Chapter 3
CLEC5A: A Promiscuous Pattern Recognition Receptor to Microbes and Beyond

Pei-Shan Sung, Wei-Chiao Chang and Shie-Liang Hsieh

Abstract CLEC5A is a spleen tyrosine kinase (Syk)-coupled C-type lectin that is highly expressed by monocytes, macrophages, neutrophils, and dendritic cells and interacts with virions directly, via terminal fucose and mannose moieties of viral glycans. CLEC5A also binds to *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) disaccharides of bacterial cell walls. Compared to other C-type lectins (DC-SIGN and DC-SIGNR) and TLRs, CLEC5A binds its ligands with relatively low affinities. However, CLEC5A forms a multivalent hetero-complex with DC-SIGN and other C-type lectins upon engagement with ligands, and thereby mediates microbe-induced inflammatory responses via activation of Syk. For example, in vivo studies in mouse models have demonstrated that CLEC5A is responsible for flaviviruses-induced hemorrhagic shock and neuroinflammation, and a CLEC5A polymorphism in humans is associated with disease severity following infection with dengue virus. In addition, CLEC5A is co-activated with TLR2 by *Listeria* and *Staphylococcus*. Furthermore, CLEC5A-postive myeloid cells are responsible for Concanavilin A-induced aseptic inflammatory reactions. Thus, CLEC5A is a promiscuous pattern recognition receptor in myeloid cells and is a potential therapeutic target for attenuation of both septic and aseptic inflammatory reactions.

Keywords CLEC5A · MDL-1 · DAP12 · ITAM · Syk

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3.1 Introduction

C-type lectins are characterized by a common structural C-type lectin domain (CTLD) that can bind glycan and non-glycan ligands in a Ca^{2+}-independent manner; CTLDs that bind glycans in Ca^{2+}-dependent manner are known as "carbohydrate recognition domains" (CRD) (Zelensky and Gready 2005). The myeloid C-type lectin CLEC5A (also known as MDL-1) (Bakker et al. 1999) is a spleen tyrosine kinase (Syk)-coupled type II membrane protein comprising a C-terminal CTLD and a short N-terminal cytoplasmic domain. Among the 15 groups of C-type lectins, CLEC5A falls into Group V (the NK cell receptor family) (Hsieh 2016), which includes CLEC7A (Dectin-1), CLEC5A, CLEC2, CLEC1, NK receptors (such as NKG2D, the NKRP1 family, the NKG2 family, CD69 and CD94), mast cell-associated functional antigen (MAFA), osteoclast inhibitory lectin (OCIL), and CD72. Similar to NKG2D, CLEC5A signals via the ITAM-containing DNAX-activating protein 12 (DAP12) when it is phosphorylated by Src upon activation (Hsieh 2016). However, the role of DAP10, a DAP12-related adaptor protein associated with NKG2D, in CLEC5A-mediated signaling remains unclear.

The human CLEC5A mRNA encodes a 165-residue polypeptide with an N-terminal signal peptide (a.a. 1–22), followed by a short intracellular cytoplasmic domain (a.a. 23–56), a transmembrane domain (a.a. 57–70) and an extracellular domain (a.a. 71–165). The transmembrane domain contains a positively charged amino acid, Lys-58, which recruits DAP10 and DAP12 to associate with CLEC5A after activation. CLEC5A is mainly expressed by myeloid cells, including monocytes, macrophages, neutrophils, and dendritic cells (Chen et al. 2008), and is further upregulated by interferon-gamma (IFN-γ)(Joyce-Shaikh et al. 2010). In addition, CLEC5A expression is under the control of the PU.1 transcription factor, which is a central regulator of myeloid cell differentiation (Batliner et al. 2011). Recent studies further demonstrate that CLEC5A expression is upregulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) (Cheng et al. 2016), suggesting CLEC5A is regulated by oxidative stress.

An X-ray crystal structure has revealed that CLEC5A is a homodimeric protein when it binds to dengue virus serotype 1–4. Moreover, the CLEC5A crystal structure revealed conformational flexibility, suggesting that CLEC5A can adopt various conformations in vivo and that its conformation is ligand-dependent (Watson et al. 2011).

While NK receptors recognize stress-associated autologous antigens and are crucial for immunosurveillance, group V spleen tyrosine kinase (Syk)-coupled C-type lectins in myeloid cells recognize diverse exogenous and endogenous antigens and are involved in host defense, aseptic inflammation, platelet activation and development (Brown et al. 2018). The ligand for CLEC5A was unknown until it was shown to interact with glycan moieties on dengue virus (DV) (Chen et al. 2008), Japanese encephalitis virus (JEV) (Chen et al. 2012) and type A influenza virus (IAV) (Teng et al. 2017). In addition, CLEC5A has been found to interact with *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) disaccharides on

gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) (Chen et al. 2017). Moreover, CLEC5A has a critical role in the inflammatory responses associated with collagen-induced rheumatoid arthritis (Joyce-Shaikh et al. 2010) and Concanavalin A-induced liver inflammation (Cheung et al. 2011). Most recently it was demonstrated that CLEC5A interacts with exosomes released from activated platelets (Sung et al. 2019), though the ligand responsible has not been identified yet. In this review, we will discuss protective and pathogenic role of CLEC5A in viral and bacterial infections, and how CLEC5A collaborates with other C-type lectins in the recognition of various antigens.

3.2 CLEC5A Is the Pattern Recognition Receptor for Viral Glycans

It has been shown that host cells contain abundant endosomal sensors (such as TLR3, TLR7, TLR8, and TLR9) and cytoplasmic sensors (such as RIG-I, MDA5, cGAS) that detect the nucleic acids of DNA and RNA viruses (Schlee and Hartmann 2016). After endocytosis, viruses release nucleic acids that trigger receptor- and sensor-mediated signaling pathways to induce the secretion of proinflammatory cytokines and interferons. These nucleic acid receptors and sensors nicely demonstrate how virus-infected cells produce interferons and inflammatory cytokines to limit viral replication and spreading. However, it is hard to explain how some viruses (such as dengue virus, H5N1 virus, SARS-Coronavirus, Ebola viruses etc.) trigger massive cytokine release from leukocytes (known as cytokine storm), thereby causing systemic permeability change and hemorrhagic shock. For example, dengue virus (DV) infection frequently causes high fever (>40 °C), rash, thrombocytopenia, back pain, retroorbital pain and joint pain (Wilder-Smith and Schwartz 2005), but can give rise to more severe symptoms, i.e., dengue hemorrhagic fever/dengue shock syndrome, due to elevated systemic vascular permeability and activation of platelets (Wilder-Smith and Schwartz 2005). In contrast, Hepatitis B virus (HBV) infection is associated with low-grade fever and persistent viremia, leading to fibrotic changes and hepatocellular carcinoma (Ganem and Prince 2004). These observations suggest that each virus must be able to trigger a distinct pathway in addition to nucleic acid receptor-/sensor-mediated signaling to modulate host immunity, thus resulting in various clinical symptoms and outcomes.

