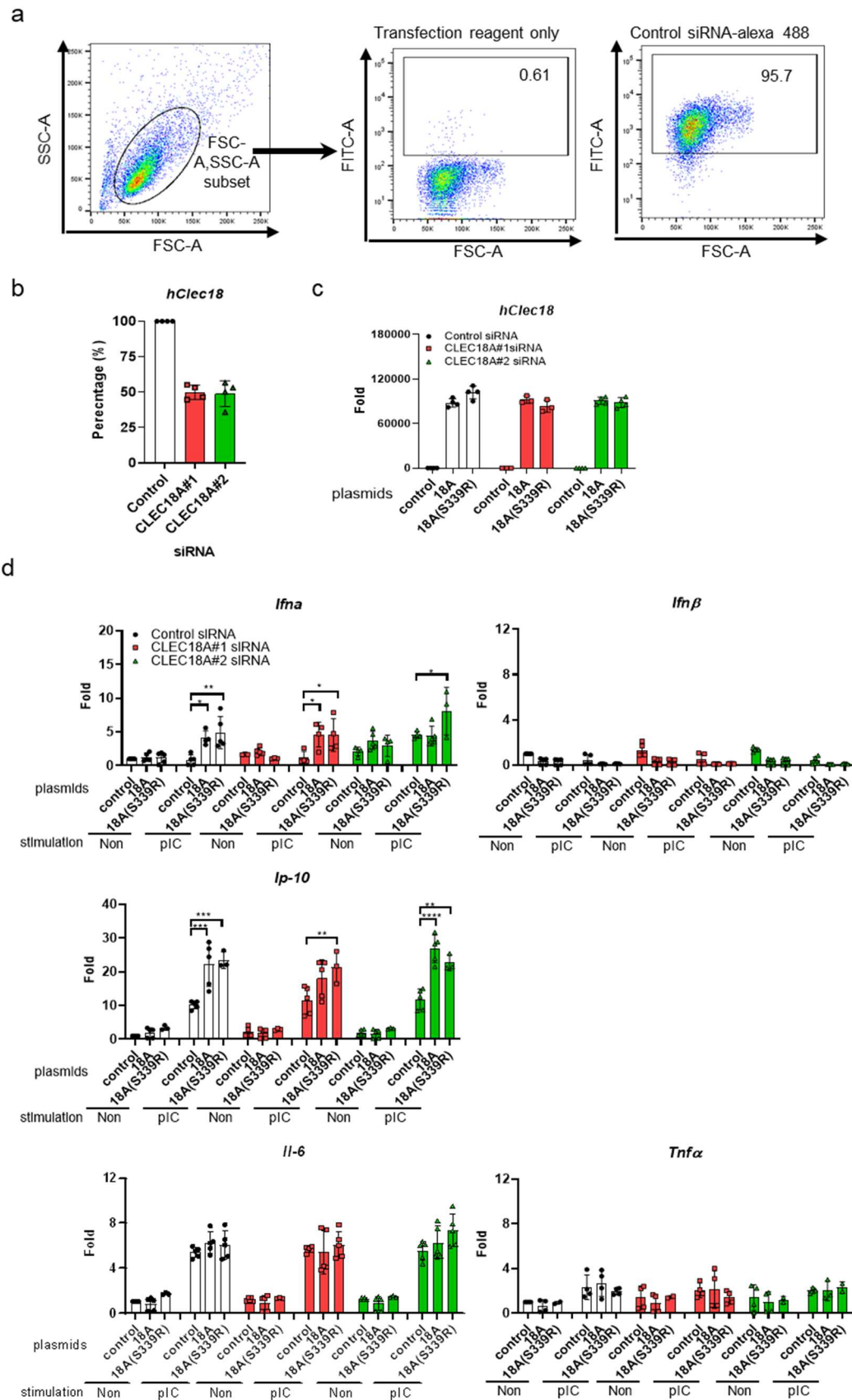
BMDMs (1×10^6 /well) from WT, ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice were incubated with various TLR ligands for 12 or 24 hr, and samples were harvested to determine cytokine expression by RT-PCR (a) and ELISA (b). One-way ANOVA was performed. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$, for WT group versus group of CLEC18A or CLEC18A(S339R). BMDMs: bone marrow derived macrophages.



Supplementary Figure 3. CLEC18 enhances the expression of IFN and proinflammatory cytokines in human macrophages

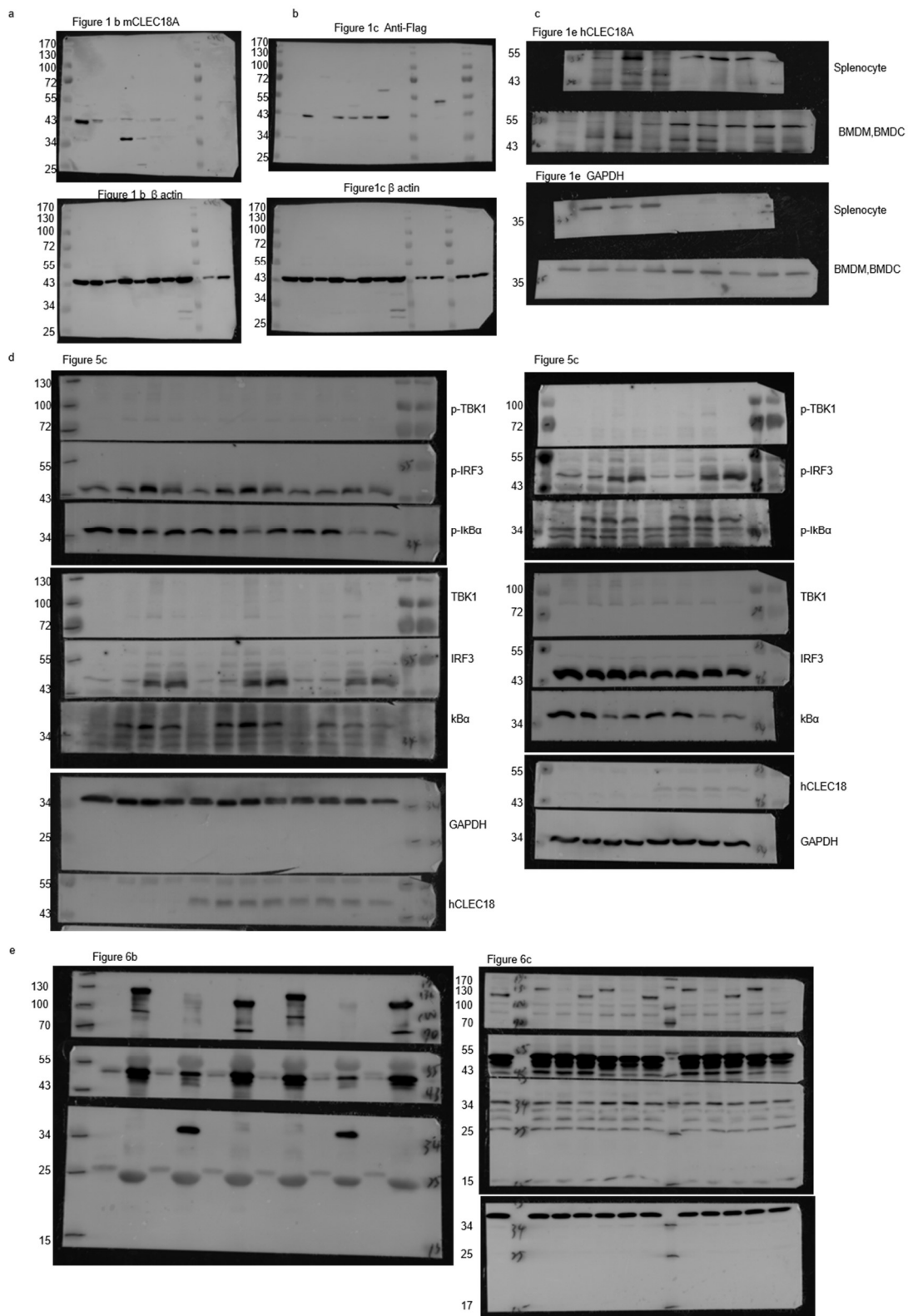
(a) The expression level of *hTlr3* and *hCLEC18* in siRNA-transfected human macrophage cells. (b&c) Human macrophages (7×10^4 /well) were transfected with human TLR3 or CLEC18 siRNA for 48 hr, followed by incubation with high

molecular weight poly (I:C) ($50 \mu\text{g/ml}$) and H5N1 virus (M.O.I. = 2) (b), or LPS ($1 \mu\text{g/ml}$) (c) for 24 hr. Samples were harvested to determine cytokine expression by ELISA. One-way ANOVA was performed. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$, for control group versus group of TLR3 or CLEC18 siRNA. pIC: poly (I:C).

