Vol 453|29 May 2008|doi:10.1038/nature07013

LETTERS

CLEC5A is critical for dengue-virus-induced lethal disease

Szu-Ting Chen[1], Yi-Ling Lin[2,3], Ming-Ting Huang[1], Ming-Fang Wu[1], Shih-Chin Cheng[1], Huan-Yao Lei[4], Chien-Kuo Lee[5], Tzyy-Wen Chiou[6], Chi-Huey Wong[3] & Shie-Liang Hsieh[1,3,7]

Dengue haemorrhagic fever and dengue shock syndrome, the most severe responses to dengue virus (DV) infection, are characterized by plasma leakage (due to increased vascular permeability) and low platelet counts[1,2]. CLEC5A (C-type lectin domain family 5, member A; also known as myeloid DAP12-associating lectin (MDL-1))[3] contains a C-type lectin-like fold similar to the natural-killer T-cell C-type lectin domains and associates with a 12-kDa DNAX-activating protein (DAP12)[4] on myeloid cells. Here we show that CLEC5A interacts with the dengue virion directly and thereby brings about DAP12 phosphorylation. The CLEC5A–DV interaction does not result in viral entry but stimulates the release of proinflammatory cytokines. Blockade of CLEC5A–DV interaction suppresses the secretion of proinflammatory cytokines without affecting the release of interferon-α, supporting the notion that CLEC5A acts as a signalling receptor for proinflammatory cytokine release. Moreover, anti-CLEC5A monoclonal antibodies inhibit DV-induced plasma leakage, as well as subcutaneous and vital-organ haemorrhaging, and reduce the mortality of DV infection by about 50% in STAT1-deficient mice. Our observation that blockade of CLEC5A-mediated signalling attenuates the production of proinflammatory cytokines by macrophages infected with DV (either alone or complexed with an enhancing antibody) offers a promising strategy for alleviating tissue damage and increasing the survival of patients suffering from dengue haemorrhagic fever and dengue shock syndrome, and possibly even other virus-induced inflammatory diseases.

Dengue is a mosquito-borne infection caused by four serotypes of dengue virus (DV1–DV4) and is currently the most common arboviral disease worldwide[1,2]. Primary infection with any of the four DV serotypes typically results in mild dengue fever (DF) and provides lifelong immunity to the infecting strain. However, secondary infection with different DV serotypes is associated with an increased risk of developing dengue haemorrhagic fever (characterized by thrombocytopenia and capillary leakage) and can progress to life-threatening hypovolaemic dengue shock syndrome[1]. The pathogenesis of dengue haemorrhagic fever and dengue shock syndrome remains unclear, but massive cytokine secretion (cytokine storm) is believed to be one of the major contributory factors[5]. Indeed, dengue is a major public health problem, with about 50 million people infected each year (of whom about 20,000 die) and about 2.5 billion people worldwide being at risk of infection. Unfortunately, no DV-specific therapies or vaccines are available[1,2].

Dendritic cells (DCs) and macrophages are the primary targets of DV infections[6–8]. Whereas infected DCs undergo apoptosis (despite the secretion of proinflammatory cytokines by bystander DCs)[9], infected macrophages survive for at least 45 days and secrete multiple cytokines and chemokines from 6 h after infection[10]. This result suggests that macrophages are the major source of proinflammatory cytokines after infection with DV, in which virions might trigger inflammatory reactions by activating pattern recognition receptors. Toll-like receptors (TLRs), C-type lectins and immunoglobulin-like (Ig-like) receptors (for example, TREMs (triggering receptors expressed on myeloid cells) and TREM-like transcript (TLT)) have been implicated as potential pattern recognition receptors[11–13]. To determine whether DV binds to and activates candidate pattern recognition receptors on immune cells, we expressed 22 fusion proteins in mammalian cells and screened for their interaction with DV2 (Supplementary Table 1). Among the receptors tested, the C-type lectin receptor DC-SIGN (dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin; also known as CLEC4L) has previously been shown to interact with glycans located on the envelope (E) protein of DV[14]. Using an enzyme-linked immunosorbent assay (ELISA) we showed that CLEC5A.Fc (in addition to DC-SIGN.Fc and DC-SIGNR.Fc) was able to capture DV2 (Fig. 1a). To confirm the specificity of the interaction between CLEC5A and DV, complexes were immunoprecipitated with Protein A–Sepharose beads and then probed with an anti-DV envelope (anti-E) monoclonal antibody (mAb). E protein was detected in the immunoprecipitates of DC-SIGN.Fc and CLEC5A.Fc, confirming that CLEC5A interacts with the dengue virion (Fig. 1b). However, whereas the binding of DC-SIGN to DV is Ca^{2+} dependent, EDTA (a Ca^{2+} chelator) had no effect on the CLEC5A–DV interaction (Fig. 1c). The Ca^{2+}-independent binding feature is in accord with that of β-glucan receptor dectin 1, which also contains a similar C-type lectin-like domain and binds zymosan independently of metal ions[15]. Furthermore, transfection of 293T cells with DC-SIGN and CLEC5A resulted in increased binding of biotinylated DV to the cells (Fig. 1d). There are two conserved N-linked glycosylation sites at Asn 67 and Asn 153 of E protein[14], and these N-glycans have been implicated in cellular attachment and viral entry[16]. To investigate the participation of glycans in the association of CLEC5A with DV, virions were incubated with fucose, mannose or mannan (the latter two sugars are ligands for DC-SIGN[17]). As expected, mannose and mannan caused dose-dependent inhibition of the interaction between DC-SIGN and DV (Fig. 1e). The binding of CLEC5A to DV was significantly reduced in the presence of fucose ($P < 0.0001$) and, to a smaller extent, mannan ($P = 0.0005$) (Fig. 1e), indicating that fucose might be more important than mannose in the CLEC5A–DV interaction.