The viral nucleocapsid is surrounded by a lipid bilayer that contains viral proteins (such as the DV E protein and HBsAg) to enable virus binding to host cells. Even though the lipid bilayer of the viral envelope is derived from host cell membranes, the envelope also contains proteins encoded by the viral genome. One of the best-studied viral envelope proteins is the E protein of dengue virus, which has two N-linked glycosylation sites at Asn-67 and Asn-153 (Pokidysheva et al. 2006), where the terminal sugars include mannose, fucose, and GluNac (Modis et al. 2003). While the glycosylation site at Asn-153 is conserved in most flaviviruses, glycosylation

at Asn-67 is unique for DV serotype 2 (DV2) (Heinz and Allison 2003). It has been shown that dengue virus interacts with the C-type lectin DC-SIGN via Asn-67 (Tassaneetrithep et al. 2003), while JEV interacts with DC-SIGNR via Asn-163 (Shimojima et al. 2014). In addition, E protein is a viral structural protein and is immunogenic; thus we speculated that this glycosylated viral envelope protein could be regarded as a "virus-associated molecular pattern" (VAMP) that triggers host immune responses.

By lectin array screening, we demonstrated that the intact dengue virion binds to DC-SIGN, mannose receptor (MR) and CLEC5A specifically(Chen et al. 2008). While DC-SIGN and MR interact with the dengue virion via mannose in a Ca^{2+}-dependent manner, CLEC5A interacts with the dengue virion via fucose in a Ca^{2+}-independent manner. Even though DC-SIGN (Tassaneetrithep et al. 2003) and MR (Miller et al. 2008) have been reported to interact with dengue viruses, both receptors lack motifs capable of transducing signals upon ligand binding. In contrast, DV activates CLEC5A to recruit DAP12, thereby triggering downstream signaling in immune cells (Chen et al. 2008).

By comparing the binding of CLEC5A to JEV mutants lacking either Asn-67 or Asn-153, we found that CLEC5A interacts with flavivirus via Asn-153 (Fig. 3.1). DC-SIGN is shown to interacts with dengue virion via Asn-67 (Mondotte et al. 2007), thus dengue virion seems interacting with DC-SIGN and CLEC5A via Asn-67 and Asn-153, respectively. Furthermore, the affinity of the CLEC5A-DV interaction is much lower than that of the DC-SIGN-DV interaction, and colocalization of CLEC5A
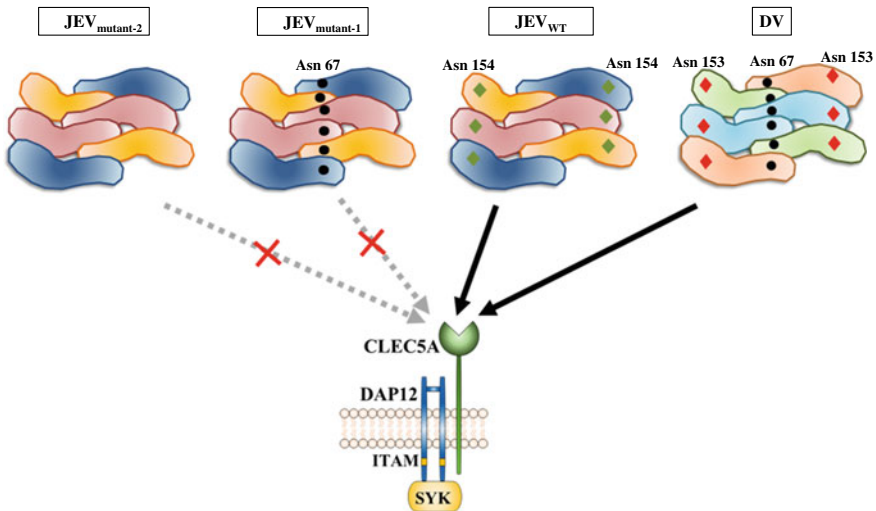

Fig. 3.1 Interaction between CLEC5A and Flaviviruses. Dengue virus (DV) contains two glycosylation sites at Asn-67 Asn-153. Japanese encephalitis virus (JEV) contains a glycosylation site at Asn-154. JEV mutant-1 contains a glycosylation site at Asn-67, while JEV mutant-1 contains no glycosylation site. Because CLEC5A interacts with both DV and wild type JEV, JEV-154 and DV-153 are crucial for the interaction between CLEC5A with JEV and DV, respectively