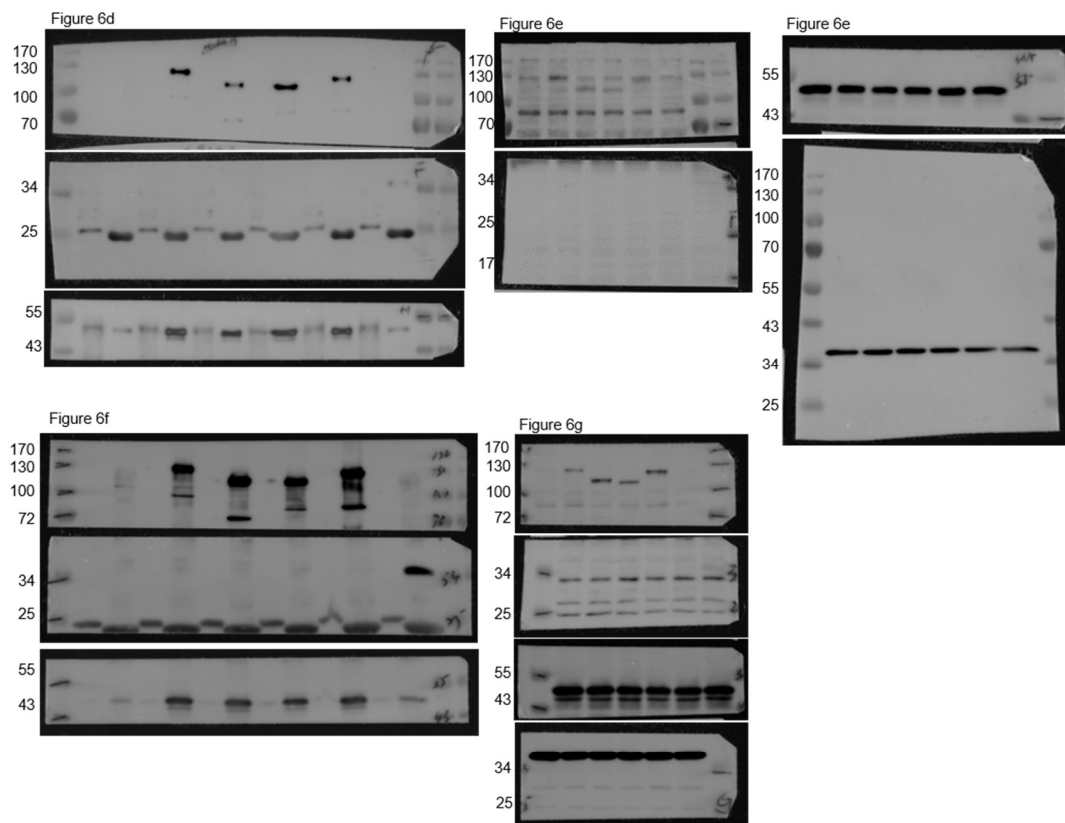


Supplementary Figure 4. CLEC18 enhances IFN cytokines production in HT1080 cell lines

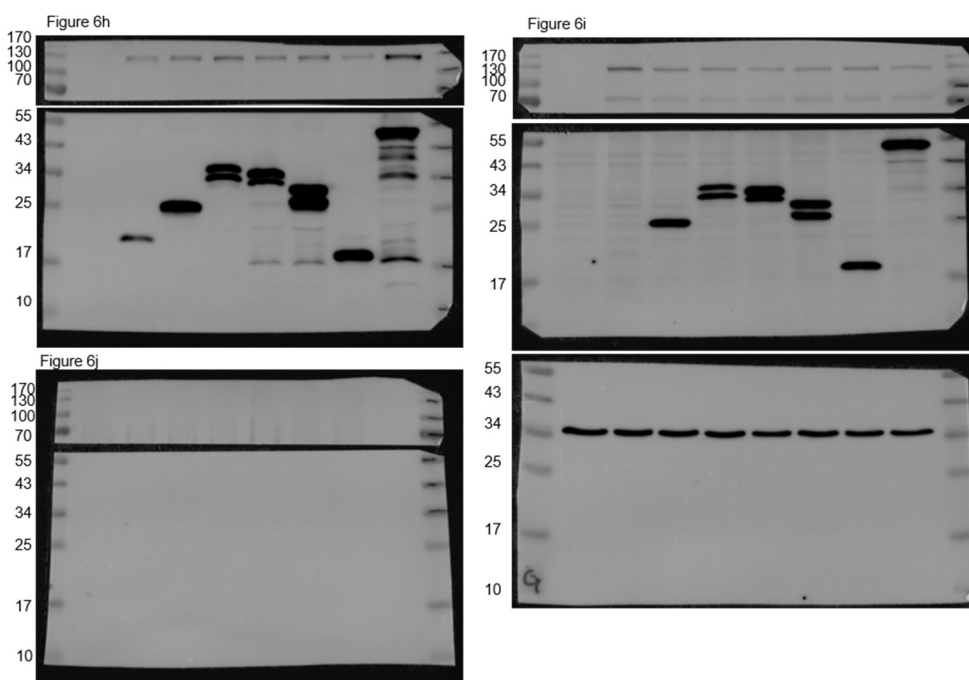
(a) The transfection efficiency of control siRNA-alexa 488 is shown by flow cytometry. (b) The expression levels of *hCLEC18* in siRNA transfected HT1080 cells were determined by RT-PCR. (c) The expression level of *hCLEC18* in plasmid transfected cells that firstly transfect with siRNA was determined by RT-PCR. (d) The siRNA-transfected HT1080 cells were further transfected with control vector, pMACS.Kk-HA(C)-hCLEC18A and pMACS.Kk-HA(C)-hCLEC18A(S339R) for 24 h, and further incubated with high molecular weight poly (I:C) (50 μ g/ml) for 8 h. Samples were harvested to determine cytokine expression by qPCR. Two-way ANOVA was performed. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$, for control vectors group versus group of hCLEC18A or hCLEC18A(S339R) plasmids. pIC: poly (I:C). White color: HT1080 transfected with control siRNA; Red color: HT1080 transfected with CLEC18A#1 siRNA; Green color: HT1080 transfected with CLEC18A#2 siRNA;



f



g



Supplementary Figure 5. Full blot shows in Fig1, Fig5, Fig6

(a~c) Blots shown in Figure 1b,c&e; (d) Blots shown in Figure 5c; (e~g) Blots shown in Figure 6 b~j.