DC-SIGN, which is expressed on DCs and macrophages (Supplementary Fig. 1a), contains three motifs in its cytoplasmic tail that are believed to be involved in endocytosis or intracellular

[1]Department and Institute of Microbiology and Immunology, National Yang-Ming University, Taipei 112, Taiwan. [2]Institute of Biomedical Sciences, and [3]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan. [4]Department of Microbiology and Immunology, National Cheng Kung University, Tainan 701, Taiwan. [5]Institute of Immunology, National Taiwan University, Taipei 100, Taiwan. [6]Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien 974, Taiwan. [7]Immunology Research Center, National Yang-Ming University & Taipei Veterans General Hospital, Taipei 112, Taiwan.

trafficking[18]. In contrast, CLEC5A was originally identified as a DAP12-associated molecule expressed exclusively on monocytes and macrophages (Supplementary Fig. 1b), although its ligand(s) and biological functions remain to be determined[3]. Infection of macrophages with DV was found to induce DAP12 phosphorylation in a dose-dependent manner (Fig. 2a). DAP12 phosphorylation peaked at 12 h after infection and persisted for at least 48 h in the presence of live DV, whereas ultraviolet-inactivated DV (UV-DV) triggered only limited DAP12 phosphorylation that lasted for 12 h (Fig. 2b). This indicates that DAP12 phosphorylation is independent of DV replication during the first 2–6 h of infection. Knockdown of CLEC5A (using the short hairpin RNA (shRNA) pLL3.7/CLEC5A), but not that of DC-SIGN (by pLL3.7/DC-SIGN), caused a substantial decrease in DAP12 phosphorylation (Fig. 2c), suggesting that DV-triggered DAP12 phosphorylation is mediated through CLEC5A. It is known that DC-SIGN participates in the infection of DCs by DV[19].

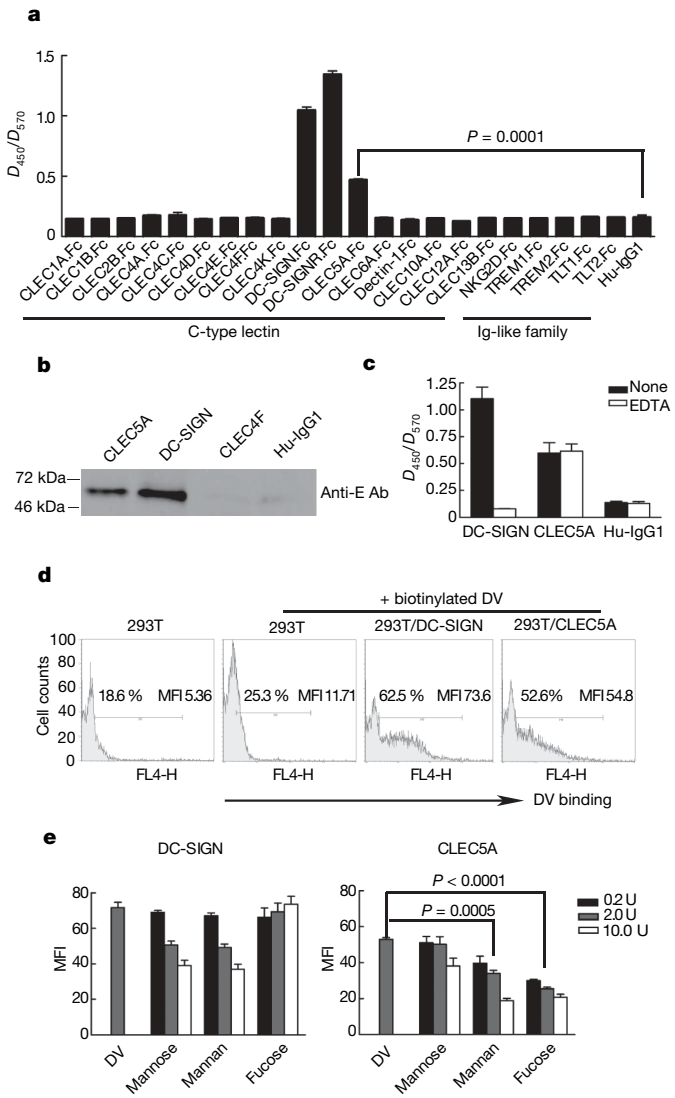

Figure 1 | DV interacts with CLEC5A. **a**, Interaction of DV with receptor.Fc was determined by ELISA. **b**, DV–receptor.Fc complexes were immunoprecipitated and detected by western blotting. **c**, Inhibition of CLEC5A–DV interaction by EDTA was determined by ELISA. **d**, Both DC-SIGN and CLEC5A increase DV binding to 293T cells. **e**, Addition of sugars inhibits binding of biotinylated DV to DC-SIGN-transfected or CLEC5A-transfected 293T cells. MFI, mean fluorescence intensity. Units (U) for monosaccharide (mannose and fucose) and polysaccharide (mannan) are mM and mg ml^{-1}, respectively. Data are expressed as means and s.d. for three independent experiments. Two-tailed Student's *t*-tests were performed.

We therefore tested the hypothesis that CLEC5A is involved in the entry of DV into macrophages by monitoring the expression of DV nonstructural protein 3 (NS3), which is expressed when DV replicates in macrophages. In contrast to that of DC-SIGN, knockdown of CLEC5A by shRNA (Fig. 2d) or blocking of the CLEC5A–DV interaction with anti-CLEC5A antibody (Fig. 2e) did not inhibit NS3 expression in macrophages as examined by flow cytometry and confocal microscopy, respectively. The shRNA pLL3.7/CLEC5A also failed to suppress the release of dengue virions into the supernatant of infected macrophages, as determined by a plaque-forming assay (data not shown). These results indicate that, whereas DC-SIGN mediates DV infection and replication, the interaction of DV with CLEC5A triggers cell signalling.

To determine whether CLEC5A is involved in DV-induced inflammation, we examined the secretion of inflammatory cytokines by macrophages after infection with DV. At 6 h after infection, we detected dose-dependent secretion of tumour necrosis factor (TNF)-α; similar levels of cytokine were secreted by macrophages infected with either DV or UV-DV (Fig. 3a, left panel). However,

