Chapter 40
Targeting C-Type Lectin for the Treatment of Flavivirus Infections

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40.1 Flavivirus-Mediated Human Diseases

The genus *Flavivirus* contains approximately 70 viruses, and the major flaviviruses that cause human diseases are yellow fever virus (YFV), dengue virus (DV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus [1]. The flaviviral particles contain single-stranded, positive-sensed RNA genome packaged within an icosahedral capsid formed by the capsid protein. The genome-containing capsid is surrounded by a host-derived lipid bilayer bearing dimers of the viral envelope protein and the membrane protein. The sizes of flavivirus virions are approximately 37–50 nm. Thus, the antigenic, genetic, and three-dimensional structures of all the flaviviruses are similar to each other.

Most flavivirus infections are relatively benign, but serious aseptic meningitis and encephalitic or hemorrhagic disease can occur. The encephalitis viruses include St. Louis encephalitis virus, WNV, JEV, Murray Valley encephalitis virus, and Russian spring-summer encephalitis virus. Approximately 20% of individuals infected with WNV develop West Nile fever, and 1% may develop encephalitis, meningitis, or meningoencephalitis [2].

The hemorrhagic flaviviruses are DV and YFV. DV is a major worldwide problem, with up to 100 million cases of dengue fever (DF) and 300,000 cases of dengue hemorrhagic fever (DHF) occurring per year. The incidence of the more serious DHF has quadrupled since 1985. DF is also known as breakbone fever; the symptoms and signs consist of high fever, headache, rash, and back and bone pain that last 6–7

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days. Secondary infection with another serotype of the four related strains of DV might cause DHF and dengue shock syndrome (DSS). Nonneutralizing cross-reactive antibodies resulting from previous DV infection promote uptake of the virus into macrophages, a phenomenon known as antibody-dependent enhancement, which is one of the major factors leading to vasculature rupture, internal bleeding, and loss of plasma [2].

YFV infections are characterized by severe systemic disease, with degeneration of the liver, kidney, and heart, as well as hemorrhaging. Liver involvement causes the jaundice from which the disease gets its name. The mortality rate associated with yellow fever during an epidemic is as high as 50%.

No treatment exists for flavivirus infection other than supportive care. A live vaccine against YFV and killed vaccines against JEV and Russian spring-summer encephalitis virus are available. A tetravalent vaccine against DV is being developed to prevent the risk of immune enhancement of the disease on subsequent challenge. However, whether it can stimulate equal responses to all four serotypes in people who have been exposed to one DV serotype is unknown, and its long-term and short-term efficacy need to be further confirmed.

40.2 Glycosylation and Flavivirus Virulence

Numerous evidences have shown that the glycosylation status of envelope (E) protein is involved in the pathogenesis of flavivirus infections. The DV E protein contains two N-linked glycosylation sites, at Asn-67 and Asn-153. The glycosylation site at position 153 is conserved in most flaviviruses while the site at position 67 is thought to be unique for DVs [3]. However, this is in conflict with previous observations that the DV serotype-2 (DV2) E protein is only glycosylated at Asn-67 while the DV1 E protein is glycosylated at both Asn-67 and Asn-153 [4]. Whether both Asn-67 and Asn-153 are glycosylated in all the DV serotypes needs to be further confirmed.

It has been shown that Asn-67 of the DV E protein is essential during viral propagation. DV2 lacking Asn-67 was able to infect mammalian cells and translate and replicate the viral genome, but the production of new infectious particles from mammalian BHK cells was dramatically impaired, while DV secretion from the mosquito cell lines C6/36 was not affected. Furthermore, DVs lacking the Asn-67 showed reduced infection of immature dendritic cells, suggesting an interaction between this glycan and the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) or C-type lectin domain family 4, member L (CLEC4L) [5]. This is in accord with the finding that CLEC4L/DC-SIGN, which is abundant in immature dendritic cells, binds to DV2 (strain PR159-S1) as determined by cryo-electron microscopy [6].

A previous study indicated that the carbohydrate moiety at Asn-153 extends across the dimer interface covering the fusion peptide [7]. An N153Q DV2 mutant, which abolishes N-glycosylation at Asn-153 on the E protein, has a deleterious

effect on viral infectivity to mammalian BHK cells (but not to mosquito C6/36 cells). This suggests that the glycan at Asn-153 has a differential modulatory effect on infectivity in mammals and mosquitoes [5]. In contrast, glycosylation at Asn-154 of WNV decreased WNV infectivity in BHK and C6/36 cells (corresponding to Asn-153 in DV) [8]. Together, the results indicate that the glycans at positions 153 and 154 play different roles in DV and WNV, respectively.