Primer	Forward	Reverse
hGapdh	5'-ccactcctccaccttgac-3'	5'-accctgttgctgtagcca -3'
hlf β	5'-aaactcatgagcagtctgca -3'	5'- actatgggtccaggcacagtg -3'
hlfna1/13	5'- tggctgtgaagaaatacttccg-3'	5'- tgtttcatgttgaccagatg-3'
hTnf α	5' acaagcctgtagcccatgtt-3'	5'- aaagtagacctgcccagact-3'
hll-6	5'-gtagccgccccacacaga-3'	5'-catgtctccttctcagggctg -3'
mgapdh	5'-gcatccactgggtgctgcc-3',	5'-tcatcatacttggcagggttc-3'
mlf β	5'-ccaccactcattctgaggca -3'	5'- atgggtgggtccgagcagagat-3'
mlfna4	5'-tactcagcagacctgaacct-3'	5'-cagtcttggcagcaagttgac -3'
mlfn λ 2/3	5' - agctgcaggccttcaaaaag -3'	5' - tgggagtgaatgtggctcag -3'
mTnf α	5'-gcctcttctcattcctgcttg -3'	5'-ctgatgagagggaggccatt -3'
mll-6	5'-ctgcaagagacttccatccagtt-3'	5'-gaagtagggaaggccgtgg-3'
mMx1	5' -aaccctgctacctttcaa-3'	5' -aagcatcgtttctctatttc-3'
mCh25h	5'-tgctacaacggttcggagc -3'	5'-agaagcccacgtaagtgatgat -3'
mlsg15	5'-tgggacctaaaggtgaagatgctg-3'	5'-tgcttgatcactgtgcactggg -3'
M gene	5'-cttctaaccgaggtcgaaacg-3'	5'-ggcattttggacaaagcgtcta-3'
NP gene	5'-tttctagcacgggtctgcactcatattg-3'	5'-cttggctgtttgaagcagtctgaaag-3'

Supplementary Table I. Primer list

protein	Protein size (MW)	loading protein (ug)	Con.(nM)
ecdTLR3-Fc	130k	1.18	45.4
CLEC18A-Fc	55k	0.5	45.4
CLEC18A(S339R)-Fc	55k	0.5	45.4
dsRNA	dsRNA size (MW)	dsRNA strength (kb)	loading dsRNA (ug)
Poly(I:C) HMW	3135k	1.5-8 (ave=4.75kb)	10
pH 6.3 condition	kon(1/Ms)	kdis(1/s)	KD (M)
ecdTLR3-Fc	8.13E+05	4.54E-03	5.59E-09
CLEC18A-Fc	2.08E+06	4.37E-02	2.10E-08
CLEC18A(S339R)-Fc	2.38E+06	1.95E-02	8.17E-09
ecdTLR3-Fc+18A-Fc	5.89E+06	2.63E-03	4.46E-10
ecdTLR3-Fc+18A(S339R)-Fc	4.69E-03	<1.0E-07	<1.0E-12

Supplementary Table II. Determination of affinity of poly (I:C)-CLEC18 versus poly (I:C)-TLR3

Bio-layer interferometry (BLI, ForteBio) binding assays show the binding affinity between CLEC18-CTLD and ecdTLR3 fusion protein to HMW poly (I:C), respectively. The biotinylated-CLEC18A-CTLD-Fc, CLEC18A(S339R)-CTLD-Fc and ecdTLR3-Fc were immobilized on streptavidin (SA)-coated biosensor tips, respectively, followed by incubation with HMW poly (I:C) in PiBS binding buffer (20 μ M Pipes, 150 mM NaCl, pH = 6.3). The processed data were fitted with the integrated fitting function by a 1:1 binding mode. The various KD values obtained by curve fitting were summarized in supplementary Table II. CTLD: C- type lectin like domain; ecd: extracellular domain; HMW: high molecular weight.

Description of Additional Supplementary Files

File name: Supplementary Data 1

Description: The source data underlying graphs shown in main figures are presented in Supplementary Data 1.

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- | | |
|--------------------------|--|
| n/a | Confirmed |
| ☐ | ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☐ | ☒ A description of all covariates tested |
| ☐ | ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐ | ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☐ | ☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☐ | ☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐ | ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	LightCycler480 software 1.5.1.62 (build-in software) GraphPad Prism 8.2.1 GPS-1350867-LKQH-F0642 BD FACSuite v1.0.6 (build-in software) FlowJo (AA040127000)
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Data analysis	LightCycler480 software 1.5.1.62 (build-in software) GraphPad Prism 8.2.1 GPS-1350867-LKQH-F0642 BD FACSuite v1.0.6 (build-in software) FlowJo (AA040127000)
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For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The sample size is determined based on statistics significance of each experiment.
Data exclusions	No data are excluded
Replication	The data are repeated at least three times by the co-authors listed in the authorship
Randomization	Animal are chosen randomly based on the ages, not specific group.
Blinding	Nil

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems		Methods	
n/a	Involved in the study	n/a	Involved in the study
☐	☒ Antibodies	☒	☐ ChIP-seq
☐	☒ Eukaryotic cell lines	☐	☒ Flow cytometry
☒	☐ Palaeontology and archaeology	☒	☐ MRI-based neuroimaging
☐	☒ Animals and other organisms		
☒	☐ Human research participants		
☒	☐ Clinical data		
☒	☐ Dual use research of concern		

Antibodies

Antibodies used	Anti-phospho-TBK1 (#5483), anti-TBK1 (#3504), anti-phospho-IRF3 (#4947), anti-IRF3 (#4302), anti-phospho-IkBα (#9246), and anti-IkBα (#4814) Abs were purchased from Cell Signaling Technology. TLR3 antibody (GTX20260) and TLR7 antibody (NBP2-24906) purchased from Genetex and Novus Biologicals. PE-conjugated anti-mouse CD4 antibody (Biolegend 100408), PerCP-Cy5.5-conjugated rat anti-mouse CD8a (BD 561109), PE/Cyanine7-conjugated anti-mouse CD11b antibody (Biolegend 101216), Alexa Fluor 488-conjugated anti-mouse Ly6G/Ly6C (Gr-1) antibody (Biolegend 108417), brilliant Violet 510-conjugated anti-mouse IA/IE antibody (Biolegend 107636), brilliant Violet 421-conjugated anti-mouse/human CD45R/B220 antibody (Biolegend 103251) were purchased from biolegend and BD.
Validation	Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb #5483 Application: Western Blotting (WB), Immunoprecipitation (IP), Immunofluorescence (IF), Flow Cytometry (F) TBK1/NAK (D1B4) Rabbit mAb #3504 Application: WB, IP Phospho-IRF-3 (Ser396) (4D4G) Rabbit mAb #4947 Application: WB IRF-3 (D83B9) Rabbit mAb #4302 Application: WB, IP Phospho-IkBα (Ser32/36) (5A5) Mouse mAb #9246 Application: WB IkBα (L35A5) Mouse mAb (Amino-terminal Antigen) #4814 Application: WB, IHC, IP, IF, F TLR3 antibody (GTX20260) Rabbit mAb Application: WB, ICC/IF, IHC, ELISA TLR7 antibody (NBP2-24906) Rabbit polyclone Ab Application: WB, DB, Flow, Flow-IC, ICC/IF, IHC, IHC-Fr, IHC-P, IP, PLA PE-conjugated anti-mouse CD4 antibody (Biolegend 100408) Application: FC PerCP-Cy5.5-conjugated rat anti-mouse CD8a (BD 561109) Application: FC PE/Cyanine7-conjugated anti-mouse CD11b antibody (Biolegend 101216) Application: FC Alexa Fluor 488-conjugated anti-mouse Ly6G/Ly6C (Gr-1) antibody (Biolegend 108417) Application: FC brilliant Violet 510-conjugated anti-mouse IA/IE antibody (Biolegend 107636) Application: FC brilliant Violet 421-conjugated anti-mouse/human CD45R/B220 antibody (Biolegend 103251) Application: FC

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	ATCC
Authentication	The cell lines are from ATCC.
Mycoplasma contamination	The cell lines are free of mycoplasma contamination. N.A.
Commonly misidentified lines (See ICLAC register)	N.A.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	Eight to twelve-week-old wild-type C57BL/6
Wild animals	N.A.
Field-collected samples	N.A.
Ethics oversight	Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at AS core (protocol ID 18-12-1243), and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Taiwanese Council of Agriculture.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Mouse peripheral blood cells, bone marrow cells, and splenocytes were isolated from WT mice, after RBC lysis, followed by incubation with fluorochrome-conjugated mAb to detect various cell surface markers and endogenous intracellular CLEC18A by flow cytometry.
Instrument	Genomics Research Center, Academia Sinica, Taipei, Taiwan
Software	BD FACSuite v1.0.6; FlowJo_V10 6.2
Cell population abundance	Mouse peripheral blood cells, bone marrow cells, and splenocytes were isolated without sorting.
Gating strategy	In this sample gating, cells were gated for total cells (FSC-A vs. SSC-A).

- ☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors present a nice study demonstrating the involvement of C-type lectin, CLEC18A in TLR3 signalling. Interestingly the expression pattern of this accessory molecule appears to be similar to others reported for TLR3 i.e. TRIL (predominantly in the brain). The study demonstrates the interaction of CLEC18A with TLR3 and poly I:C through a variety of techniques. The authors take this further by identifying a Serine residue that lies in the C-Type lectin domain, CTLD of CLEC18A important for the binding of CLEC18 to TLR3. The study also demonstrates the importance of CLEC18A and the serine mutant form CLEC18A-1 in the induction of IFNs following viral infection (H5N1 IAV and Dengue Virus) whereby they decreases viral titres, viral protein levels and mortality in vivo. Collectively, the study highlights the importance of this accessory molecule and specifically the serine residue within the C-type lectin domain of CLEC18A for adequate IFN responses following TLR3 engagement most likely in the brain. This work would be of significant interest to researchers in the field with limited publications available on this molecule. Some major and minor comments that may improve the manuscript prior to publication are detailed below.

Major

1. Line 233- 235: Authors state that unlike IFN α and IFN β , ROSA-CLEC18A and ROSA-CLEC18A-1 BMDMs do not upregulate TNF- α and IL-6 in Fig. 2a. However, TNF and IL-6 are both clearly upregulated on the RNA (Fig. 2a) and protein level (Supp Fig. 1a) the difference is simply that expression is not significantly increased compared to WT BMDM levels. This is important to note and suggests that the mouse model in which human CLEC18A is overexpressed may not match what is known to be true for TLR3 responses in human macrophage. Importantly however, this does not negate the significant amount of data in the remainder of the paper demonstrating the interaction of CLEC18A/A-1 with poly I:C and TLR3.
2. It is interesting that in this system CLEC18A-1 seems to be inhibitory on the TRAF6/NF- κ B side of the TLR3 pathway following polyI:C stimulation (Supp Fig 1a), yet enhances the TRIF side. On the other hand in CLEC18A BMDM, 'endogenous' CLEC18A appears important for both. Have the authors identified the residue responsible for the differences we observe between human and murine signalling in response to poly I:C?? What's the conservation in sequence between human and mouse. Data in supplemental Fig1a only appears to have one data point. Can authors please clarify if this is the mean of technical triplicate, how many experiments or perform additional experiments to satisfy n number requirements for the conclusions drawn.
3. The TLR3/dsRNA inhibitor suppresses IFN β at the same concentration in WT and CLEC18 and CLEC18A-1 cells (100uM) (Fig. 4c). This is also the case for IFN α . This result weakens the claim that C18A is infact a co-receptor for TLR3. Could the TLR3/dsRNA inhibitor be added to FRET experiments to definitively prove C18A/C18A-1 as co-receptors of TLR3? If not perhaps remove. The bio-layer interferometry data is much more convincing.
4. The confocal data in supplemental showing EEA1, TLR3 and CLEC18 co-localisation is really nice and could perhaps be moved to the main figures.
5. Figure 5b – it is unfortunate that the authors present the input blots for the immunoprecipitation studies on separate gels/western blots. It is generally expected that the Input/expression of plasmids in both the Isotype control lane and the Flag lanes be shown to confirm adequate expression of plasmid in these samples and in order to confirm a legitimate interaction. Blots in Figure 5d with deletion clones shows this appropriately.
6. Stats are needed in figure 7 for survival. Number of mice not indicated in text or figure legend. This should definitively be included prior to publication.

Minor

1. The labelling of human Macrophages as M-M in figure legends is very confusing for the reader. Prior to reading the legend the reader may think this data represents values from Mouse Macrophages (M-M). Can authors please explain or change this to H-M or other?
2. Figure 1, Expression in mouse DC is not as convincing as in BMDM however, I appreciate the antibodies may not be great and the majority of the data in the paper is in BMDM.

Reviewer #2 (Remarks to the Author):

The manuscript by Ya-Lang Huang et al. roles of human CLEC18A function in TLR3 dependent signaling event. They generated transgenic mice expressing human CLEC18A and mutant CLEC18A S339R for ex vivo and in vivo experiment. Author firstly demonstrated Ifn and other cytokines expression in BMDM and alveolar epithelial cells and found that hCLEC18A and mutant CLEC18A expression enhances expression of Ifnb and after poly IC stimulation and IAV and DV infection. Then author demonstrated TLR3 and CLEC18a associated each other. Author performed in vivo inflammatory model using poly I:C and found that CLEC18A and mutant CLEC18A mice showed severe inflammation. In contrary, IAV infection to CLEC18A and mutant CLEC18A mice showed reduced inflammatory response and prolonged survival comparing to wild mice.

Author tried to explained the discrepancy of TLR3 innate immune response between mice and human, and CLEC18A may causes changes of TLR3 response in human and mice. However, logics of manuscript and these results are not fully support the author's point. In addition, control of experiments in each experiments and references are unsuitable.

The concept is certainly intriguing and is of interest. However, there are several limitations in the data as following.

1. In the introduction, explanation of mutant CLEC18A-1 is unclear. Author should what are causes by the mutation in CREC18A function. Mutant for CLEC18A-1 is should show like "CLEC18A S339R".
2. Ref 6 is not suitable. Ref 6 did not include the results of infection by H3N2 IAV.
3. Explanation of Ref8 is incorrect. Ref 8 is results of human cells.
4. Author mentioned that Clec18A is low expression in BMDM, however expression level of endogenous clec18a is unclear in Fig1.
5. In Fig2a and b, author included results of human macrophages. These are completely different cell line from mouse cells, therefore it is not suitable to put in the same panel and cannot discuss the production level of Ifns in between mouse and human by these results.
6. Poly I:C treatment in vivo showed increase of inflammation in CLEC18A and mutant CLEC18A mice compared with wild type mice, however IAV treatment in these mice showed reduces of inflammation. Why these difference induced by these stimulation?
7. It is unclear why author use CLEC18A mutant mice to show difference of TLR3 responses between human and mice.
8. Author tied to explain the discrepancy of TLR3 innate immune response between mice and human using transgenic mice which expressed human CLCE18A. However, ectopic expression in mice to human gene does not help to explain the difference of human and mice. It is hardly compared with wild type and human gene transgenic mice. Author should compare mice gene and human gene transgenic mice. If author say the expression of Clec18a in mice is low, author show the evidence of expression level in human and mice and use knockdown or knockout technique in human cells line.

Overall, the data shown do not support the main conclusions drawn by the authors.

Reviewer #3 (Remarks to the Author):

In this study, Huang et al. described essential roles of human CLEC18A and CLEC18A-1 in TLR3 signaling pathway by utilizing the ROSA-CLEC18A and ROSA-CLEC18A-1 mice. Their results suggested that human CLEC18A and CLEC18A-1 directly interacted with TLR3 and poly(I:C), enhancing IFN production without the enhancement of pro-inflammatory cytokine production. ROSA-CLEC18A and ROSA-CLEC18A-1 mice were more resistant to H5N1 IAV infection compared to WT mice, suggesting that these proteins are important in antiviral immune responses in human. Overall, their findings that human CLEC18A and CLEC18A-1 act as a co-receptor with TLR3 is particularly interesting in view of differential roles of them between human and mouse, in TLR3-mediated antiviral responses. However, data presented in this manuscript is not sufficient to support their conclusion. Furthermore, there seems to be over-interpretation throughout their manuscript. To strengthen their conclusion, many concerns listed below should be addressed.