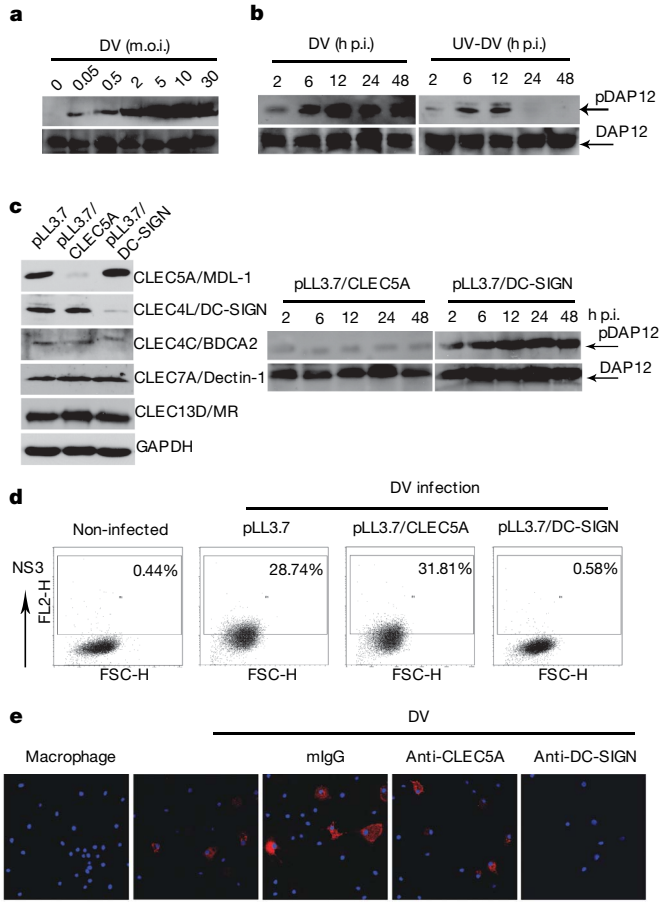

Figure 2 | CLEC5A is essential for DV-induced DAP12 phosphorylation, but not for DV replication. **a**, DV-induced DAP12 phosphorylation (2 h after infection) in human macrophages was determined by western blotting. m.o.i., multiplicity of infection. **b**, Kinetics of DAP12 phosphorylation induced by DV and ultraviolet-inactivated DV (UV-DV) was determined by western blotting. p.i., after infection. **c**, **d**, Effects of shRNAs on protein expression and inhibition of DV-mediated DAP12 phosphorylation (**c**) and DV entry and replication in macrophages (**d**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **e**, The effects of antagonistic mAbs in inhibiting NS3 expression (red) in DV-infected macrophage at 48 h after infection. Cells were counterstained with Hoechst 33342 (blue) and were examined with a confocal microscope (m.o.i. = 5). The second panel shows the cells (macrophages) infected with dengue virus (DV) only, without any other treatment.

at 12 h after infection, TNF-α secretion was further increased by DV but not by UV-DV (Fig. 3a, right panel). Over a 48-h time course, TNF-α secretion continually increased for macrophages infected with DV, whereas at 24–48 h after infection with UV-DV this cytokine was barely detectable (Fig. 3b). These data correlate with the kinetics of DAP12 phosphorylation (Fig. 2b), indicating that DV-mediated secretion of TNF-α is related to DAP12 activation. We also observed that knockdown of CLEC5A suppressed the release of TNF-α, interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1α and interferon-inducible protein (IP)-10 by DV-infected macrophages to a much greater extent than knockdown of DC-SIGN (Fig. 3c and Supplementary Fig. 2). However, whereas pLL3.7/DC-SIGN mildly suppressed interferon (IFN)-α secretion ($P = 0.048$), pLL3.7/CLEC5A did not affect this cytokine (Fig. 3c). To further dissect the DV-activated signalling pathways leading to cytokine secretion, macrophages were transfected with shRNAs to knock down CLEC5A, DC-SIGN, TLR4, TLR7 or MyD88, before DV infection. The data indicate that DV-induced IFN-α secretion occurs through the TLR7–MyD88 pathway ($P = 0.0016$), whereas TNF-α secretion is mediated through both CLEC5A ($P = 0.003$) and TLR7–MyD88 ($P = 0.013$) (Fig. 3d). We generated a panel of anti-CLEC5A mAbs with differential antagonistic effects on the four serotypes of DV (DV1–DV4; Supplementary Table 2), which were determined by inhibition of TNF-α secretion from DV-infected macrophages (Fig. 3e). Although different epitopes of CLEC5A may mediate their individual interactions, all antibodies that inhibit the CLEC5A–DV interaction suppress the inflammatory response by macrophages infected with relevant DV serotype(s). The differential antagonistic effects of anti-CLEC5A mAbs might be related to heterogeneous glycosylation among DV serotypes[20]. Additionally, an anti-CLEC5A mAb may inhibit the binding of a specific DV serotype whose binding site overlaps with that of anti-CLEC5A mAb.

It has previously been shown that non-neutralizing anti-DV Abs promote DV entry into target cells by means of FcR receptors and thereby enhance cytokine release[6,21], a phenomenon termed antibody-dependent enhancement (ADE) of infection. For example, anti-prM and anti-E mAbs have been shown to induce this effect *in vitro*[22]. Here we investigated whether blockade of the CLEC5A–DV interaction can inhibit ADE. Primary human macrophages were infected with DV alone or with anti-prM–DV or anti-E–DV immunocomplexes, in the presence of anti-CLEC5A mAb (or isotype control) for 36 h. Anti-prM–DV and anti-E–DV immunocomplexes (ADE)

