INSIGHTS

A dark side to NS1 antibodies?

Davide F. Robbiani[1] and Daniel Růžek[2,3]

The NS1 protein of flaviviruses is taking center stage. Recent work has made it an attractive target for development of vaccines and immunotherapeutics. Cavazzoni and colleagues (2021. _J. Exp. Med._ https://doi.org/10.1084/jem.20210580) now reveal a dark side to NS1, linking it to the development of self-reactive antibodies.

Flaviviruses are typically transmitted by arthropods. For example, dengue, Zika, and West Nile viruses are transmitted by mosquitoes, while tick-borne encephalitis and Powassan viruses go through ticks (Gould and Solomon, 2008). Although infection is often unnoticed, it can cause symptoms ranging from mild to severe and can even be lethal. For example, it is estimated that up to nearly half a billion people are infected with dengue virus (DENV) every year, of which several hundreds of thousands develop severe disease with a fatality rate of ~5% in treated patients (Gyawali et al., 2016). Acute infection by some flaviviruses can additionally be associated with autoimmune manifestations of neurological (Guillain-Barré syndrome [GBS]) and other natures that typically emerge a few weeks after recovery (Grijalva et al., 2020).

Flaviviruses have single-stranded RNA genomes encoding a single polyprotein subsequently cleaved into three structural and seven nonstructural proteins (Lindenbach and Rice, 2003). The structural proteins (capsid, premembrane/membrane and envelope [C-prM/M-E]) are those that form the viral particle. C packages the viral genome, forming a nucleocapsid, which is surrounded by a lipid bilayer containing M and E. Translation of the viral genome by infected cells produces, in addition to C-prM/M-E, the nonstructural proteins (NS1–NS5). Intracellular NS1 plays essential roles in virus replication. A fraction of NS1 is trafficked to the infected cell surface, where it associates with lipid rafts. Furthermore, NS1 is secreted in the form of soluble hexamers by the infected cells, resulting in it being detected in the blood of infected individuals. Several functions have been attributed to extracellular NS1. For example, it has the ability to damage endothelial cells, leading to vascular leakage (e.g., in dengue; Beatty et al., 2015), and it is involved in host immune evasion by triggering degradation of the complement protein C4 (Avirutnan et al., 2010). It can also induce autoantibodies that cross-react with host cell components (Lin et al., 2008). Thus, at least for some flaviviruses, NS1 may contribute to pathogenesis (Carpio and Barrett, 2021).

Over 30 flaviviruses can cause disease in humans, but vaccines are available against only a few of them (Gould and Solomon, 2008; Ishikawa et al., 2014). Vaccine development has been hindered by safety concerns linked to the fact that flaviviruses are structurally similar to each other. Vaccination (or primary flavivirus infection) will induce antibodies that are protective if exposed to the same flavivirus in the future. However, if the exposure is not to the same, but to a related flavivirus, then some of the antibodies may still bind but fail to sufficiently block the infection, paradoxically leading to more severe disease, a phenomenon referred to as antibody-dependent enhancement (ADE). Based on in vitro experiments, the proposed mechanism underlying ADE involves Fc-receptor bearing monocytic cells that are not infectable unless the virus is bound by anti-E antibodies. Since E mediates virus entry and is the target of neutralizing antibodies, the common approach has been to include E as a vaccine antigen, despite the risk of predisposing to ADE of heterologous flaviviruses (Rey et al., 2018).

Pioneering work in the 1980s (Gould et al., 1986; Schlesinger et al., 1985) demonstrated that passive transfer of NS1-specific monoclonal antibodies or vaccination with purified NS1 is efficacious against disease in lethally infected mice, supporting the applicability of NS1 as vaccine immunogen. NS1 vaccination is not sterilizing (it does not prevent infection). However, it appears efficacious, presumably because it enables rapid clearance of infected cells and/or it combats the pathogenic effects of NS1, such as through the endothelial damage mentioned earlier, through complement-mediated lysis of the infected cells after antibody recognition of cell surface–associated NS1, and likely through additional mechanisms. Importantly, since anti-NS1 antibodies are unable

Insights from Davide F. Robbiani and Daniel Růžek.

[1]Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland; [2]Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Ceske Budejovice, Czech Republic; [3]Veterinary Research Institute, Brno, Czech Republic.

Davide F. Robbiani: drobbiani@irb.usi.ch; Daniel Růžek: ruzekd@paru.cas.cz.

to bind to the virion, NS1-based vaccines would circumvent the safety concerns related to ADE. Moreover, anti-NS1 monoclonal antibodies have considerable potential as therapeutic in symptomatic individuals (Biering et al., 2021; Modhiran et al., 2021).

The study by Cavazzoni and colleagues in this issue of *JEM* extends our knowledge about the immune response to NS1, while at the same time casting some shadows on NS1-based vaccination strategies (Cavazzoni et al., 2021). The authors first infected immunocompetent mice with high-dose Zika virus (ZIKV; 10^6–10^7 PFUs) and measured serum antibody reactivity over time. The response to NS1 was immunodominant and depended on virus replication (as one would expect). Strikingly, infection was also accompanied by the appearance in serum of IgG antibodies reactive against multiple self-antigens in brain and muscle. Rather than being transient, the self-reactivity increased over the 7 wk of observation, paralleling the increase of anti-NS1 antibodies. Confirming this finding, autoreactivity was also observed in antibodies derived from ex vivo cultured B cells isolated from the infected mice germinal centers (microanatomical structures in the spleen and lymph nodes where the antibodies "mature" through somatic hypermutation and isotype switching).