and DC-SIGN on macrophages was detected upon engagement with DV (Lo et al. 2016). All these observations suggest that DV form a multivalent hetero-complex with CLEC5A and DC-SIGN to activate macrophages via Syk-coupled CLEC5A. Because DC-SIGN is crucial for virus entry into dendritic cells and other DC-SIGN-positive myeloid cells (such as macrophages), DV2 is more efficient than other DV serotypes (DV1, DV3, DV4) and flaviviruses at infecting and replicating in dendritic cells and macrophages. On the other hand, CLEC5A recognizes all flaviviruses via the conserved Asn-153, supporting the notion that CLEC5A is a pan-pattern recognition receptor for all the flaviviruses. These observations suggest that viruses activate host defense mechanisms not only via nucleic acids (denoted as virus-associated molecular pattern-1, VAMP-1), but also activate innate immune receptors via their surface glycans (denoted as virus-associated molecular pattern-2, VAMP-2) (Fig. 3.2). While nonmyeloid cells are only activated by VAMP-1, macrophages and other myeloid cells are activated by both VAMP-1 and VAMP-2 via nucleic acid receptors/sensors and Syk-coupled C-type lectin, respectively; this enhances NFkB-mediated proinflammatory cytokine release to induce a severe cytokine storm. This dual activation pathway provides an explanation for why viruses capable of activating or replicating in macrophages (such as dengue virus, Ebola virus, Bunya virus, H5N1 IAV) frequently cause cytokine storms.
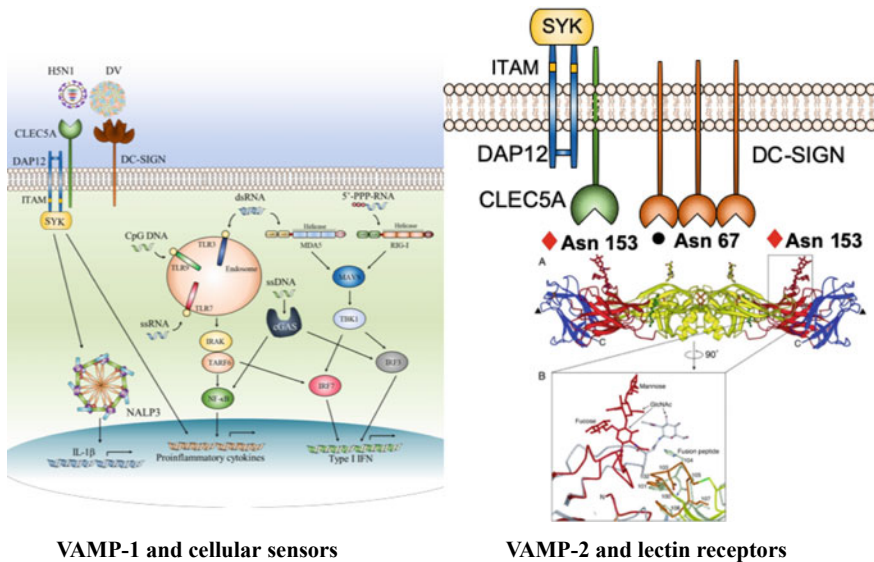

VAMP-1 and cellular sensors   **VAMP-2 and lectin receptors**

Fig. 3.2 Activation of CLEC5A and nucleic acid sensors by viruses. Dengue virus infection of dendritic cells results in release of viral nucleic acids (virus-associated molecular pattern-1/VAMP-1) that interact with endosomal TLRs and cellular sensors to activate IRAK and TBK1 and induce the production of proinflammatory cytokines and interferons. In addition, dengue virus activates CLEC5A via viral glycans (virus-associated molecular pattern-2/VAMP-2) to trigger NALP3-inflammasome formation and NFkB activation. Similar pathways are associated with H5N1 IAV infection

3.3   CLEC5A in Flaviviral Infections

The genus *Flavivirus* contains a group of single-stranded, enveloped RNA viruses that cause serious diseases in humans and animals, including DV, JEV, WNV, and ZIKA virus (ZIKV). Even though the RNA structures of these four flaviviruses are similar, their clinical symptoms are very diverse. CLEC5A has been shown to be critical in the pathogenesis of dengue hemorrhagic fever, dengue shock syndrome and JEV-induced encephalitis and neuronal inflammation.

3.3.1   CLEC5A in Dengue Virus Infection

Compared to other flaviviruses (i.e., JEV, WNV, ZIKV), higher viral titers and longer viremia durations have been noted in DV infection. The clinical symptoms of DV infection include high fever (frequently >40 °C), severe headache, retro-orbital pain, back pain, and petechia. In more severe cases, severe thrombocytopenia with increased systemic vascular permeability leads to dengue hemorrhagic fever and shock syndrome. These observations suggest that systemic viremia correlates with viral potency to trigger more severe inflammatory reactions than is the case for other flaviviruses.

3.3.1.1   CLEC5A in Virus-Induced Cytokine Release

DV infection activates both endosomal TLR7 and CLEC5A to induce the secretion of interferons (IFNs) and proinflammatory cytokines, such as TNF-α, IL-6, MIP1-α, IL-8, and IP-10. While knockdown of DC-SIGN inhibits DV entry into macrophages with mild impairment on cytokine production, blockade of CLEC5A attenuates the release of proinflammatory cytokines, such as TNF-α, IL-6, MIP1-α, IL-8, and IP-10 dramatically, without affecting IFN production. There is evidence (Chen et al. 2008) that all four serotypes of dengue virus (DV1-4) bind and activate CLEC5A to secrete proinflammatory cytokines. Furthermore, CLEC5A is also responsible for enhanced inflammatory cytokine release from macrophages incubated with immune complexes containing antibodies to DV E protein and DV pre-membrane protein (an in vitro system to mimic antibody-dependent enhancement of DV infection). This observation demonstrates that DV can activate dual receptor pathways; i.e., endosomal TLR7 and other nucleic acid sensors via viral RNA (VAMP-1), and CLEC5A via glycans and proteins on the viral envelope (VAMP-2). Thus, it should be possible to attenuate virus-induced inflammatory reactions without suppressing IFN-mediated anti-viral immunity. This was confirmed in vivo using an anti-CLEC5A mAb to treat DV-infected mice. Compared to isotype control, anti-CLEC5A mAb inhibited DV-induced plasma leakage, as well as subcutaneous and vital-organ hemorrhaging, thereby reducing mortality by about 50% in STAT1-deficient mice. Thus, blockade

of CLEC5A offers a novel strategy for alleviating tissue damage and increasing survival rate in patients suffering from dengue hemorrhagic fever and shock syndrome (Chen et al. 2008).

3.3.1.2   CLEC5A-Mediated NALP3 Inflammasome Activation

Fever is caused by endogenous pyrogens produced by stimulated leukocytes (or other cell types) upon pathogen invasion. The most abundant endogenous pyrogens are TNF-α and IL-1β derived from activated macrophages (Netea et al. 2000; Dinarello 2004), both of which can regulate local and systemic inflammation by activating lymphocytes and promoting leukocyte infiltration. In contrast to TNF-α, IL-1β production relies on the activation of the NACHT, LRR and PYD domain-containing protein 3 (NALP3) inflammasome and caspase-I (Schroder and Tschopp 2010). Thus, it is crucial to understand how DV activates inflammasomes to release IL-1β, which contributes significantly to high fever and inflammatory reactions in dengue patients.