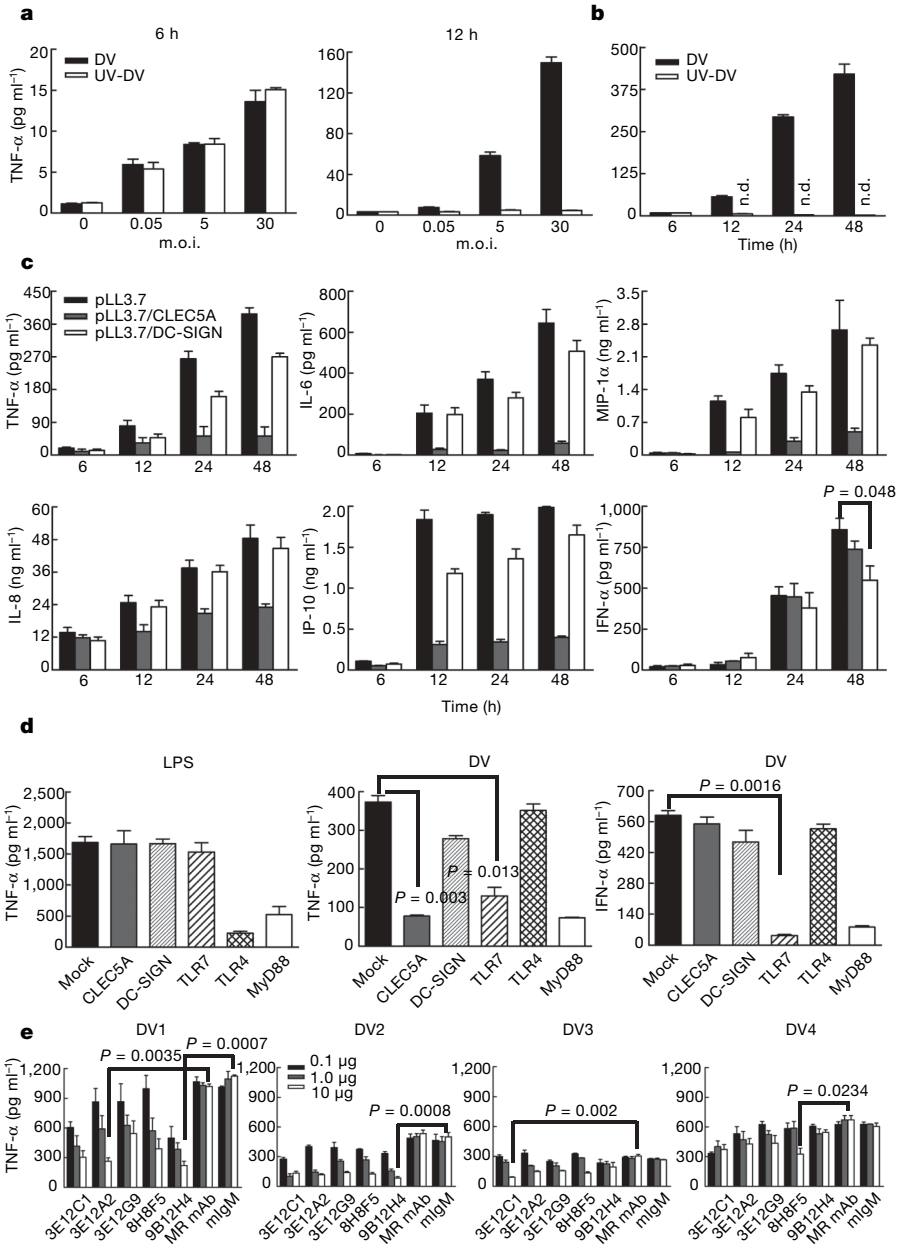

Figure 3 | CLEC5A is critical for DV-mediated secretion of TNF-α but not that of IFN-α. **a**, The dose dependence of DV-induced and UV-DV-induced TNF-α secretion by macrophages was measured by ELISA at 6 and 12 h after infection. **b**, Kinetics of TNF-α expression after DV infection. n.d., not detectable. **c**, Effects of CLEC5A and DC-SIGN shRNAs on the secretion of cytokines from DV-infected macrophages. **d**, Knockdown experiments with specific shRNAs show that DV-induced IFN-α secretion is via the TLR7–MyD88 pathway. **e**, TNF-α secretion in response to DV serotypes 1–4 is inhibited by antagonistic anti-CLEC5A mAbs (Supplementary Table 2), M.R. (mannose receptor); mIgM (murine IgM isotype control). (m.o.i. = 5). Data are expressed as means and s.d. for three independent experiments.

increased the expression of NS3 (Supplementary Fig. 3a) and the levels of TNF-α and IFN-α secretion in comparison with DV alone (Supplementary Fig. 3b, c). However, whereas anti-CLEC5A mAb significantly inhibited TNF-α release from macrophages infected with DV, or from macrophages incubated with anti-prM–DV or anti-E–DV immunocomplexes (Supplementary Fig. 3c), the secretion of IFN-α was not affected, suggesting that ADE-mediated IFN-α secretion is independent of CLEC5A. This result supports the notion that CLEC5A acts in a signalling capacity but does not alter relative infection levels.

We found that inhibition of the CLEC5A–DV interaction attenuated DV-induced permeability changes (Supplementary Fig. 4) *in vitro*. We therefore further investigated whether blockade of the CLEC5A–DV interaction can rescue mice from DV-induced lethality *in vivo*. We found that murine CLEC5A (mCLEC5A) binds to DV with an affinity similar to that of human CLEC5A (Supplementary Fig. 5a) and is expressed on myeloid lineages ($CD11b^+$, $F4/80^+$), bone marrow-derived macrophages and murine macrophage-like Raw264.7 cells (Supplementary Fig. 5b, c). Furthermore, DV replicated more efficiently and stimulated greater TNF-α secretion in bone marrow-derived macrophages derived from STAT1-deficient ($STAT1^{-/-}$)[23] mice than in wild-type mice (Supplementary Fig. 6a, b), and blockade of mCLEC5A–DV interaction by antagonistic mAbs (Supplementary Table 3) abolished DV-induced TNF-α secretion by bone marrow-derived macrophages in a dose-dependent manner (Supplementary Fig. 6c). This indicates that the function of mCLEC5A is similar to that of human CLEC5A.

IFN-α functions to inhibit viral replication in both infected and uninfected cells, and IFN-mediated responses to DV infection involve both the STAT1-dependent (essential in the control of viral replication) and STAT1-independent (essential for the resolution of infection) pathways[24]. Although wild-type mice were resistant to infection with DV, $STAT1^{-/-}$ mice were sensitive to lethality induced by DV2 (strain New Guinea C-N; Supplementary Fig. 7). We tested the potential therapeutic effects of the antagonistic mAbs on $STAT1^{-/-}$ mice. DV-challenged $STAT1^{-/-}$ mice showed ruffled fur and mild paralysis in addition to subcutaneous and intestinal haemorrhaging at 8 days after infection (Fig. 4a), and they all died within 7–14 days of infection (Fig. 4e). Five doses of antibodies (100 μg per mouse, intraperitoneally) or TNFR2.Fc (100 μg per mouse, intraperitoneally) were administered on days 0, 1, 3, 5 and 7 after infection. At 9 days after infection, leakage of Evans blue into the kidney, liver, stomach, small intestine, large intestine and spleen of DV-challenged mice was significantly decreased in mice treated with anti-mCLEC5A mAbs in comparison with controls (Fig. 4b, c). Anti-mCLEC5A mAbs also effectively lowered the serum levels of TNF-α and IP-10 (Fig. 4d, top and middle panels), without suppressing viral replication, at day 7 after infection (Fig. 4d, bottom panel), and protected mice from lethality at day 14 after infection (70% protection rate). The overall survival rate of anti-mCLEC5A-treated mice was 48% at day 21 after infection (Fig. 4e), with DV being cleared from serum of the surviving mice at day 23 after infection (data not shown). Thus, blocking the CLEC5A–DV interaction seems to prevent the DV-associated complications of haemorrhaging and plasma leakage. It also suppresses the macrophage inflammatory response without impairing virus clearance by the adaptive immune response. In contrast, TNFR2.Fc neither reduced