Specific comments

1. Figure S1A, S2B and S2C should be explained in their manuscript.
2. In figure 2A, data showing IP-10 production in response to poly(I:C) (1 microG/mL) is missing.
3. In figure 2A and S1A, there seems to be some discrepancy, especially data on pro-inflammatory cytokines, they should kindly explain about that. Furthermore, they should place ELISA data but not qPCR data onto the main figure.
4. Figure 2a did not correspond to supplementary fig. 1a. While mRNA level of TNF α showed no significant difference between WT and CLEC18 expressing cells, the protein level was increased in CLEC18 expressing cells.
5. They should describe about how they prepared human macrophages in [materials and methods] section.
6. In figure 4D, to support their conclusion, they should perform statistical analysis.
7. To strengthen their conclusion, they should perform knockdown experiment using human cells.
8. In figure 6 and 7, they should show data using control (untreated) mice.
9. In figure 1A, because the expression level of mRNA does not always reflect that of protein, they should show CLEC18 protein expression in each tissues and cells (especially immune cells). Same for figure 1B.
10. Because their previous paper (ref. 1) also suggests that CLEC18 is secreted by cells, they should clearly refer it in their manuscript.
11. In figure 1C, they should clearly describe about the antibody they used in figure legend. Furthermore, in case that they used antibody against endogenous human CLEC18 protein but not against tags, they should clearly describe about the information of the antibody used.
12. There are many typos and grammatical errors throughout the manuscript that should be corrected (e.g. [purchased] at line 101, [C57BL/J] at line 118 and [analysis] at line 133 should be [were purchased], [C57BL/6] and [analysis of], respectively.).
13. At line 133 and 134, they should not use the abbreviation [AEC] and [ATII] without prior explanation.
14. Although they claim that CLEC18A-1 is more potent to induce interferon response, this reviewer could not find any statistical analysis between CLEC18A and CLEC18A-1.
15. In figure 2A and 2B, they claim that CLEC18A and CLEC18A-1 increased type I interferon expression when even 1.1-1.2 fold. On the other hand, they claim that, in figure S1B, S1C and S1D, CLEC18 did not affect to ifna or ifnb mRNA expression even 2-4 fold. This is quite strange. They should perform statistical analysis in figure S1 and precisely interpret their data.
16. In figure 4C, statistical analysis seems to be strange. The difference in ifnb between WT (pIC 0) and WT (pIC 30) was significant, but that between WT (pIC 0) and WT (pIC 10) was not significant. If

the figure is correct, latter also seems to be significant. They should confirm the method of statistical analysis throughout their manuscript.

17. In figure 5D, the means of [+] and [-] is not clear.

18. In figure 5D, it is interesting to investigate which domain of CLEC18 is involved in its binding to poly(I:C).

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Major

1. Line 233-235: Authors state that unlike IFN α and IFN β , ROSA-CLEC18A and ROSA-CLEC18A-1 BMDMs do not upregulate TNF- α and IL-6 in Fig. 2a. However, TNF and IL-6 are both clearly upregulated on the RNA (Fig. 2a) and protein level (Supp Fig. 1a) the difference is simply that expression is not significantly increased compared to WT BMDM levels. This is important to note and suggests that the mouse model in which human CLEC18A is overexpressed may not match what is known to be true for TLR3 responses in human macrophage. Importantly however, this does not negate the significant amount of data in the remainder of the paper demonstrating the interaction of CLEC18A/A-1 with poly I:C and TLR3.

Answer:

- 1) *We agree with reviewer's comment and change the description accordingly. The original Fig. 2a is mRNA level, and we change in to protein level in this revised version. In addition, we also repeat this experiment with two different doses of poly (I:C) to show dose-dependent effect. We also compared the effect of poly (I:C) with other TLR ligand in cytokine expression in mRNA and protein levels (Supplementary Fig. 2) in this revised version.*
- 2) *To confirm the effect of CLEC18 in human macrophages, we further knock down endogenous CLEC18 in human macrophages (Supplementary Fig. 3). We found that CLEC18 knockdown in human cells only affects poly (I:C) -, but not LPS-, induced cytokine production (Supplementary Fig. 3). This observation further supports the argument that CLEC18 is the co-receptor of TLR3.*

2. It is interesting that in this system CLEC18A-1 seems to be inhibitory on the TRAF6/NF-kB side of the TLR3 pathway following poly (I:C) stimulation (Supp Fig 1a), yet enhances the TRIF side. On the other hands in CLEC18A BMDM, 'endogenous' CLEC18A appears important for both. Have the authors identified the residue responsible for the differences we observe between human and murine signaling in response to poly I:C? What's the conservation in sequence between human and mouse

Answer:

- 1) *As we show in Figure 1 a~c, mouse leukocytes do not express endogenous CLEC18A, neither can we detect CLEC18A in mouse lung tissue. Thus, the cytokine secretion in wild type mouse immune cells and lung tissue is only via TLR3, without the involvement of mouse endogenous CLEC18A.*
- 2) *The pairwise alignment of human and mouse CLEC18 is as shown in the following page.*
- 3) *The identity and consensus position of human and mice CLEC18A CTLD is 91.2% and 94.2%, respectively. The corresponding amino acid at position 339 is S in both human and mouse CLEC18A. We do not find R339 in available mouse CLEC18 sequence in database.*

Human CLEC18A ₃₃₁GGVLAQIKSQKVQDILAFYL₃₄₀

Mouse CLEC18A ₃₃₁GGVLAQIESQKVQDILAFYL₃₄₀

	(1)	10	20	30	40	50	60	70	81	Section 1	
NP_853527-mCLEC18A	(1)	MIQPEPSLWIGSQSRWLGLLLLLLLSLGITWTEVQPPQ	PKQDPTLQALS	SRKESFLIITAHNRLRSRVHP	PPAANMQMDWS						
NP_872425-hCLEC18A	(1)	MLHPETSPGRG-----	HLALVLLALLGTAAEYVMP	POLEQAPMAGALNRKES	FLLLSLHNRLRSWV	OPPAADMRRLDWS					
Consensus	(1)	MI PE S G	LL LLLALLG W EV PPQ	Q P	AL KRKESFLILS	HNRLRS V PPAA M RLWS					
	(81)	82	90	100	110	120	130	140	150	162	Section 2
NP_853527-mCLEC18A	(81)	ESLAQLAEARAALCVTVTPNLASTPGHNSHVGWNVQLMPHMGSA	SFVEVVNLWFAEG	GLQYRHGDAEACHNATCAHYT	QLVW						
NP_872425-hCLEC18A	(76)	DSLAQLAQAARAALCGT-PTPSLAAGLWRTLVQVGNMQLLPAGLV	SFVEVVSLWFAEG	QORYSHAAQECARNATCTHYT	QLVW						
Consensus	(82)	DSLAQLA ARAALC T TP LAS	VGNMQLLP G	SFVEVV LWFAEG Y HA RECA NATC	HYTQLVW						
	(163)	163	170	180	190	200	210	220	230	243	Section 3
NP_853527-mCLEC18A	(162)	ATSSQLGCGGRCPCFVDOEAMEAFVCAYSPPGNWDINGKTVAPYKKG	TWCSLCTARVSSGCFKAWDHAGGLCEV	PRNPRCMSC							
NP_872425-hCLEC18A	(156)	ATSSQLGCGGRHLCISAGAAIEAFVCAYSPPGNWVEVNGKTI	VPYKKGAWCSLCTAGVSSGCFKAWDHAGGLCEV	PRNPRCMSC							
Consensus	(163)	ATSSQLGCGGR C Q AIEAFVCAYS P GNWDINGKTI PYKKG	WCSLCTA VSGCFKAWDHAGGLCEV	PRNPRCMSC							
	(244)	244	250	260	270	280	290	300	310	324	Section 4
NP_853527-mCLEC18A	(243)	RNLGHLNISTCRCHCPCPGYTGRYQVRCRSVQCVHGFPRKEEC	SCICDVGYGGAQCATKVF	FFPHTCDLRIDGDCFMVSE							
NP_872425-hCLEC18A	(237)	QNHGHLNISTCHCHCPCPGYTGRYQVRCRSQCVRHGRFREEC	SCVCDIGYGGAQCATKVF	FFPHTCDLRIDGDCFMVSE							
Consensus	(244)	N G L NISTC CHC PGYTGRYQVRCRSQCVRHGR FREEC	SCVCDIGYGGAQCATKVF	FFPHTCDLRIDGDCFMVSE							
	(325)	325	330	340	350	360	370	380	390	405	Section 5
NP_853527-mCLEC18A	(324)	DTYYGAKMKCGGKGGVLAQIESQRVQDILAFYLGRL	LETTNEVTDSD	DFETKNFVIGLTYKAAKDSFRW	TGEHQSF	TSFAFG					
NP_872425-hCLEC18A	(318)	DTYYARMKCKQRKGGVLAQIKSRVQDILAFYLGRL	LETTNEVIDSD	DFETKNFVIGLTYKAKDSFRW	TGEHQSF	TSFAFG					
Consensus	(325)	DTYY AKMKCG KGGVLAQI	SRVQDILAFYLGRL	LETTNEV DSD	DFETKNFVIGLTYK AKDSFRW	TGEHQSF	TSFAFG				
	(406)	406	420	430	440	453					Section 6
NP_853527-mCLEC18A	(405)	QPDNQGFGNCVEMQASAAAFNWNDDQCKTRNRYICQ	FAQKHYSRWEPGP								
NP_872425-hCLEC18A	(399)	QPDNQGFGNCVCLQASAAAFNWNDDQCKTRNRYICQ	FAQEHISRWEPGGS								
Consensus	(406)	QPDN GFGNCVELQASAAAFNWNDDQCKTRNRYICQ	FAQ HSRW PG								