Although both M-CSF and GM-CSF contribute to macrophage differentiation, during infection this is primarily regulated by GM-CSF, which is upregulated during inflammatory responses (Wu et al. 2013) and can cause massive expansion of the macrophage population in the spleen leading splenomegaly (Krakowski et al. 2002). In addition, CLEC5A expression is much higher in GM-CSF-derived macrophages (GM-Mφ), compared to M-CSF-derived macrophages (M-Mφ) (Gonzalez-Dominguez et al. 2015). We found that DV activates CLEC5A to induce the activation of the NALP3 inflammasome and caspase-1, leading to abundant release of IL-1β from GM-Mφ (Wu et al. 2013). Moreover, blockade of CLEC5A by an antagonistic mAb inhibits DV-induced NALP3 inflammasome activation and IL-1β secretion. While serum IL-1β is elevated after DV infection in $stat1^{-/-}$ mice, it is almost undetectable in $stat1^{-/-} clec5a^{-/-}$ mice. These observations help to explain how DV binding to CLEC5A contributes to the severe inflammatory reaction during DV infection (Wu et al. 2013).

3.3.1.3   CLEC5A in DV-Induced Osteoclast Activation

In addition to high fever, the clinical symptoms of dengue infection include severe headache and retro-orbital pain, arthralgia, myalgia, anorexia and abdominal discomfort. Therefore, dengue fever is also known as "break-bone" disease. However, the underlying mechanism for the pain experienced by dengue patients is still unclear. Because increased osteoclastic bone resorption is associated with pain, we asked whether DV infects osteoclasts and increases their osteolytic activity. We found that osteoclasts are highly susceptible to DV infection and that they produce high levels of TNF-α and IL-6 following infection. In contrast, osteoclasts are resistant to WNV and JEV infection, and do not produce detectable proinflammatory cytokines

in response to these viruses. Furthermore, DV activates NFATc1 to upregulate osteolytic activity via CLEC5A, and DV infection causes bone tissue inflammation and disturbance of bone homeostasis in *stat1*$^{-/-}$ mice. While CLEC5A-induced proinflammatory cytokine release is via DAP12-mediated signaling, CLEC5A-induced osteolytic activity requires the formation of a CLEC5A/DAP12/DAP10 tri-molecular complex (Inui et al. 2009). Nevertheless, blockade of CLEC5A is able to suppress DV-induced bone inflammation and osteolytic activity in vivo (Huang et al. 2016), confirming the critical role of CLEC5A in osteoclast activation. It would be interesting to explore whether blockade of CLEC5A is able to reduce pain in dengue patients in the future.

3.3.2   CLEC5A in JEV-Induced Neuronal Inflammation

Even though JEV is structurally similar to DV its tropism and the clinical symptoms it induces are distinct. JEV is less effective at infecting peripheral macrophages and inducing proinflammatory cytokines and, while DV is detectable in sera following infection, JEV is almost undetectable in patients' sera. However, JEV infects neuronal cells efficiently, and JEV-induced neuronal inflammation associated with microglia activation. In contrast to the transient nature of DV-induced hemorrhagic fever/shock syndrome, JEV-infected victims experience permanent neuropsychiatric sequelae, including persistent motor defects and severe cognitive and language impairments (Mackenzie et al. 2004). However, it is unclear whether neuronal death after JEV infection is due to a direct cytotoxic effect of JEV on infected neuronal cells, or is via inflammatory mediators released from microglia cells.

We found that supernatants from JEV-infected mouse glial cells have potent neurotoxic effects. Incubation of macrophages and microglia with JEV induces proinflammatory cytokine and chemokine release, where addition of an anti-CLEC5A mAb inhibits JEV-induced proinflammatory cytokine release, and attenuates the neurotoxic effects of supernatants from JEV-infected mixed glial cells in vitro. Although blockade of CLEC5A cannot inhibit JEV infection of neurons and astrocytes, anti-CLEC5A mAb inhibits JEV-induced proinflammatory cytokine release from microglia and prevents bystander damage to neuronal cells. These observations suggest that JEV activates CLEC5A to induce the release of neurotoxic substances from activated microglia. Moreover, injection of anti-CLEC5A mAb into JEV-infected mice maintains the integrity of the blood–brain-barrier, attenuates infiltration of CD45$^+$Ly6G$^+$ and CD45$^+$Ly6C$^+$ inflammatory cells into the brain, and protects mice from JEV-induced lethality; surviving mice develop protective humoral and cellular immunity against JEV infection. Thus, it is evident that CLEC5A plays a critical role in the pathogenesis of JEV-induced neurotoxicity, where blockade of CLEC5A protects against JEV-induced brain damage (Chen et al. 2012).

3.4 CLEC5A in Type A Influenza Virus (IAV) Infection

Influenza virus is the most common pathogen associated with human respiratory infections, and causes high morbidity and mortality in susceptible hosts who are unable to tolerate the negative consequences of the immune response during the acute stage or reduce pathogen burden in later stages. Innate immune receptors and sensors involved in recognition of influenza viruses include Toll-like receptors TLR3, TLR7, TLR8, retinoic acid-inducing gene (RIG-I), NOD-like receptor family members and NALP3. However, there are no published data to evidence a direct interaction between the NALP3 inflammasome and influenza virus.

As seen in DV infection, C-type lectins expressed on myeloid cells, including DC-SIGN (CLEC4L), DC-SIGNR (CLEC4M), macrophage mannose receptor (MMR) and macrophage galactose-type lectin (MGL), have been shown to mediate influenza virus internalization in a sialic acid-independent manner (Londrigan et al. 2011; Ng et al. 2014; Reading et al. 2000; Upham et al. 2010). However, it is still unclear whether influenza virus can bind and trigger signaling via myeloid C-type lectins to further modulate the host immune response.

We employed a lectin Fc array to identify pattern recognition receptors for H5N1 and H1N1 influenza viruses, using pseudotyped lentiviral particles with surface expression of influenza HA proteins derived from H5N1 or H1N1 isolates. We found that both H1N1 and H5N1 bind to DC-SIGN (CLEC4L) via mannose moieties associated with the influenza virus membrane protein (Londrigan et al. 2011). Moreover, DC-SIGNR (CLEC4M), Mincle (CLEC4E) and CLEC5A also interact with H1N1 and H5N1, where the highest affinity interaction is between CLEC5A and H5N1 (Teng et al. 2017). We further found that CLEC5A plays a critical role in H5N1 IAV-induced release of proinflammatory cytokine (TNF-α), chemokines (IP-10, MCP-1, MIP-1α) and IFN-α (Londrigan et al. 2011). Similar observations were made when human macrophages were incubated with H1N1 and H5N7 IAVs.