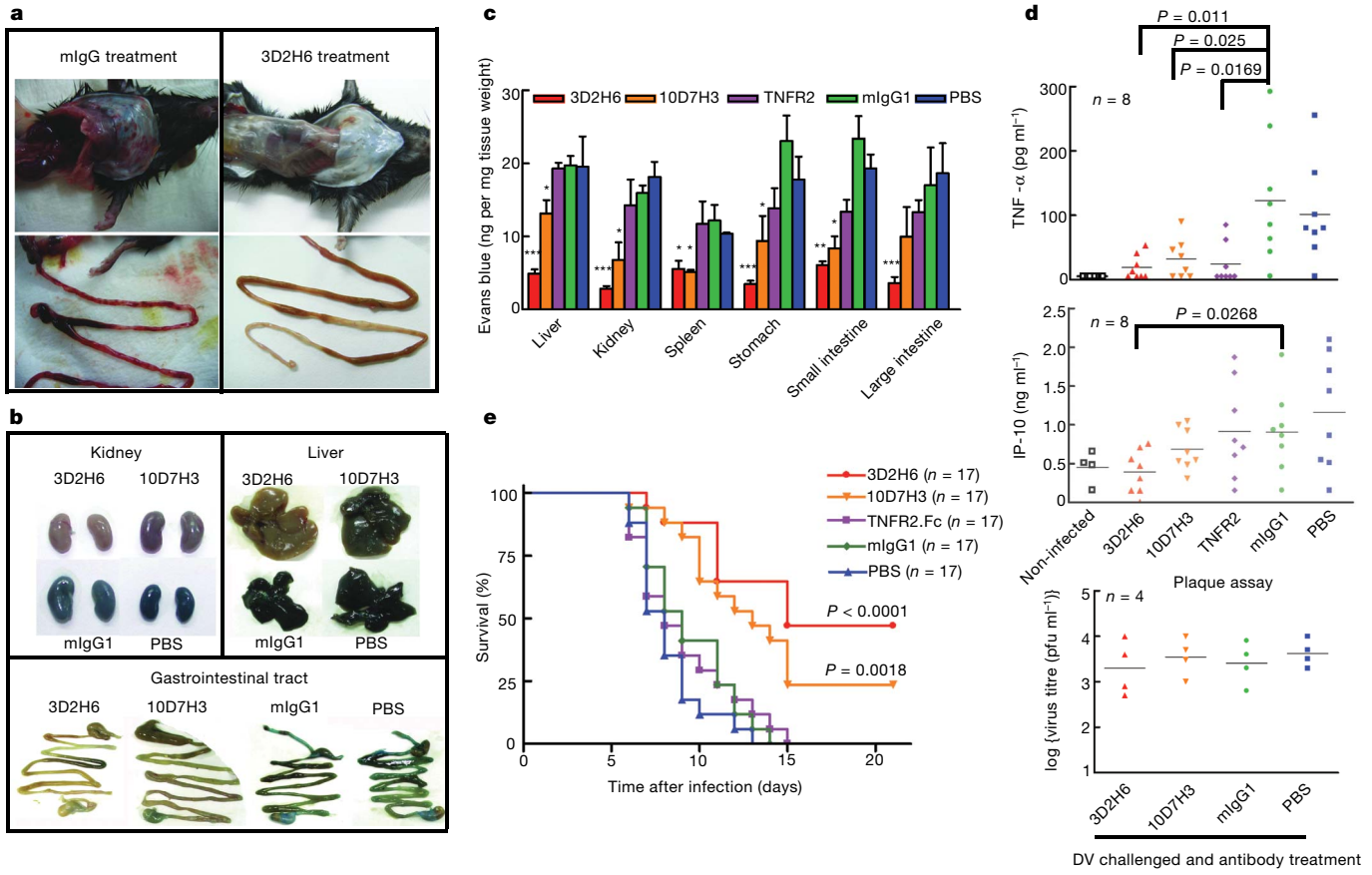

Figure 4 | Anti-mCLEC5A mAbs prevents DV-induced vascular leakage and lethality in STAT1-deficient mice. **a**, **b**, Anti-murine CLEC5A mAb inhibits subcutaneous and intestinal haemorrhaging (**a**) and plasma leakage (**b**) of DV-challenged mice. **c**, Quantification of Evans blue extracted from organs. Data are expressed as means and s.d. for three independent experiments. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$ (Student's *t*-test). **d**, Serum levels of cytokines and virus titres at day 7 after infection (two-tailed Student's *t*-tests). p.f.u., plaque-forming units. **e**, Survival curve of mice challenged with DV2 (New Guinea C-N). Data were collected from four independent experiments and are shown as Kaplan–Meier survival curves with log rank test.

vascular permeability (Fig. 4c) nor protected mice from lethality (Fig. 4e), despite effectively lowering the level of TNF-α in serum (Fig. 4d).

We have thus demonstrated that CLEC5A is an important pattern recognition receptor in the context of dengue virus infection. It is clear that although DV-triggered cytokine release from macrophages involves both CLEC5A and TLR7 pathways, only the blockade of CLEC5A–DV interactions can attenuate inflammation and maintain host immunity so as to clear virus. Even though the direct relevance of this *STAT1*$^{-/-}$ mouse model in relation to human infection with DV remains unclear, our *in vivo* protection data of CLEC5A mAbs still shed light on its therapeutic potential for blocking the progression of severe dengue disease in patients with dengue haemorrhagic fever or dengue shock syndrome. It will be interesting to test whether this strategy can be applied to the identification of receptors interacting with other pathogenic viruses, and to the treatment of the severe inflammatory consequences of infection.