For DV4, the mouse-adapted strain H241 is highly neurovirulent for mice, whereas its non-mouse-adapted parent is rarely neurovirulent. The single substitution of Ile for Thr-155 or Ala-144, which ablated the glycosylation of Asn-153 in the E protein of the parent strain, yielded a virus that was almost as neurovirulent as the mouse-adapted mutant [9]. This indicates that Asn-153 is also involved in viral virulence in mice.

For WNV, the E protein glycosylation status of the New York strain is an important determinant of virus neuroinvasiveness. To elucidate the determinant of the difference between E protein-glycosylated and nonglycosylated WNV infections, the cytokine expression of murine peritoneal macrophages infected with each virus was examined. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β were upregulated with replication of the E protein-glycosylated virus, suggesting that TNF-α and IL-1β expression are related to the virulence of E protein-glycosylated WNV [10]. It is interesting to note that the virions and subviral particles are bound to CLEC4L/DC-SIGN and CLEC4M/DC-SIGN receptor (DC-SIGNR) [11]. Unlike the two glycosylation sites found in the DV E protein, the WNV E protein only contains Asn-153, indicating that the interaction of WNV with CLEC4L and CLEC4M might be via Asn-153. Thus, DV and WNV seem to use distinct glycans to interact with DC-SIGN and DC-SIGNR.

40.3 C-Type Lectins and Flavivirus Infection

Even though in vitro studies demonstrate that the target cells of flaviviruses include monocytes, macrophages, dendritic cells, endothelial cells, and others, the information regarding in vivo replication of flaviviruses in host cells is very limited. Recently, several studies demonstrated the importance of myeloid cells in the pathogenesis of dengue infection. DV was shown to replicate in human dermal dendritic cells, the Langerhans cells [12], and monocytes/macrophages [13] isolated from human samples during acute infectious status. Replication of the flavivirus produces a double-stranded RNA replicative intermediate, which then stimulates dendritic cells and macrophages to secret both inflammatory cytokines and interferons (IFN). However, the details about how DV enters dendritic cells and monocytes/macrophages, and the machinery involved in DV recognition, were unknown until very recently.

Several C-type lectin receptors, such as CLEC4L/DC-SIGN/CD209, CLEC4M/DC-SIGNR/CD299 [14, 15], and CLEC13D/mannose receptor/CD206 [16], have been shown to interact with DV directly and play important roles in DV's entry into

dendritic cells and macrophages. To further investigate the role of the innate immunity receptors involved in DV-mediated disease, 22 fusion proteins comprising the Fc portion of human immunoglobulin G1 and the extracellular domain of innate immunity receptors – including Toll-like receptors (TLRs), C-type lectins, triggering receptor expressed on myeloid cells (TREMs), and TREM-like transcripts (TLTs) – were tested for their binding specificity to DV (Fig. 40.1). In addition to CLEC4L and CLEC4M, CLEC5A was found to bind to DV specifically in a Ca^{++}-independent manner [17].