The cytokine profile observed for $Clec5a^{-/-}$ mouse bone marrow-derived macrophages (mBMDM) was slightly different from that seen for human macrophages blockaded with anti-CLEC5A mAb, when these cells were infected with IAV. While the production of TNF-α and IP-10 was downregulated in $Clec5a^{-/-}$ mBMDM, IFN-α/β production was upregulated. Since mice lack orthologues of DC-SIGN and DC-SIGNR, which are involved in IAV infection of human macrophages, the differential responses of mouse and human macrophages to IAV infection can be attributed to the presence or absence of DC-SIGN/DC-SIGNR. Nevertheless, $Clec5a^{-/-}$ mice were protected from the sublethal challenge of H5N1 virus by reducing the level of proinflammatory cytokine in the lungs, suggesting activation of CLEC5A plays a pathogenic role in IAV infection.

3.5 CLEC5A in Gram-Positive Bacterial Infection

While TLR2 and TLR4 recognize PAMPs expressed on gram-positive and gram-negative bacteria, respectively (Medzhitov 2001; Akira 2001), TLR2-deficient mice are not always more susceptible than wild type to Gram-positive infection, e.g., in the case of *Listeria* infection (Edelson and Unanue 2002). These observations suggest that other receptors/sensors may play more important roles than TLR2 in host defense against some gram-positive bacterial infection.

3.5.1 CLEC5A Is Critical for Bacteria-Induced NET Formation and Proinflammatory Cytokine Production

Neutrophils clear invading bacteria by phagocytosis and production of reactive oxygen species (ROS) to kill engulfed bacteria. In addition, neutrophils produce neutrophil extracellular traps (NETs) to ensnare a variety of microbes (Brinkmann et al. 2004). However, the key receptors that trigger bacteria-induced NET formation are not clearly defined. It has been shown that free radical production is required for NET formation; however, TLR-mediated signaling does not contribute to ROS production (Gantner et al. 2003). In contrast, Syk-mediated signaling leads to ROS production in the cytosol (Mocsai et al. 2010), thus we asked whether CLEC5A contributes to bacteria-induced NET formation.

We demonstrated that CLEC5A is required for *Listeria*- and *Staphylococcus*-induced NOD-like receptor (NLR) 4 inflammasome activation and IL-1β production in macrophages and also to restrict bacteria-spreading and NET formation in vivo (Chen et al. 2017). While TLR2 binds to teichoic acids, CLEC5A binds to *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) disaccharides of the *Listeria* cell wall. Thus, engagement of *Listeria* by neutrophils is associated with co-activation of TLR2 and CLEC5A to induce ROS and proinflammatory cytokine production. Moreover, $Clec5a^{-/-}$ mice are more susceptible to infection than $Tlr2^{-/-}$ mice after intravenous inoculation with *Listeria monocytogenes*; $Clec5a^{-/-}Tlr2^{-/-}$ mice are extremely susceptible to infection with all mice dying within 5 days. These observations suggest co-activation of CLEC5A and TLR2 is critical to host defense against Listeria and Staphylococcus infections. Moreover, CLEC5A deficiency is associated with impaired production of TNF-α and IL-1β, and reduced the numbers of IL-17A-producing γδ T cells, which are critical for immunity to *L. monocytogenes* (Romagnoli et al. 2016) as well as contributing to autoimmune diseases (Papotto et al. 2018; Akitsu and Iwakura 2018). Therefore, CLEC5A/TLR2 activation is not only central to bacteria-induced inflammatory responses, but may also play a key role in the pathogenesis of autoimmunity.

3.6 Multivalent Binding Between Microbes and C-Type Lectins

Using genetic approaches, we have clearly demonstrated critical roles for CLEC5A in viral and bacterial infections as mentioned above. Immunofluorescent staining revealed that CLEC5A colocalizes with mannose receptor (MR) and DC-SIGN in the presence of DV infection, where both MR and DC-SIGN display higher binding affinities for DV compared to CLEC5A (Lo et al. 2016). This observation suggests that CLEC5A and other C-type lectins form a hetero-multivalent receptor complex to engage with DV (Fig. 3.3a). This model is in accord with our observation that dengue virions bind to DC-SIGN/CLEC5A via N-linked glycans associated with Asn-67 (mannose) and Asn-153 (fucose) on DV E protein (VAMP-2), thus activating CLEC5A to trigger downstream signaling. In addition, dengue virus enters macrophages via DC-SIGN and activates TLR7 by interaction with viral RNA (VAMP-1). Co-activation of CLEC5A and endosomal TLR7 leads to enhanced proinflammatory cytokine release from DV-infected macrophages (Chen et al. 2008). These observations suggest that viruses are captured by high-affinity receptors such as DC-SIGN, DC-SIGN and DC-SIGNR, and form a hetero-multivalent receptor
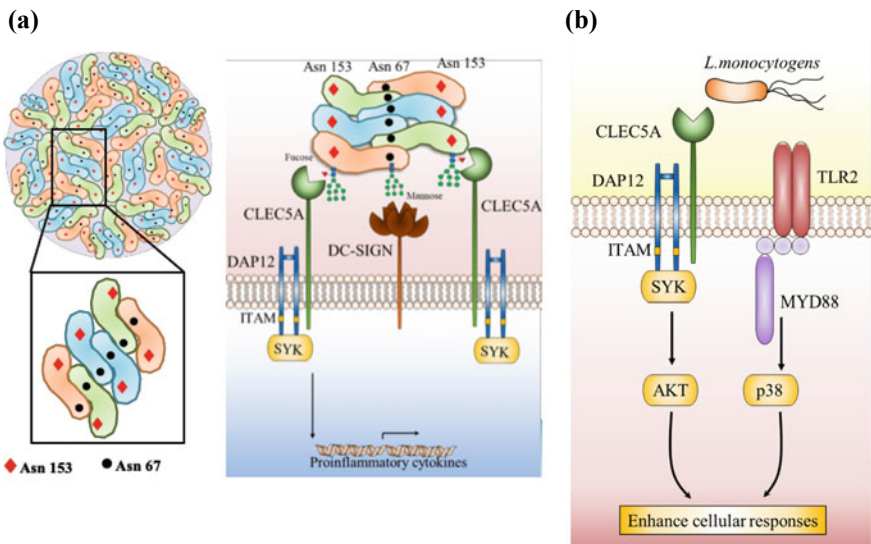