METHODS SUMMARY

Reagents. Human MyD88, TLR4 and TLR7 shRNA expression vectors were from InvivoGen. The DC-SIGN shRNA expression vector was provided by P. Vincet[25] and TNFR2.Fc (Enbrel; Wyeth) was provided by H.-Y. Lin. Anti-CLEC4L/DC-SIGN mAb (clone 120507), anti-CLEC4C/BDCA2 and anti-CLEC7A/dectin-1 polyclonal antibody were purchased from R&D Systems. Anti-human CD206 (mannose receptor; clone 15-2; IgG1) was purchased from BioLegend.

Virus stock. Four DV serotypes, namely DV1/Hawaii, DV2/PL046, DV3/H-087 and DV4/866146A (ref. 26), were used for *in vitro* studies. For animal studies, mouse-adapted neurovirulent DV2 (strain New Guinea C-N) was provided by C. J. Lai. Virus propagation was performed in C6/36 cells, and virus titres were determined by plaque-forming assays with BHK-21 cells. Unless otherwise specified, DV2/PL046 was used throughout the study.

Transfection of macrophages. Macrophages were transfected by electroporation with the Amaxa Human Monocyte Neucleofector kit (Amaxa Inc.) in accordance with the manufacturer's instructions. In brief, macrophages (6×10^6) were harvested and resuspended in 100 μl of nucleofector solution. After addition of appropriate shRNA expression vector or control vector (5 μg), cells were electroporated with Amaxa program Y-001 and recovered for 16 h before infection with DV. The transfection efficiency was determined by fluorescence microscopy, and the transfected (enhanced green fluorescent protein-positive) cells were sorted by FACSAria (Becton Dickinson) before cytokine assay.

Permeability assay. Vascular leakage was examined by intravascular administration of Evans blue into mice, as described previously[27]. Uptake of Evans blue by organs was quantified by extracting with formamide and measuring the attenuance at 610 nm (D_{610}).

Inoculation of virus. Mice were challenged intraperitoneally with 10^5 plaque-forming units of DV2 (New Guinea C-N) in 300 μl of PBS and simultaneously injected intracranially (i.c.) with 30 μl of PBS into the right hemisphere of mouse brains.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 29 February; accepted 18 April 2008.
Published online 21 May 2008.

1. Wilder-Smith, A. & Schwartz, E. Dengue in travelers. *N. Engl. J. Med.* **353,** 924–932 (2005).
2. Mackenzie, J. S., Gubler, D. J. & Petersen, L. R. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nature Med.* **10,** S98–S109 (2004).
3. Bakker, A. B., Baker, E., Sutherland, G. R., Phillips, J. H. & Lanier, L. L. Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. *Proc. Natl Acad. Sci. USA* **96,** 9792–9796 (1999).
4. Lanier, L. L., Corliss, B. C., Wu, J., Leong, C. & Phillips, J. H. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* **391,** 703–707 (1998).
5. Pang, T., Cardosa, M. J. & Guzman, M. G. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever–dengue shock syndrome (DHF/DSS). *Immunol. Cell Biol.* **85,** 43–45 (2007).
6. Halstead, S. B. & O'Rourke, E. J. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* **146,** 201–217 (1977).
7. Palucka, A. K. Dengue virus and dendritic cells. *Nature Med.* **6,** 748–749 (2000).
8. Wu, S. J. *et al.* Human skin Langerhans cells are targets of dengue virus infection. *Nature Med.* **6,** 816–820 (2000).
9. Palmer, D. R. *et al.* Differential effects of dengue virus on infected and bystander dendritic cells. *J. Virol.* **79,** 2432–2439 (2005).
10. Chen, Y. C. & Wang, S. Y. Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. *J. Virol.* **76,** 9877–9887 (2002).
11. Cook, D. N., Pisetsky, D. S. & Schwartz, D. A. Toll-like receptors in the pathogenesis of human disease. *Nature Immunol.* **5,** 975–979 (2004).
12. Klesney-Tait, J., Turnbull, I. R. & Colonna, M. The TREM receptor family and signal integration. *Nature Immunol.* **7,** 1266–1273 (2006).
13. Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S. & Reis e Sousa, C. Myeloid C-type lectins in innate immunity. *Nature Immunol.* **7,** 1258–1265 (2006).
14. Pokidysheva, E. *et al.* Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN. *Cell* **124,** 485–493 (2006).
15. Brown, G. D. & Gordon, S. A new receptor for β-glucans. *Nature* **413,** 36–37 (2001).
16. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J. Virol.* **79,** 1223–1231 (2005).
17. Mitchell, D. A., Fadden, A. J. & Drickamer, K. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. *J. Biol. Chem.* **276,** 28939–28945 (2001).
18. Lozach, P. Y. *et al.* Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. *J. Biol. Chem.* **280,** 23698–23708 (2005).
19. Tassaneetrithep, B. *et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med.* **197,** 823–829 (2003).
20. Johnson, A. J., Guirakhoo, F. & Roehrig, J. T. The envelope glycoproteins of dengue 1 and dengue 2 viruses grown in mosquito cells differ in their utilization of potential glycosylation sites. *Virology* **203,** 241–249 (1994).
21. Goncalvez, A. P., Engle, R. E., St Claire, M., Purcell, R. H. & Lai, C. J. Monoclonal antibody-mediated enhancement of dengue virus infection *in vitro* and *in vivo* and strategies for prevention. *Proc. Natl Acad. Sci. USA* **104,** 9422–9427 (2007).
22. Huang, K. J. *et al.* The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J. Immunol.* **176,** 2825–2832 (2006).
23. Durbin, J. E., Hackenmiller, R., Simon, M. C. & Levy, D. E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84,** 443–450 (1996).
24. Shresta, S. *et al.* Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice. *J. Immunol.* **175,** 3946–3954 (2005).
25. Arrighi, J. F. *et al.* Lentivirus-mediated RNA interference of DC-SIGN expression inhibits human immunodeficiency virus transmission from dendritic cells to T cells. *J. Virol.* **78,** 10848–10855 (2004).
26. Lin, Y. L. *et al.* Study of Dengue virus infection in SCID mice engrafted with human K562 cells. *J. Virol.* **72,** 9729–9737 (1998).
27. Shresta, S., Sharar, K. L., Prigozhin, D. M., Beatty, P. R. & Harris, E. Murine model for dengue virus-induced lethal disease with increased vascular permeability. *J. Virol.* **80,** 10208–10217 (2006).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank W.-C. Yeh, C. Milner and J. Paulson for critical comments; N.-J. Chen, C.-H. Lin, Y-L. Lee and J.-J. Liang for technical assistance. Resources and collaborative efforts were provided by the RNAi Consortium, Academia Sinica, Taiwan, and the Consortium for Functional Glycomics funded by the National Institute of General Medical Sciences (GM62116). This work was supported mainly by the National Research Program for Genomic Medicine, National Science Council, Taiwan (NSC-95-3112-B-010-0171 and NSC 96-3112-B-010-2), and in part by the National Yang-Ming University, Taiwan (96A-D-D132 from the Ministry of Education), Taipei Veterans General Hospital (V97S5-001), and Academia Sinica.