Answer:

4. The TLR3/dsRNA inhibitor suppresses IFN β at the same concentration in WT and CLEC18 and CLEC18A-1 cells (100uM) (Fig. 4e). This is also the case for IFN α . This result weakens the claim that C18A is in-fact a co-receptor for TLR3. Could the TLR3/dsRNA inhibitor be added to FRET experiments to definitively prove C18A/C18A-1 as co-receptor of TLR3? If not perhaps remove. The bio-layer interferometry data is much more convincing.

1. We performed FRET experiments in Figure 4f. The high concentration of TLR3/dsRNA inhibitor also disrupts the association between poly(I:C)-CLEC18A as well as poly(I:C)-CLEC18A-1. The FRET assay is in accord with what we observed in cytokine production (Figure 4e).
2. Lower concentration of inhibitor (10 μ M and 30 μ M) can only suppress cytokine release from WT, but not CLEC18A/18A-1 KI mice. However, when the inhibitor is up to 100 μ M, it also disrupts the interactions between TLR3 and poly (I:C). This observation is in accord with our claim that the CLEC18 act as a TLR3 co-receptor and can increase binding affinity between poly (I:C)/TLR3-CLEC18A complex.

5. The confocal data in supplemental showing EEA1, TLR3 and CLEC18 co-localization is really nice and could perhaps be moved to the main figures.

Answer:

Yes, we move these supplementary figures to figure 4c & d in this revised version.

6. Figure 5b – it is unfortunate that the authors present the input blots for the immunoprecipitation studies on separate gels/western blots. It is generally expected that the Input/expression of plasmids in both the Isotype control lane and the Flag lanes be shown to confirm adequate expression of plasmid in these samples and in order to confirm a legitimate interaction. Blots in Figure 5d with deletion clones shows this appropriately.

Answer:

- 1) Because the input gel represents the total lysates (right), and the proteins amounts in the immunoprecipitation (left) is far more than total lysates, therefore we need to separate the samples into separate gels and transfer blots, otherwise all signals in total input (right) will be far less than the immunoprecipitation (left). As far as I know, most of the papers present the total lysates and immunoprecipitation results in two sperate gels.*
- 2) We did show the results of isotype and flag lane side by side in Figure 5b.*
- 3) Thank you for your comment on Figure 5d that our presentation is OK. But I would like to say that the total input and IP are also developed separately in western blot analysis.*

7. Stats are needed in figure 7 for survival. Number of mice not indicated in text or figure legend. This should definitively be included prior to publication.

Answer:

We add the symbols to represent statistical significance in the figure7b now. Number of mice was shown in figure7b.

Minor

1. The labelling of human Macrophages as M-M in figure legends is very confusing for the reader. Prior to reading the legend the reader may think this data represents values from Mouse Macrophages (M-M). Can authors please explain or change this to H-M or other?

Answer:

We present the cytokines production profile of human and mice macrophages in separate panels (Figure 2, mouse BMDM) and Supplementary Figure 3 (human macrophages) to prevent confusion. °

2. Figure 1, Expression in mouse DC is not as convincing as in BMDM however, I appreciate the antibodies may not be great and the majority of the data in the paper is in BMDM.

Answer:

We try a couple of times and find that human CLEC18 expression in mouse DC are much weaker than that BMDM. It may due to the differential promoter activity in mouse DC and BMDM.

Reviewer #2 (Remarks to the Author):

Author tried to explained the discrepancy of TLR3 innate immune response between mice and human, and CLEC18A may causes changes of TLR3 response in human and mice. However, logics of manuscript and these results are not fully support the author`s point. In addition, control of experiments in each experiments and references are unsuitable.

The concept is certainly intriguing and is of interest. However, there are several limitations in the data as following.

1. In the introduction, explanation of mutant CLEC18A-1 is unclear. Author should what are causes by the mutation in CLEC18A function. Mutant for CLEC18A-1 is should show like “CLEC18A S339R”.

Answer:

- 1) *When we initiated the research of CLEC18, we found two CLEC18A polypeptides (NP_872425 and AAI41809) which are identical except polymorphic amino acids in CTLD and SCP domain. To understand the functions of polymorphic amino acid residue located at 339, we mutate S339 into R339 in to generate CLEC18A (S339R) mutant.*
- 2) *We agree with reviewer`s comment and change CLEC18A-1 into CLEC18A(S339R).*
- 3) *We also add a paragraph to describe difference between CLEC18A and CLEC18A(S339R mutant in the Introduction session (line 46-51).*

2. Ref 6 is not suitable. Ref 6 did not include the results of infection by H3N2

IAV.?? **Answer:** *we remove reference 6 accordingly.*

3. Explanation of Ref 8 is incorrect. Ref 8 is results of human cells. ???

Answer: *we remove reference 8 accordingly.*

4. Author mentioned that Clec18A is low expression in BMDM, however expression level of endogenous clec18a is unclear in Fig. 1.

Answer:

Mouse BMDM does not express endogenous CLEC18A mRNA (first lane from left, Fig 1 a).

5. In Fig2a and b, author included results of human macrophages. These are completely different cell line from mouse cells, therefore it is not suitable to put in the same panel and cannot discuss the production level of Ifns in between mouse and human by these results.

Answer:

We present the cytokine production profile of human (Supplementary Fig. 3) and mice (Fig. 2a) macrophages in separate figures.

6. Poly I:C treatment in vivo showed increase of inflammation in CLEC18A and mutant CLEC18A mice compared with wild type mice, however IAV treatment in these mice showed reduces of inflammation. Why the difference induced by these stimulations?