Fig. 3.3 a Formation of a hetero-multivalent receptor complex upon engagement with DV. Acrophages express abundant DC-SIGN and CLEC5A, which capture dengue virions via mannose and fucose moieties associated with glycans on Asn-67 and Asn-153 of DV E protein to form a hetero-multivalent receptor complex. **b** Co-activation of CLEC5A and TLR2 by Listeria. Neutrophils and macrophage express abundant TLR2 and CLEC5A, which interact with teichoic acid and GlcNAc/MurNAc disaccharides on bacterial cell walls and are co-activated leading to production of free radicals and NET formation by neutrophils, as well as inducing proinflammatory cytokine and chemokine expression by macrophages

complex with the low-affinity receptor CLEC5A via fucose on the viral envelope. This model is consistent with our observations in flaviviral and H5N1 viral infections and also predicts that CLEC5A on macrophages and dendritic cells will be activated by other microbes that harbor mannose and fucose moieties on their surfaces.

In addition to C-type lectins, we have demonstrated that TLR2 and CLEC5A bind teichoic acid and GlcNAc-MurNAc disaccharides of Listeria cell wall, thereby forming CLEC5A-TLR2 heterocomplex upon engagement with *Listeria monocytogenes* (Chen et al. 2017) (Fig. 3.3b). Because microbial surfaces are rich in various glycans with terminal mannose and fucoses, CLEC5A may also be co-activated with other TLRs during microbial infections. Because activation of CLEC5A induces the production of chemokines and free radicals by macrophages and neutrophils, blockade of CLEC5A may represent a means of attenuating inflammation due to excessive NET formation.

3.7   CLEC5A Polymorphism and Disease Susceptibility

Associations between *CLEC5A* polymorphisms and disease susceptibility have been investigated in previous clinical studies. Furthermore, in vitro studies have been conducted to assess the relationship between *CLEC5A* genetic variants and the expression levels of *CLEC5A* and various immunological mediators (Xavier-Carvalho et al. 2013, 2017).

3.7.1   CLEC5A Polymorphism and Dengue Fever

Xavier-Carvalho et al. recruited 88 patients with severe dengue virus infection in Brazil, along with 335 healthy controls who shared the same living environment as the patients. The authors studied the association between susceptibility to severe dengue fever and selected single nucleotide polymorphisms (SNPs) located in candidate genes, including *TNF*, *IL-10*, *MIF*, *DC-SIGN*, *CLEC5A*, *NOD2*, *CCR5* and *MRC1*. The most significant association was found between the TT genotype of *CLEC5A* (rs1285933 C > T) and severe dengue susceptibility (OR = 2.25; $p = 0.03$). In addition, the authors measured the serum levels of TNF, IL-10, IL-13 and IFN-γ at a critical disease phase in patients with different rs1285933 genotypes. Those with CT or TT genotypes exhibited higher levels of serum TNF than CC individuals, suggesting that *CLEC5A* variants might increase the risk of disease susceptibility and serum TNF level in dengue patients. In a subsequent analysis, the control cohort was divided into groups according to their IgG status, where IgG[+] implied recent infection with mild to no symptoms and without hospital admission; here, only borderline significance was reported for the association between rs1285933 genotype and dengue severity (Xavier-Carvalho et al. 2013, 2017). To validate their findings and to decipher the role of *CLEC5A* genetic variants in dengue viral infection, Xavier-Carvalho

et al. conducted a follow-up study. They recruited 213 hospitalized dengue patients and investigated the association of rs1285933 with disease severity. Consistent with their previous study, T carriers at rs1285933 were associated with a more severe manifestation in dengue fever. Furthermore, to investigate the role of *CLEC5A* after DV infection, the authors collected monocytes from healthy individuals and treated the cells with DV. The DV-infected monocytes showed increased *CLEC5A* mRNA and protein expression after infection. Among the groups, monocytes from CC genotype individuals showed higher CLEC5A protein expression 24 h after infection. Furthermore, a positive correlation was found between TNF concentration in supernatants and CLEC5A protein 48 h after infection. However, there was no difference in dengue viral load or TNF expression among the different genotypes. Moreover, the authors observed only borderline significance for the association between *CLEC5A* mRNA levels in patients with mild dengue infection and the CC genotype; TNF mRNA expression showed no variation among mild dengue patients with different genotypes (Xavier-Carvalho et al. 2017).

There is evidence that dengue patients with different ethnicities exhibit different levels of clinical symptoms. For example, migrant Chinese have a higher risk of severe dengue fever than local Chinese in Singapore (Xu et al. 2018). Furthermore, African ethnicity has been identified as a protective factor for severe symptoms in Cuban dengue patients (Sierra et al. 2017). Using data from the 1000 Genomes project, we screened the frequency of rs1285933 alleles of *CLEC5A* in the African, European, American, South Asian and East Asian populations. As shown in Fig. 3.4, the frequency of the T allele, which is associated with increased risk of severe dengue fever, has the lowest frequency in Africans (AFR, 34.9%), followed by the Europeans (EUR, 46.8%), Americans (AMR, 58.1%), South Asians (SAS, 60.9%) and East Asians (EAS, 77.2%). The results indicate that the highly polymorphisms CLEC5A
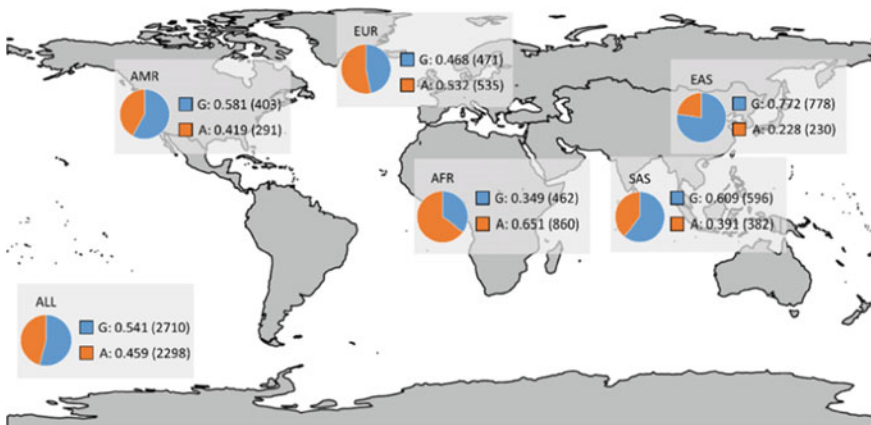

Fig. 3.4 Worldwide allele frequency at rs1285933 in CLEC5A in different ethnicities (shown as frequency (numbers)). ALL: all populations; EUR: Europeans; AMR: Americans; SAS: south Asians; EAS: east Asians

may associate with human migration distance as well as susceptibility of infectious diseases such as dengue fever.