Author Contributions S.-T.C. designed, performed and analysed experiments, and wrote the paper. Y.-L.L. designed, analysed experiments and wrote the paper. M.-T.H., M.-F.W. and S.-C.C. performed experiments. H.-Y.L., C.-K.L. and T.-W.C. provided materials and reagents. C.-H.W. analysed experiments. S.-L.H. designed and analysed experiments, and wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S-L H. (slhsieh@ym.edu.tw or slhsieh@gate.sinica.edu.tw).

METHODS

Reagents. Evans blue and human IgG1 mAbs were purchased from Sigma. Human macrophage-colony-stimulating factor (M-CSF) and TNF-α were purchased from R&D Systems. Protein A–Sepharose beads, horseradish peroxidase (HRP)-conjugated second antibody and enhanced chemiluminescence reagents were from Amersham Biosciences. Proteinase inhibitor cocktail tablets were from Roche. Anti-CD14 microbeads were from Miltenyi Biotec GmbH. Anti-phosphotyrosine mAb (clone 4G10) was purchased from Upstate Biotechnology. The polyclonal antibody against human DAP12 was from Santa Cruz Biotechnology.

Biotinylation of dengue virions. Sucrose-cushion-purified DV2 (about 5×10^9 plaque-forming units) were resuspended in PBS, followed by the addition of 80 μl of 10 mM biotin solution (EZ-Link Sulfo-NHS-SS-Biotin; Cat. 21331; Pierce) and incubated on ice for 2 h. After removal of the unconjugated biotin by dialysis, the biotinylated virions were stored at 4 °C, and the efficiency of biotinylation was determined by flow cytometry with allophycocyanin-conjugated streptavidin.

Receptor-virus interaction. Receptor.Fc constructs in pcDNA3.1 (Invitrogen) were transfected into 293 FreeStyle cells (Invitrogen) and expressed proteins were purified with Protein A beads. Fusion proteins (1 μg per well) were coated on microtitre plates to capture DV2 (5×10^6 plaque-forming units per well), and the bound dengue virions were detected by biotinylated anti-DV envelope protein antibody[26] and HRP-conjugated streptavidin with 3,3′,5,5′-tetramethylbenzidine (TMB) (BD Pharmingen) substrate.

Sugar competition assay. Human 293T cells overexpressing human DC-SIGN or CLEC5A were preincubated with 15 U of heparin for 15 min, followed by the addition of biotinylated dengue virus (m.o.i. = 20) in conjunction with monosaccharides (mannose and fucose) or polysaccharide (mannan) and incubated at 4 °C for 30 min. After being washed with PBS, cells were incubated with allophycocyanin-conjugated streptavidin at 4 °C for 30 min, and the biotinylated dengue virus adsorbed on cell surfaces was detected by flow cytometry.

Preparation of PBMC and *in vitro* differentiation of monocytes into macrophage and DCs. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy human donors by standard density-gradient centrifugation with Ficoll-Paque (Amersham Biosciences). Purified neutrophils were resuspended in PBS (pH 7.4) and hypotonic lysis of erythrocytes was performed to improve the purity of polymorphonuclear cells. $CD14^+$ cells were purified from PBMCs by high-gradient magnetic sorting, using the VarioMACS technique with anti-CD14 microbeads (Miltenyi Biotec GmbH). Cells were then cultured in complete RPMI 1640 medium (Life Technologies) supplemented with 10 ng ml^{-1} human M-CSF (R&D Systems) for 6 days (ref. 28). DCs were generated from adherent PBMCs by incubation in RPMI 1640 medium supplemented with 10% FCS, 800 U ml^{-1} human GM-CSF (Leucomax; Schering-Plough) and 500 U ml^{-1} human IL-4 (R&D Systems) for 6 days (immature DCs). To prepare mature activated DCs, immature DCs were further incubated for 36 h with γ-irradiated (5,500 rad) CD40 ligand (CD40L)-expressing L cells (DNAX Research Institute) at a ratio of 3:1 (ref. 29).

Flow cytometry analysis. Human polymorphonuclear cells and PBMCs were isolated from the whole blood of healthy donors; macrophages and DCs were generated from $CD14^+$ monocytes as described above. To characterize the expression pattern of CLEC5A and DC-SIGN, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CLEC5A mAb (clone 283834; R&D Systems) or FITC-conjugated anti-DC-SIGN mAb (BD Pharmingen) in conjunction with phycoerythrin (PE)-conjugated mAbs against CD3, CD19, CD56, CD14 or CD66 (BD Pharmingen). Results were compared with those obtained using isotype-matched controls (IgG2b for anti-CLEC5A mAb, IgG1 for anti-DC-SIGN; Sigma). To detect intracellular DV antigens, infected cells were fixed with 1% (v/v) paraformaldehyde and permeabilized with 0.1% (w/v) saponin, followed by staining with NS3 mAb or an isotype-matched control (mIgG1; Sigma). After incubation for 1 h, PE-conjugated goat F(ab)′ anti-mouse IgG secondary antibody was added to detect emitted fluorescence by FACSCalibur (Becton Dickinson) with CellQuest software (Becton Dickinson).