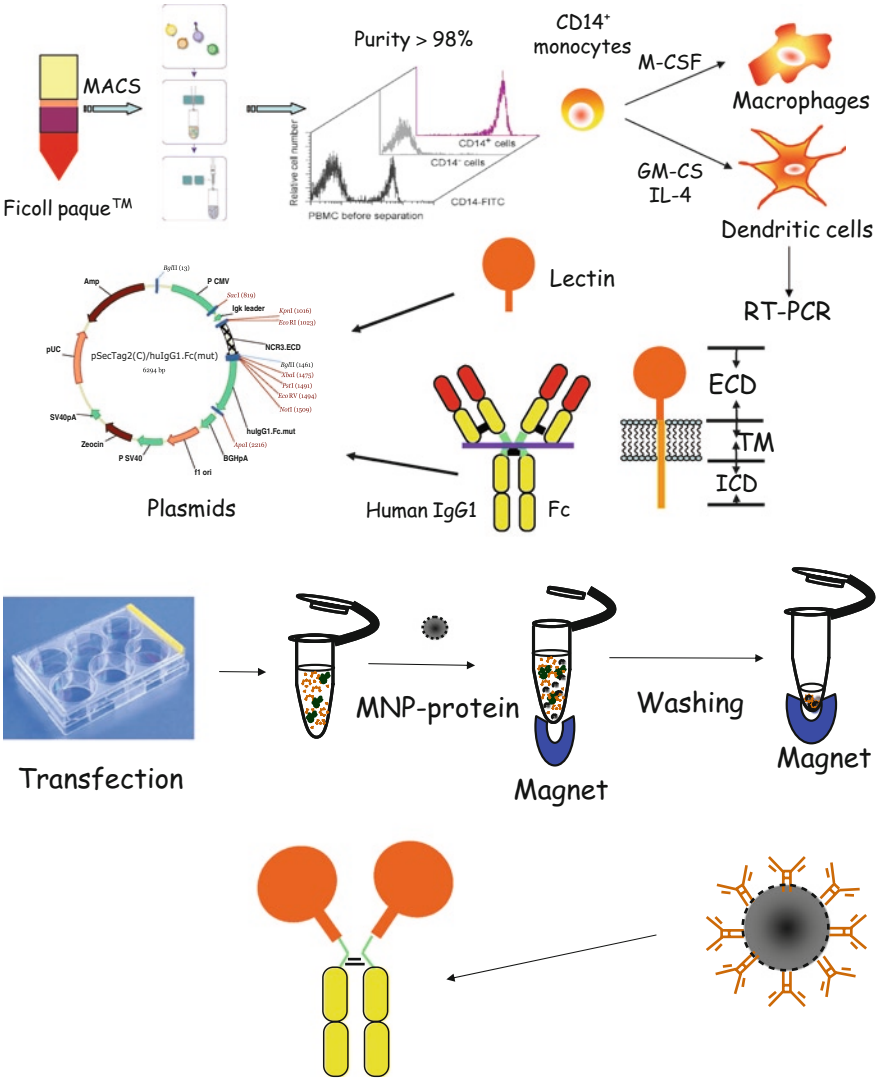

Fig. 40.1 Flow chart for the production of innate receptor Fc fusion proteins

CLEC5A is a type II transmembrane protein belonging to the C-type lectin superfamily and contains a charged residue in the transmembrane region that enables it to pair with a 12-kDa DNAX-activating protein (DAP12). DAP12 contains an immunoreceptor tyrosine-based activation motif-bearing signaling molecule that is noncovalently associated with activating isoforms of major histocompatibility complex class I receptors on natural killer cells [18]. Unlike the classic C-type lectin domain, CLEC5A contains a "natural killer T-cell C-type lectin domain" that binds sugar independently of Ca^{++} [17]. It is interesting to note that CLEC7A/Dectin-1, a C-type lectin receptor for fungal cell wall β-glucan that plays an essential role in immune responses against fungi [19], also contains a natural killer T-cell C-type lectin domain and binds to glycans independently of Ca^{++} and Mg^{++} [20].

In contrast to CLEC4L and CLEC4M, the CLEC5A–DV interaction does not result in viral entry. In contrast, DV induces DAP12 phosphorylation and stimulates the release of proinflammatory cytokines via CLEC5A. Blockade of the CLEC5A–DV interaction suppresses the secretion of proinflammatory cytokines without affecting the release of IFN-α, supporting the notion that CLEC5A acts as a signaling receptor for proinflammatory cytokine release.

To study the role of CLEC5A in the pathogenesis of DHF/DSS, DV2 (either New Guinea C-N or PL046 strain) was inoculated into signal transducer and activator of transcription-1 (STAT1)-deficient mice to induce a severe inflammatory reaction and a massive cytokine release (also called a cytokine storm), which is believed to be one of the major contributory factors for the pathogenesis of DHF/DSS [21]. Severe subcutaneous hemorrhage, neuronal damage, and shock were observed. The severe symptoms observed in STAT1-deficient mice can be attributed to at least three aspects: (1) STAT1 is essential for the activation of IFN signaling [22]; (2) the negative feedback of the TAM receptor tyrosine kinase is STAT1 dependent [23]; and (3) the binding of the WNV E protein to DC-SIGN-downregulated TLR3 expression and reduced cytokine secretion via a STAT1-mediated pathway; thus, the deficiency of STAT1 enhances the TLR3-mediated signaling cascade to augment cytokine release [24].

Under such severe inflammatory reactions, injection of anti-CLEC5A monoclonal antibodies (mAbs) still inhibits DV-induced plasma leakage, as well as subcutaneous and vital-organ hemorrhaging, and reduces the mortality of DV infection by about 50% in STAT1-deficient mice. This observation indicates that blockade of CLEC5A-mediated signaling attenuates the production of proinflammatory cytokines by macrophages infected with DV (either alone or complexed with an enhancing antibody), without damping the secretion of IFN-α, which is essential for antiviral immunity [17].

40.4 C-Type Lectin and the Severity of DV Infections

After incubation of the DV2 PL046 strain with $CD14^{+}$-derived macrophages randomly obtained from the blood bank, variations in the infection rate and cytokine release were observed. Among the 200 samples tested, the infection rates can be

classified into three categories: (1) 80–50% (approximately 50 out of 200 cases); (2) 20–40% (approximately 100 out of 200 cases); and (3) less than 10% (approximately 50 out of 200 cases). This suggests that certain unidentified host factors might determine the different DV infection rates in human macrophages.