3.7.2   CLEC5A Polymorphism and Kawasaki Disease

Kawasaki disease (KD) is an acute systemic childhood vasculitis without clear etiology. The clinical symptoms include fever, enlargement of lymph nodes, rash in the genital area and sore throat. The most severe complications of KD are aseptic meningitis and aortic aneurysm (Romagnoli et al. 2016). An association study based on 381 KD patients and 664 normal controls was conducted to identify SNPs associated with KD. In that study, four tagging single nucleotide polymorphisms (tSNPs) (i.e., rs1285968, rs11770855, rs1285935, rs1285933) were selected for genotyping. However, no clear association was found between the four tSNPs and susceptibility of coronary artery lesion formation or response to intravenous immunoglobulin treatment (Yang et al. 2012). Since only the SNP with a minor allele frequency of more than 10% were selected for testing, in this study, the effects of the rare causal SNPs are very likely to be underestimated. To understand the genetic functions of CLEC5A in the pathogenesis of KD, a large-scale DNA sequencing to CLEC5A is needed.

3.8   The Role of CLEC5A in Aseptic Inflammatory Reactions

CLEC5A is not only involved in host recognition to viruses and bacteria, but has also been implicated in the recognition of endogenous ligands during aseptic inflammation. The collagen antibody-induced arthritis (CAIA) model in mice is initiated by passive transfer of type II collagen-specific antibody and is dependent on myeloid cell activation. It is interesting to note that CD11b$^+$Ly6G$^+$CLEC5A$^+$ cell numbers are increased after arthritis induction, and that injection of an agonistic anti-CLEC5A mAb further increases CD11b$^+$Ly6G$^+$CLEC5A$^+$ cell infiltration into joint and aggravates disease severity. In contrast, injection of CLEC5A.Fc inhibits collagen-induced autoimmune arthritis. These observations suggest that activation of CLEC5A can enhance the pathogenic effect of CD11b$^+$Ly6G$^+$CLEC5A$^+$ cells in autoimmune arthritis (Joyce-Shaikh et al. 2010). This argument is supported by the observation that CLEC5A is highly upregulated in monocytes of rheumatoid arthritis patients (Chen et al. 2014). In addition, Concanavalin A-induced hepatitis is associated with infiltration of CD11b$^+$GR1$^+$Ly6G$^+$Ly6C$^+$CLEC5A$^+$ immature myeloid cells into the liver, and activation of this cell population by an agonistic mAb activates eNOS leading to high levels of NO and TNF-α, thereby resulting in shock syndrome in this mouse model (Cheung et al. 2011).

Recent studies have demonstrated that DV infection activates nuclear factor Nrf2, which further upregulates CLEC5A and TNF-α expression (Cheng et al. 2016). These observations suggest that CLEC5A expression is upregulated under oxidative stress. Thus, overactivation of CLEC5A$^+$ immature myeloid cells by endogenous ligands under stressful conditions can lead to systemic inflammatory response syndrome (SIRS), and blockade of CLEC5A may represent a novel strategy for prevention of septic and aseptic shock syndrome.

3.9 Summary

CLEC5A is a myeloid Syk-coupled C-type lectin, which preferentially binds to fucose and mannose. As microbial surfaces are rich in glycans, microbes are captured by lectins, including CLEC5A. Thus, CLEC5A can form multivalent heterocomplex with other C-type lectins or TLRs, resulting in receptor activation, upon engagement with microbial and nonmicrobial antigens. Moreover, CLEC5A is involved in the pathogenesis of aseptic inflammation, suggesting that it may also recognize various fucosylated endogenous danger signals. Blockade of CLEC5A attenuates inflammatory reactions, but without downregulating antiviral immunity (since it does not influence TLR-mediated IFN production), thereby protecting the host from virus-induced systemic inflammatory reactions. In addition to infectious diseases, CLEC5A$^+$ cells mediate aseptic inflammation, e.g., in the contexts of collagen-induced arthritis and cigarette smoke-induced chronic obstructive pulmonary disease (COPD); blockade of CLEC5A inhibits the onset of arthritis and COPD. Thus, CLEC5A is a potential therapeutic target for various inflammatory diseases, and blockade of CLEC5A and associated TLRs may provide a means of protecting against both septic and aseptic inflammatory diseases.

References

Akira S (2001) Toll-like receptors and innate immunity. Adv Immunol 78:1–56

Akitsu A, Iwakura Y (2018) Interleukin-17-producing gammadelta T (gammadelta17) cells in inflammatory diseases. Immunology 155(4):418–426

Bakker AB et al (1999) Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. Proc Natl Acad Sci U S A 96(17):9792–9796

Batliner J et al (2011) CLEC5A (MDL-1) is a novel PU.1 transcriptional target during myeloid differentiation. Mol Immunol 48(4):714–719

Brinkmann V et al (2004) Neutrophil extracellular traps kill bacteria. Science 303(5663):1532–1535

Brown GD, Willment JA, Whitehead L (2018) C-type lectins in immunity and homeostasis. Nat Rev Immunol 18(6):374–389

Chen ST et al (2008) CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453(7195):672–676

Chen ST et al (2012) CLEC5A regulates Japanese encephalitis virus-induced neuroinflammation and lethality. PLoS Pathog 8(4):e1002655

Chen DY et al (2014) A potential role of myeloid DAP12-associating lectin (MDL)-1 in the regulation of inflammation in rheumatoid arthritis patients. PLoS ONE 9(1):e86105

Chen ST et al (2017) CLEC5A is a critical receptor in innate immunity against Listeria infection. Nat Commun 8(1):299