Given that self-reactivity was observed only in the presence of virus replication, which is required for nonstructural protein production, the authors hypothesized that NS1 alone could be sufficient to induce autoantibodies. To test this, mice were vaccinated with recombinant ZIKV NS1 protein or with the NS1 protein of DENV as control. Several features of the antibodies derived from germinal center B cells that were induced by vaccination were similar between the two groups: V_H gene usage, number of mutations, length of CDRH3 (complementarity determining region 3 of the antibody heavy chain). Differences were also noted: more charged amino acids at CDRH3 with ZIKV NS1, a feature previously associated with self-reactivity (Radic and Weigert, 1994). The CDRH3 is the most variable portion of the antibody and mediates binding to the antigen.

Single germinal center B cell cultures were then derived, which enabled them to assay both the antibody's reactivity and sequence from each individual B cell. Out of the nearly 300 monoclonal antibodies that were derived from ZIKV NS1 vaccinated mice, a large fraction bound to the immunogen (30% on day 10, 50% on day 21 after infection). In agreement with the earlier analysis, the CDRH3s bearing charged amino acids were more frequent upon ZIKV NS1 compared with DENV NS1 vaccination, regardless of whether the clone was binding to NS1 or not. How about self-reactivity? None of the monoclonal antibodies derived from DENV NS1 vaccinated mice were autoreactive, and neither were those from mice immunized with an irrelevant antigen (OVA). In contrast, a sizeable fraction of those induced by ZIKV NS1 vaccination recognized self-antigens (~20% on day 14 and 40% on day 21 after immunization). Most of the autoreactive antibodies were ZIKV NS1 binders, and self-reactivity was observed also in antibodies that were not highly charged at the CDRH3.

Although generally rare, infection-related autoimmunity is being recognized during or following an increasing number of viral and nonviral diseases, including COVID-19. The mechanisms underlying the breakage of tolerance are only partly understood but may result in the development of autoantibodies. Cavazzoni and colleagues contribute a novel experimental system to study autoantibody development, a system in which self-reacting antibodies are induced by infection with a virus, Zika, that is well-known to cause post-infectious autoimmune disease, particularly GBS. Importantly, the authors show that infection is not required, since immunization with a single viral protein, NS1, is sufficient. This work raises several questions. How does ZIKV NS1 do this? Is it homology to endogenous mouse proteins? Does it interfere with the germinal center reaction or with the checkpoints at which self-reactivity is censored? Does the genetic background contribute?

The relevance of the findings for human disease remains unclear at this point. There is no evidence that the autoreactive mouse antibodies are pathogenic, nor that the same antibodies cross-react with human tissue extracts (although they do cross-react with the Hep-2 human cell line). It will be interesting to determine if similar tissue cross-reactivity occurs with convalescent human sera, comparing ZIKV to DENV infection, and if the cross-reactivity is higher or the pattern different in those patients that develop GBS.

Changes in climate are facilitating the spread of mosquitoes and ticks to new regions, bringing with them the disease agents that they carry. As demonstrated by the recent outbreaks of West Nile and Zika viruses in the Americas, flaviviruses bear epidemic potential and are therefore a present and growing threat to global health. Still, many flaviviruses lack effective medical countermeasures or vaccines. Flavivirus NS1 represents a promising candidate immunogen for the next generation of flaviviral vaccines. However, the recent work by Cavazzoni and colleagues indicates that the development of NS1-based vaccine candidates may not be without significant difficulties and that the potential risk for induction of self-reactive antibodies needs to be properly addressed.

References

Avirutnan, P., et al. 2010. *J. Exp. Med.* https://doi.org/10.1084/jem.20092545

Beatty, P.R., et al. 2015. *Sci. Transl. Med.* https://doi.org/10.1126/scitranslmed.aaa3787

Biering, S.B., et al. 2021. *Science.* https://doi.org/10.1126/science.abc0476

Carpio, K.L., and A.D.T. Barrett. 2021. *Vaccines (Basel).* https://doi.org/10.3390/vaccines9060622

Cavazzoni, C.B., et al. 2021. *J. Exp. Med.* https://doi.org/10.1084/jem.20210580

Gould, E.A., and T. Solomon. 2008. *Lancet.* https://doi.org/10.1016/S0140-6736(08)60238-X

Gould, E.A., et al. 1986. *J. Gen. Virol.* https://doi.org/10.1099/0022-1317-67-3-591

Grijalva, I., et al. 2020. *PLoS Negl. Trop. Dis.* https://doi.org/10.1371/journal.pntd.0008032

Gyawali, N., et al. 2016. *J. Vector Borne Dis.* 53: 293–304.

Ishikawa, T., et al. 2014. *Vaccine.* https://doi.org/10.1016/j.vaccine.2014.01.040

Lin, C.F., et al. 2008. *Lab. Invest.* https://doi.org/10.1038/labinvest.2008.70

Lindenbach, B.D., and C.M. Rice. 2003. *Adv. Virus Res.* https://doi.org/10.1016/S0065-3527(03)59002-9

Modhiran, N., et al. 2021. *Science.* https://doi.org/10.1126/science.abb9425

Radic, M.Z., and M. Weigert. 1994. *Annu. Rev. Immunol.* https://doi.org/10.1146/annurev.iy.12.040194.002415

Rey, F.A., et al. 2018. *EMBO Rep.* https://doi.org/10.15252/embr.201745302

Schlesinger, J.J., et al. 1985. *J. Immunol.* 135: 2805–2809.