Answer:

- 1) Poly (I:C) is able to induce inflammation, so enhanced TLR3 signaling will cause further inflammatory reactions.*
- 2) However, TLR3 is essential for anti-viral infection (Julia Zinngrebe et al. J Exp Med. 2016), so enhanced TLR3-mediated signaling will help to clear virus infection. In addition, the antiviral genes (Ch25h, Mx1) are upregulated in CLEC18A/CELC18A(S339R) KI cells. This is why IAV infection cause less severe inflammation in the CLEC18A and CELC18A(S339R) KI mice.*

7. It is unclear why author use CLEC18A mutant mice to show difference of TLR3 responses between human and mice.

Answer:

We noticed the presence of CLEC18A(S339R) in the human genome database when we initiated the projects, so we are curious to understand whether the S339→R339 has any effect in host response to TLR3 ligand and IAV infection. Because mice do not express endogenous CLEC18A in immune cells (Figure 1), so we take this advantage to compare the differential response of CLEC18A and CLEC18A(S339R) in knock-in mice.

8. Author tried to explain the discrepancy of TLR3 innate immune response between mice and human using transgenic mice which expressed human CLCE18A. However, ectopic expression in mice to human gene does not help to explain the difference of human and mice. It is hardly compared with wild type and human gene transgenic mice. Author should compare mice gene and human gene transgenic mice. If author say the expression of Clec18a in mice is low, author should show the evidence of expression level in human and mice and use knockdown or knockout technique in human cells line.

Answer:

- 1) As we show in Figure 1 a~c & Supplementary Fig. 1, mouse leukocytes do not express endogenous CLEC18A, neither can we detect CLEC18A in mouse lung tissue. Thus, the cytokine secretion in wild type mouse immune cells and lung tissue is only via TLR3, without the involvement of mouse endogenous CLEC18A.*
- 2) To confirm the effect of CLEC18 in human macrophages, we further knock down endogenous CLEC18 in human macrophages (Supplementary Fig. 3). We found that*

CLEC18 knockdown in human cells only affects poly (I:C)-, but not LPS-, induced cytokine production (Supplementary Fig. 3). This observation further supports the argument that CLEC18 is the co-receptor of TLR3.

Reviewer #3 (Remarks to the Author):

Overall, their findings that human CLEC18A and CLEC18A-1 act as a co-receptor with TLR3 is particularly interesting in view of differential roles of them between human and mouse, in TLR3-mediated antiviral responses. However, data presented in this manuscript is not sufficient to support their conclusion. Furthermore, there seems to be over-interpretation throughout their manuscript. To strengthen their conclusion, many concerns listed below should be addressed.

Specific comments

1. Figure S1A, S2B and S2C should be explained in their manuscript.

Answer: *We follow the suggestion, and add explanation for what we observed in this revised manuscript. The S1A, S2B and S2C in previous version are moved to Fig. 2A, S2A, S2B, respectively, in this revised version. Description of these findings is shown in section 2 (line 256-266).*

2. In figure 2A, data showing IP-10 production in response to poly(I:C) (1 microG/mL) is missing.

Answer: *We present the IP-10 production in Figure 2A*

3. In figure 2A and S1A, there seems to be some discrepancy, especially data on pro-inflammatory cytokines, they should kindly explain about that. Furthermore, they should place ELISA data but not qPCR data onto the main figure.

Answer:

1) *We follow the suggestion and present the new ELISA results on the main figure (Figure 2a in this revised version).*

2) *We describe the results in in section 2 (line 256-266) of this revised version.*

4. Figure 2a did not correspond to supplementary fig. 1a. While mRNA level of TNF-alpha showed no significant difference between WT and CLEC18 expressing cells, the protein level was increased in CLEC18 expressing cells.

Answer:

We present the new ELISA results in Figure 2a and supplementary Figure 2. No significant change in TNF- α production between WT and CLEC18 mice at protein (determined by ELISA) and RNA (determined by RT-PCR) levels.

5. They should describe about how they prepared human macrophages in [materials and methods] section.

Answer:

The description of human macrophage preparation is shown in line 96-100 of this revised version.

6. In figure 4D, to support their conclusion, they should perform statistical analysis.

Answer:

We add the symbols to represent statistical significance in the figure 4D.

7. To strengthen their conclusion, they should perform knockdown experiment using human cells.

Answer:

We perform this experiment and show the data in Supplementary figure 3. We found that knockdown of CLEC18 in human macrophages only affects poly (I:C)-, but not LPS-, induced cytokine production. This observation further supports the argument that CLEC18 is the co-receptor of TLR3.

8. In figure 6 and 7, they should show data using control (untreated) mice.

Answer:

We show the results of control mice in Figure 6 & 7.

9. In figure 1A, because the expression level of mRNA does not always reflect that of protein, they should show CLEC18 protein expression in each tissues and cells (especially immune cells). Same for figure 1B.

Answer:

We analyze the expression of endogenous CLEC18 in mice tissue and immune cells by WB (Figure 1 b&c) and flow cytometry (Supplementary Figure 1). Mouse endogenous CLEC18A is only expressed in brain, but is undetectable in heart and kidney based on western blot analysis (Fig. 1b), though low levels of mRNA are detectable in mouse kidney and heart (Fig. 1a).

We also try to detect endogenous CLEC18 by flow cytometry, but it is not detectable in T cell, B cells, neutrophils, or other myeloid cells (Supplementary Figure 1). This result is in accord with what we observed in Figure 1a&b.

3. Because their previous paper (ref. 1) also suggests that CLEC18 is secreted by cells, they should clearly refer it in their manuscript.

Answer:

We address this issue and discuss the potential role of soluble CLEC18 in the Discussion Session (line 422-431) in this revised version.

4. In figure 1C, they should clearly describe about the antibody they used in figure legend. Furthermore, in case that they used antibody against endogenous human CLEC18 protein but not against tags, they should clearly describe about the information of the antibody used.

Answer:

Description of the antibodies used in Figure 1C is shown in line 128-143 of this revised version. Because the commercially available antibody (Proteintech Catalog number: 21013-1-AP) is

unable to detect flag-tagged mouse CLEC18 (as we show in Fig. 1B) and endogenous CLEC18, we screen human phage library to identify phage clone which can recognize recombinant mouse CLEC18A. We are negotiating with Biolegend and Minipore to license this mAb now.

12. There are many typos and grammatical errors throughout the manuscript that should be corrected (e.g. [purchased] at line 101, [C57BL/J] at line 118 and [analysis] at line 133 should be [were purchased], [C57BL/6] and [analysis of], respectively.).

Answer:

We correct those typos and grammatical errors accordingly.

13. At line 133 and 134, they should not use the abbreviation [AEC] and [ATII] without prior explanation.

Answer:

We change those labeling accordingly.

14. Although they claim that CLEC18A-1 is more potent to induce interferon response, this reviewer could not find any statistical analysis between CLEC18A and CLEC18A-1.

Answer:

- 1) *We add the symbols to represent statistical significance in the figures in this revised version.*
- 2) *We rename CLEC18A-1 as CLEC18A(S339R) in this revised version.*

15. In figure 2A and 2B, they claim that CLEC18A and CLEC18A-1 increased type I interferon expression when even 1.1-1.2 fold. On the other hand, they claim that, in figure S1B, S1C and S1D, CLEC18 did not affect to ifna or ifnb mRNA expression even 2-4 fold. This is quite strange. They should perform statistical analysis in figure S1 and precisely interpret their data.

Answer:

We repeat the experiment and present the data with statistical analysis in Figure 2a and Supplementary Figure 2. Compared to WT BMDMs, higher expression of type I interferons was in CLEC18A and CLEC18A(S339R) cells after poly (I:C) stimulation. In contrast, other TLR ligand has no obvious effect to upregulate cytokine production.

16. In figure 4C, statistical analysis seems to be strange. The difference in ifnb between WT (pIC 0) and WT (pIC 30) was significant, but that between WT (pIC 0) and WT (pIC 10) was not significant. If the figure is correct, latter also seems to be significant. They should confirm the method of statistical analysis throughout their manuscript.

Answer:

I think the reviewer may misunderstand what we present in Figure 4c (changed to Figure 4e in this revised version). In this experiment, all groups were incubated with the same concentration of the poly (I:C), but with the different concentration of TLR3/dsRNA complex inhibitor as indicated. We re-analyze data by one-way ANOVA method.