Given that the glycosylation status of DV influences virus replication and propagation; that members of C-type lectins (CLEC4L [14], CLEC4M [15], and CLEC13D [16]) are involved in DV entry; and that CLEC5A is responsible for DV-induced inflammatory reactions [17], it is reasonable to speculate that the polymorphism of C-type lectins might play an important role in DV infections.

It has been demonstrated that variation of the CLEC4L promoter (G allele at 336) correlates with dominant protection against DF but not DHF. The expected decrease in expression of CLEC4L carrying the G allele at CLEC4L-336 may result in a lower susceptibility of dendritic cells to DV in the early stages of infection [25]. However, the correlation between dengue infections and CLEC4M, CLEC13D, and CLEC5A has not been investigated yet.

40.5 Targeting CLEC5A for the Treatment of DV Infections

The dilemma to control severe inflammatory reactions is that suppression of inflammatory cytokine release also attenuates antiviral immunity. Steroid treatment is ineffective at controlling viral spread and decreasing mortality in victims of severe acute respiratory syndrome. TLRs are involved in the production of both proinflammatory (such as TNF-α) and antiviral (such as IFN) cytokines, thus apparently they are not ideal candidates for controlling virus-induced inflammatory reactions. In contrast, members of C-type lectins are not involved in IFN production. Therefore, blockade of the virus–lectin interaction offers a promising strategy for alleviating tissue damage and increasing the survival of patients suffering from DHF and DSS, and possibly even other virus-induced inflammatory diseases (Fig. 40.2).

40.6 Summary

It becomes clear that multiple receptors and intracellular sensors are involved in host–pathogen interactions. In addition to TLRs, the non-TLRs – including C-type lectins, TREMs, and TLTs – are promising candidates for recognizing pathogen-associated molecular patterns. Considering that glycosylation is one of the most common and complex posttranslational modifications and that pathogens are covered by glycans, it is obvious that the first contact between innate immunity receptors and pathogens depends on a protein–carbohydrate interaction. Among the acute viral infections, the pathogenesis of viral hemorrhagic fever is still unclear, but a cytokine storm is believed to play a critical role in such a highly lethal disease. Our recent experiments demonstrate that the dengue virion acts as a ligand to bind CLEC5A (also know as myeloid DAP-12-associating lectin), and possibly other

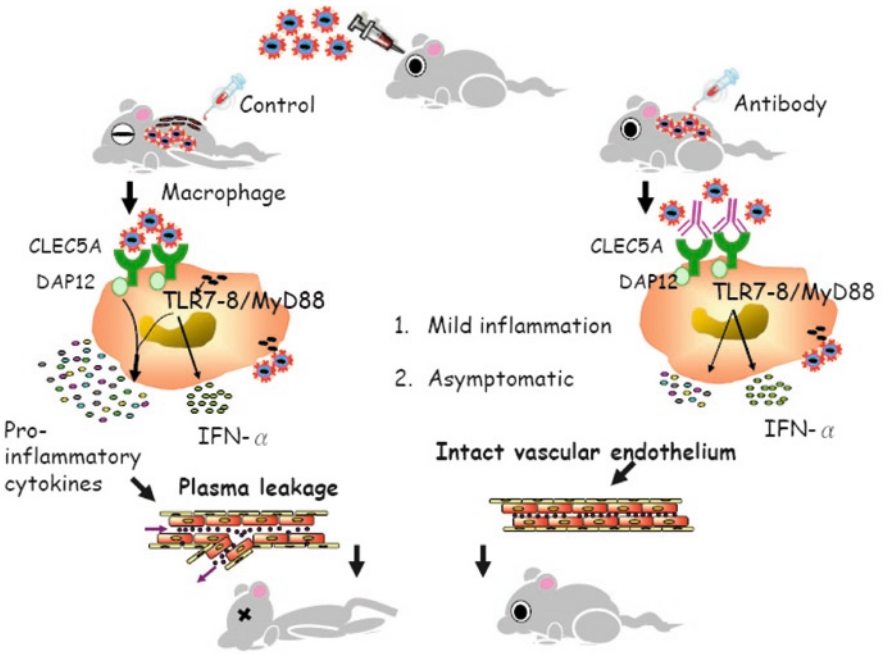

Fig. 40.2 Inhibition of proinflammatory cytokine secretion by antagonistic anti-CLEC5A mAb without attenuating IFN-α secretion

non-TLRs, directly. DV induces massive cytokine secretion via CLEC5A, and blockade of CLEC5A attenuates inflammatory reactions without damping antiviral immunity. Moreover, anti-CLEC5A antagonistic mAbs protect mice from DV-induced hemorrhaging and shock syndrome. The segregation of proinflammatory reaction from antiviral immune response provides a great chance to design a better strategy for treating viral infections in the future.

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