Detection of NS3 in DV-infected cells by immunofluorescence assay. To determine the blocking effects of mAbs on DV entry to macrophages, cells were cultured on glass coverslips and preincubated with anti-DC-SIGN mAb (50 μg ml^{-1}; clone 120507; R&D Systems), anti-CLEC5A mAb (50 μg ml^{-1}; clone 8H8F5), or mouse IgG1 (50 μg ml^{-1}; Sigma-Aldrich) for 2 h at 4 °C, before the addition of DV2. After incubation with DV2 for 2.5 h, and unbound virus was removed by washing the cell monolayers with serum-free RPMI and then with complete RPMI medium containing blocking antibodies. After washing, cells were fixed with 4% (v/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min, and then incubated with blocking buffer [10% (w/v) BSA in PBS), before the addition of anti-NS3 mAb (20 μg ml^{-1}). After being washed, cells were incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson Immuno) and Hochest 33342 to detect NS3 and nuclei, respectively. Slides were mounted and observed by fluorescence microscopy (AX-70 laser scanning microscope; Olympus).

Detection of DAP12 by immunoprecipitation and immunoblotting. Macrophages were stimulated with dengue virus, followed by resuspension in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, and proteinase inhibitor cocktail tablet (Roche)). Equal amounts of total cell extracts (100 μg) were incubated with rabbit anti-DAP12 polyclonal antibody (Santa Cruz Biotechnology Inc.) at 4 °C for 4 h and then with 15 μl of Protein A–Sepharose (Amersham Biosciences AB) for 2 h. The immunocomplex was washed three times before fractionation on SDS–PAGE, followed by transfer to nitrocellulose membrane and probing with anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Inc.). Immunoblots were developed with HRP-conjugated anti-mouse IgG antiserum (Cat. AP181P; Chemicon) followed by enhanced chemiluminescence detection reagents (Amersham). To detect the total amount of DAP12 in the blot, the membrane was stripped with Re-Blot Plus Strong solution (Cat. 2504; Chemicon), and probed with rabbit anti-DAP12 antibody. The effects of pLL3.7/DC-SIGN and pLL3.7/CLEC5A at 24 h after transfection were determined by probing the blot with anti-CLEC5A and anti-DC-SIGN mAbs, respectively (R&D Systems).

RNA interference. The coding region of human CLEC5A was targeted with the sequence 5′-TTGTTGGAATGACCTTAT-3′ (clone 5), which was adapted by addition of a loop sequence (5′-TTCAAGAGA-3′) to create a shRNA. The polymerase III terminator stretch used here was 5′-TTTTTT-3′. The sequences of scrambled RNAs of clone 5, as well as other targeting sequences (clones 1, 2, 3, 4 and 6) is shown in Supplementary Fig. 2. The pLL3.7 gene silencing vector was provided by V. Parijs. For the human DC-SIGN shRNA, the backbone of the pSUPER-siDC-SIGN construct (provided by P. Vincet) was used to target the sequence 5′-AAGGCTGCAGTGGGTGAGCTT-3′. The shRNA was amplified by PCR, using sense and antisense primers tagged with *Hpa*I and *Xho*I sites, respectively, and was subcloned into the pLL3.7 vector. Human MyD88 and TLR7 shRNA expression vectors were purchased from InVivoGen; the target sequences are 5′-AACTGGAACAGACAAACTATC-3′ (ref. 30) and 5′-GGCAGACCTTGGATCTAAGTA-3′, respectively, where these are cloned in the psiRNA-h7SKGFPzeo backbone.

Generation and characterization of anti-CLEC5A mAbs and selection of antagonistic antibody. Breeder mice (BALB/c strain) were maintained in the standard animal facility of the National Yang-Ming University. For the production of mAbs, mice were immunized with purified recombinant CLEC5A.Fc fusion protein as antigen. The most suitable mouse was selected for administration of the final boost. Lymphocytes from the immunized mouse spleen were fused with mouse myeloma NS-1 cells in the presence of 50% (v/v) polyethylene glycol (PEG1450; Sigma). Fused cells were cultured in HAT selection medium and the medium was refreshed after one week. About 2 weeks after fusion, culture supernatants were screened by ELISA to identify the candidate clones for further analysis by limiting dilution. Anti-CLEC5A mAbs were selected by ELISA-based differential screening, and only those recognizing recombinant CLEC5A.Fc, but not human IgG1, were regarded as positive clones. A similar strategy was used to generate anti-mCLEC5A mAbs. To select antagonistic mAbs against human CLEC5A and murine CLEC5A, mAbs were incubated with human macrophages (6×10^4 per well) and Raw264.7 cells transfected with human DC-SIGN, respectively, in 96-well plates for 30 min at 37 °C, before the addition of DV2 (m.o.i. = 5) and incubation for 2.5 h. After washing out unbound virus, cells were cultured for a further 36 h before harvesting the supernatants to determine TNF-α levels by ELISA.

Preparation of murine bone marrow-derived macrophages. Bone marrow cells were isolated from femurs and tibias and were incubated in RPMI complete medium supplemented with 10% fetal calf serum (Gibco-BRL) and 10 ng ml^{-1} recombinant mouse M-CSF (R&D) for 6–8 days. At day 7, the expression of F4/80 (murine macrophage marker) was examined by fluorescence-activated cell sorting. More than 90% of cells were $F4/80^+$ under this culture condition.

Quantification of vascular permeability, serum TNF-α and virus titre in infected mice. Vascular leakage was examined by intravascular administration of Evans blue (Sigma-Aldrich) as described previously[27]. In brief, Evans blue (0.15 ml, 0.5% (v/v) in PBS) was injected intravenously into NGC-N-infected $STAT1^{-/-}$ mice at day 8 or 9 after infection; this was the point at which control mice began to exhibit signs of illness, such as ruffled fur and lethargy. At 2 h after injection, mice were killed and extensively perfused with PBS; tissues were harvested and weighed before the addition of formamide (2 ml of formamide per gram of tissue). Samples were incubated at 55 °C for 2.5 h, after which the Evans blue concentrations in formamide extracts were quantified by measuring A_{610}. Data are presented as A_{610} per gram tissue weight. Blood samples were collected

from tail veins, and the levels of TNF-α and IP-10 and the titre of infectious virus in the sera were determined by ELISA (R&D Systems) and plaque assay in BHK-21 cells, respectively.

28. Chang, Y. C. *et al.* Modulation of macrophage differentiation and activation by decoy receptor 3. *J. Leukoc. Biol.* **75,** 486–494 (2004).
29. Hsu, T. L. *et al.* Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J. Immunol.* **168,** 4846–4853 (2002).
30. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. & Seya, T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nature Immunol.* **4,** 161–167 (2003).