17. In figure 5D, the means of [+] and [-] is not clear.

Answer:

We describe the means of [+] in legend (line 584-585) in this revised version.

18. In figure 5D, it is interesting to investigate which domain of CLEC18 is involved in its binding to poly (I:C).

Answer:

In Figure 5D, we want to identify CLEC18 domain responsible for association with TLR3. Based on our BLI binding study, CLEC18 interacts with poly (I:C) via CTLD domain (Table II).

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I commend the authors in their efforts to address all of the reviewers comments and perform additional experiments.

I am satisfied that authors have answered the concerns of all three reviewers.

I would suggest minor amendments:

In supplemental figure 3 for the knockdown of CLEC18A by two different shRNA can authors please include data demonstrating sufficient knockdown.

In the relevant text and discussion regarding comments on the induction of phospho-TBK1 and phospho-IRF3 between CLEC18A and CLEC18A(S339R) expressing BMDM and WTs(Figure 4g). It is clear that the induction of p-TBK1 and p-IRF3 is enhanced in CLEC18A(S339R) compared to WT cells. However this does not appear as clear for the results presented for CLEC18A compared to WT. This simply adds to the overall argument in the discussion that the ROSA-CLEC18A(S339R) allele may be more beneficial following viral infection than CLEC18A and therefore warrants further investigation. I think the overall text should reflect this. Or do the others have other blots for CLEC18A and pTBK1/p-IRF3? n numbers are not indicated in the figure legend for these experiments.

The manuscript reads well but requires additional proof reading for grammar and typos prior to publication. For example lines [36-37], [234-239], [256-266], [96 - 100].

Reviewer #2 (Remarks to the Author):

In this manuscript, Huang et al, mentioned mainly two points. The one is human CLEC18A support TLR3 function to enhance inflammatory response, and the other is hCLEC18A mutation at S339R further enhance inflammatory response by regulating TLR3-poly IC binding. Author greatly improved in the revised manuscript, however there are several discrepancies.

To investigate hCLEC18A function in TLR3 dependent pathway, author showed ectopic human CLEC18A expression in mouse cells causes TLR3 response, human genes expression in mouse cells is unlikely to be occurred in natural condition. Although author newly included knockdown experiment by using human cells, author should show the role of CLEC18A and CLEC1A(S339R) function in human cells. For example, Author should express CLEC1A(S339R) in knock down cells and investigate inflammatory and ifn genes expression and poly IC binding.

Author showed the role of mutation in S339R, however several Type I IFN and inflammatory genes expression are comparable between 18A and 18A(S339R) in Figure 2 and 3, and hCLEC 18A(S339R) mice only showed protection from IAV infection in Figure 7, and hCLEC 18A(S339R) mice showed enhanced inflammation in Figure 6. There are several discrepancy in the role of S339R in those figures.

Reviewer #3 (Remarks to the Author):

The authors adequately addressed all the concerns raised by this reviewer. I think the revised manuscript is suitable for the publication.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

1. In supplemental figure 3 for the knockdown of CLEC18A by two different shRNA can authors please include data demonstrating sufficient knockdown.

Answer:

The knock-down efficiency of two siRNAs is shown in the supplementary figure 3(a) of this revised manuscript, and the legend is highlighted in yellow.

2. In the relevant text and discussion regarding comments on the induction of phospho-TBK1 and phosphor-IRF3 between CLEC18A and CLEC18A(S339R) expressing BMDM and WTs(Figure 4g). It is clear that the induction of p-TBK1 and p-IRF3 is enhanced in CLEC18A(S339R) compared to WT cells. However this does not appear as clear for the results presented for CLEC18A compared to WT. This simply adds to the overall argument in the discussion that the ROSA-CLEC18A(S339R) allele may be more beneficial following viral infection than CLEC18A and therefore warrants further investigation. I think the overall text should reflect this. Or do the others have other blots for CLEC18A and pTBK1/p-IRF3? n numbers are not indicated in the figure legend for these experiments.

Answer:

- 1) *We agree the comment and address the beneficial role of CLEC18A(S339R) vs CLEC18A in Discussion session (line 415-429) of this revised manuscript.*
 - 2) *We add the 'n' numbers in line 603 of this revised manuscript.*
3. The manuscript reads well but requires additional proof reading for grammar and typos prior to publication. For example lines [36-37], [96 - 100], [234-239], [256-266],

Answer:

We revised these sentences and corrected the typos in this revised manuscript. All the newly added sentences are highlighted in yellow. lines [36-37], [93-100], [244-250], [265-274].

Reviewer #2 (Remarks to the Author):

In this manuscript, Huang et al, mentioned mainly two points. The one is human CLEC18A support TLR3 function to enhance inflammatory response, and the other is hCLEC18A mutation at S339R further enhance inflammatory response by regulating TLR3-poly IC binding. Author greatly improved in the revised manuscript, however there are several discrepancies.

1. To investigate hCLEC18A function in TLR3 dependent pathway, author showed ectopic human CLEC18A expression in mouse cells causes TLR3 response, human genes expression in mouse cells is unlikely to be occurred in natural condition. Although author newly included knockdown experiment by using human cells, author should show the role of CLEC18A and CLEC18A(S339R) function in human cells. For example, Author should express CLEC18A(S339R) in knock down cells and investigate inflammatory and ifn genes expression and poly IC binding.

Answer:

We perform this experiment accordingly, and the results are shown in supplementary figure 4. Because primary macrophages become fragile after siRNA transfection, cells died after following plasmid transfection to express human CLEC18A and CLEC18A(S339R). Thus, we transect siRNA to human HT1080 cells to knockdown endogenous CLEC18, followed by transfecting plasmids to overexpress CLEC18A and CLEC18A(S339R). The description of this experiment is shown in the materials and methods (line 122-133), and text (line 278-284) (highlighted in yellow).

2. Author showed the role of mutation in S339R, however several Type I IFN and inflammatory genes expression are comparable between 18A and 18A(S339R) in Figure 2 and 3, and hCLEC18A(S339R) mice only showed protection from IAV infection in Figure 7, and hCLEC18A(S339R) mice showed enhanced inflammation in Figure 6. There are several discrepancy in the role of S339R in those figures.

Answer:

We summarize the differential responses between CLEC18A and hCLEC18A(S339R) allele and discuss the potential role of CLEC18A(S339R) allele in host defense against various microbial infections in the DISCUSSION session of this revised manuscript (line 415-429).

a) The results of Figure 2 and 3 are from in vitro studies.

We did find differential response of hCLEC18A and hCLEC18A(S339R) BMDMs (Fig. 2) and epithelial cells (Fig. 3) to viral infections in vitro, and compared with cytokine production and mice survival rates in vivo (Fig. 7). We found CLEC18A(S339R) is more potent than CLEC18A to respond to viral infections and poly. (I:C) stimulation.

b) Figure 6 is based on the stimulation of poly (I:C) in vivo.

Because CLEC18A and hCLEC18A(S339R) enhance host response to poly (I:C), so ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice display more severe inflammatory reactions than WT mice in tissue section (Fig. 6a). These observations are consistent with our observations that CLEC18A and CLEC18A(S339R) enhance cytokine productions to poly (I:C) in vitro.

c) Figure 7 is based on IAV infection in vivo.

These results are in accord with our claim that hCLEC18A and hCLEC18A(S339R) enhance the expression of interferon and ISG to suppress virus infection. Therefore, less cell infiltration and lower virus titer are found in ROSA-CLEC18A and ROSA-CLEC18A(S339R) mice.

Reviewer #3 (Remarks to the Author):

The authors adequately addressed all the concerns raised by this reviewer. I think the revised manuscript is suitable for the publication.

Answer: Thank you very your nice comment

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addresses all of this reviewers concerns. The manuscript has also been corrected for typos and the discussion improved as requested.

I believe the manuscript is now suitable for publication

Reviewer #2 (Remarks to the Author):

Authors address the concern. I think the revised manuscript is suitable for the publication.