Cheng YL et al (2016) Activation of Nrf2 by the dengue virus causes an increase in CLEC5A, which enhances TNF-alpha production by mononuclear phagocytes. Sci Rep 6:32000

Cheung R et al (2011) Activation of MDL-1 (CLEC5A) on immature myeloid cells triggers lethal shock in mice. J Clin Invest 121(11):4446–4461

Dinarello CA (2004) Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. J Endotoxin Res 10(4):201–222

Edelson BT, Unanue ER (2002) MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. J Immunol 169(7):3869–3875

Ganem D, Prince AM (2004) Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 350(11):1118–1129

Gantner BN et al (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 197(9):1107–1117

Gonzalez-Dominguez E et al (2015) CD163L1 and CLEC5A discriminate subsets of human resident and inflammatory macrophages in vivo. J Leukoc Biol 98(4):453–466

Heinz FX, Allison SL (2003) Flavivirus structure and membrane fusion. Adv Virus Res 59:63–97

Hsieh SL (2016) The DAP12-assoiced myeloid C-type lectin. In: C-type lectin receptors in immunity, pp 35–49 (Chapter 3)

Huang YL et al (2016) CLEC5A is critical for dengue virus-induced osteoclast activation and bone homeostasis. J Mol Med (Berl) 94(9):1025–1037

Inui M et al (2009) Signal adaptor DAP10 associates with MDL-1 and triggers osteoclastogenesis in cooperation with DAP12. Proc Natl Acad Sci U S A 106(12):4816–4821

Joyce-Shaikh B et al (2010) Myeloid DAP12-associating lectin (MDL)-1 regulates synovial inflammation and bone erosion associated with autoimmune arthritis. J Exp Med 207(3):579–589

Krakowski M et al (2002) Granulocyte macrophage-colony stimulating factor (GM-CSF) recruits immune cells to the pancreas and delays STZ-induced diabetes. J Pathol 196(1):103–112

Lo YL et al (2016) Dengue virus infection is through a cooperative interaction between a mannose receptor and CLEC5A on macrophage as a multivalent hetero-complex. PLoS ONE 11(11):e0166474

Londrigan SL et al (2011) N-linked glycosylation facilitates sialic acid-independent attachment and entry of influenza A viruses into cells expressing DC-SIGN or L-SIGN. J Virol 85(6):2990–3000

Mackenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10(12 Suppl):S98–S109

Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1(2):135–145

Miller JL et al (2008) The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog 4(2):e17

Mocsai A, Ruland J, Tybulewicz VL (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol 10(6):387–402

Modis Y et al (2003) A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci U S A 100(12):6986–6991

Mondotte JA et al (2007) Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. J Virol 81(13):7136–7148

Netea MG, Kullberg BJ, Van der Meer JW (2000) Circulating cytokines as mediators of fever. Clin Infect Dis 31(Suppl 5):S178–S184

Ng WC et al (2014) The macrophage galactose-type lectin can function as an attachment and entry receptor for influenza virus. J Virol 88(3):1659–1672

Papotto PH et al (2018) Innately versatile: gammadelta17 T cells in inflammatory and autoimmune diseases. J Autoimmun 87:26–37

Pokidysheva E et al (2006) Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN. Cell 124(3):485–493

Reading PC, Miller JL, Anders EM (2000) Involvement of the mannose receptor in infection of macrophages by influenza virus. J Virol 74(11):5190–5197

Romagnoli PA et al (2016) IL-17A-producing resident memory γδ T cells orchestrate the innate immune response to secondary oral Listeria monocytogenes infection. Proc Natl Acad Sci U S A 113(30):8502–8507

Schlee M, Hartmann G (2016) Discriminating self from non-self in nucleic acid sensing. Nat Rev Immunol 16(9):566–580

Schroder K, Tschopp J (2010) The inflammasomes. Cell 140(6):821–832

Shimojima M et al (2014) Distinct usage of three C-type lectins by Japanese encephalitis virus: DC-SIGN, DC-SIGNR, and LSECtin. Arch Virol 159(8):2023–2031

Sierra B et al (2017) OSBPL10, RXRA and lipid metabolism confer African-ancestry protection against dengue haemorrhagic fever in admixed Cubans. PLoS Pathog 13(2):e1006220

Sung PS, Huang TF, Hsieh SL (2019) Extracellular vesicles from CLEC2-activated platelets enhance dengue virus-induced lethality via CLEC5A/TLR2. Nat Commun 10(1):2402

Tassaneetrithep B et al (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 197(7):823–829

Teng O et al (2017) CLEC5A-mediated enhancement of the inflammatory response in myeloid cells contributes to influenza virus pathogenicity in vivo. J Virol 91(1)

Upham JP et al (2010) Macrophage receptors for influenza A virus: role of the macrophage galactose-type lectin and mannose receptor in viral entry. J Virol 84(8):3730–3737

Watson AA et al (2011) Structural flexibility of the macrophage dengue virus receptor CLEC5A: implications for ligand binding and signaling. J Biol Chem 286(27):24208–24218

Wilder-Smith A, Schwartz E (2005) Dengue in travelers. N Engl J Med 353(9):924–932

Wu MF et al (2013a) CLEC5A is critical for dengue virus-induced inflammasome activation in human macrophages. Blood 121(1):95–106

Wu MF, Chen ST, Hsieh SL (2013b) Distinct regulation of dengue virus-induced inflammasome activation in human macrophage subsets. J Biomed Sci 20:36

Xavier-Carvalho C et al (2013) Single nucleotide polymorphisms in candidate genes and dengue severity in children: a case-control, functional and meta-analysis study. Infect Genet Evol 20:197–205

Xavier-Carvalho C et al (2017) Association of rs1285933 single nucleotide polymorphism in CLEC5A gene with dengue severity and its functional effects. Hum Immunol 78(10):649–656

Xu C et al (2018) Differences in clinical features and dengue severity between local and migrant Chinese with dengue infection in Singapore. PLoS ONE 13(8):e0201441

Yang YL et al (2012) Lack of association between CLEC5A gene single-nucleotide polymorphisms and Kawasaki disease in Taiwanese children. J Biomed Biotechnol 2012:398628

Zelensky AN, Gready JE (2005) The C-type lectin-like domain superfamily. FEBS J 272(24):6179–6217