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An Overview of the Immune Evasion Strategies Adopted by Different Viruses with Special Reference to Classical Swine Fever Virus

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1. Introduction

Viruses are considered as extremely successful predators as they can replicate and control the host cell synthesizing machinery. Viruses have coevolved with their hosts and thus have limited pathogenicity in any immunocompromised natural host. Viruses can exist in two forms: extra cellular virion particles and intracellular genomes. Virions are more resistant to physical stress than genomes but are susceptible to humoral immune control. Nevertheless, to exist as a species, virus replication and transfer to a new host are essential. These processes are associated with the production of antigenic proteins that make the virus vulnerable to immune control mechanisms 'warning' the host of the presence of an invader [1]. There are two classes of viral immunoregulatory proteins: the proteins encoded by genes having sequence similarity with cellular genes and those coded by genes without any sequence similarity to cellular genes. The second class of protein may represent a paradigm for co-evolution [2]. During the period of coexistence with their hosts, viruses have learned how to manipulate host immune control mechanism. It is well established that the viruses have evolved wide variety of immune evasion strategies *viz.*, evasion by noncytotoxic infection (Arena and Hanta viruses), evasion by cell to cell spread (Canine distemper virus and cytomegalovirus), evasion by infection of nonpermissive, resting or undifferentiated cells (herpes virus induced latency), evasion by infection with restricted viral gene expression by destruction of immune effector cells and macrophages (destruction of CD4⁺ T lymphocytes by HIV 1 and 2 viruses), evasion by downregulation of MHC – antigen expression (betaherpesviruses), evasion from cytokine action (Adenoviral infected cells evade the action of TNF through viral gene products), masking of epitopes and immune decoy (Ebola virus), evasion by induction of nonneutralizing antibodies (Aleutian Mink

disease virus), evasion by induction of immunologic tolerance (congenital infections like Bovine Viral diarrhea, arena virus infections and some retro virus infections), evasion by sequestration in immunologically privileged tissues (replication of cytomegaloviruses in the kidney, salivary glands and mammary glands), evasion by integration of viral genome into host cell genome (induction of prophage in case of retro viral infection) and evasion by genetic drift (Maedi/Visna, Equine Infectious Anaemia) [2, 3]. The present review will highlight the different complex mechanisms associated with the host immune evasion by the viruses with special reference to the Classical Swine Fever Virus.

2. Newer concepts in the evasion of host defense by viruses

The main sensors of the innate immune response are pattern recognition receptors (PRR) which can recognize pathogen associated molecular patterns (PAMPs). This recognition leads to the expression of cytokines, chemokines and co-stimulatory molecules that eliminate pathogens like viruses for the activation of antigen presenting cells and for the activation of specific adaptive response [4]. Among the PRRs, there are Toll Like Receptors (TLRs) that can be either endosomal or extracellular [5, 6] and retinoic acid-inducible gene-(RIG-I)/MDA5 (melanoma differentiation-associated gene) [7] known as RNA helicase-like receptors (RLRs). Further, Double-stranded RNA-dependent protein kinase (PKR), 2', 5'-oligoadenylate synthetase (2'- 5' OAS), and adenosine deaminase acting on RNA (ADAR), known as effector proteins, complement the function of PRRs. All these proteins are responsible for recognizing viral components and induce proinflammatory cytokine expression or interferon (IFN) response factors. There are certain cellular components which are manipulated by viruses to evade the innate immune response. Expression of type-I IFN depends on the activation of Interferon Regulatory Factor - 3 (IRF3) and IRF7 via I kappa B kinase (IKK) epsilon and Tank Binding Kinase 1 (TBK1). The genome of Rabies virus, Borna disease virus and Ebola virus code for the P phosphoprotein and VP35 that can block the antiviral response induced by IFN [8, 9, 10]. In contrast, the human herpes simplex virus 8 encodes different analogs of IRF with negative dominant activity, allowing it to interfere with the activity of cellular IRFs [11]. The infected cell polypeptide 0 (ICP0) from Bovine herpes virus can interact with IRF3 and induce its proteasome-dependent degradation [12]. Similarly, the V protein of paramyxoviruses interacts with MDA5- α and inhibits IFN- α expression [13].

One of the major non-specific humoral defense mechanisms of the body for combating and clearing the infectious agents is complement system [14, 15, 16]. Viruses encode homologs of complement regulatory proteins that are secreted and block complement activation and neutralization of virus particles. The cowpox virus (CPV) complement inhibitor, termed inflammation modulatory protein (IMP), blocks immunopathological tissue damage at the site of infection, presumably by inhibiting production of the macrophage chemo attractant factors C3a and C5a. Viruses protect the membranes of infected cells and the lipid envelopes of virus particles from complement lysis by encoding homologs of inhibitors of the membrane-attack complex. Human cytomegalovirus (HCMV), HIV and vaccinia virus (VV) used to borrow different host cellular factors, such as CD59, to protect from complement action. Moreover, some viruses encode Fc receptors [17], thus inducing antibody response.

These antibodies may kill infected cells by complement-mediated cytolysis or by antibody-dependent cell-mediated cytotoxicity (ADCC).

In case of FMD virus, following a 5' untranslated region known as the S fragment, there is poly "C" tract comprising over 90 per cent 'C' residues [18]. The length of this tract is extremely variable [19]. There are some evidences that length of this tract is associated with virulence and persistence of infections [20].

There is also evidence of viral interference with interferons. Interferons were discovered because of their ability to protect cells from viral infection. The key role of both type I (α and β) and type II (γ) IFNs as one of the first anti-viral defense mechanisms is indicated by the fact that anti-IFN strategies are present in most viruses. Viruses block IFN-induced transcriptional responses and the Janus Kinase (JAK) / signal transducers and activators of transcription (STAT) signal transduction pathways also inhibit the activation of IFN effector pathways that induce an anti-viral state in the cell and limit virus replication. This is mainly achieved by inhibiting double-stranded (ds)-RNA-dependent protein kinase (PKR) activation. Once active, the PKR causes phosphorylation of eukaryotic translation initiation factor 2a (eIF-2a) and the RNase L system, which are responsible for degrading viral RNA and translation in the host cell. Moreover, active PKR is also able to mediate the activation of the transcription factor NF κ B which upregulates the expression of interferon cytokines, which work to spread the antiviral signal locally. In addition, active PKR is also able to induce cellular apoptosis. All these mechanisms due to PKR activation ultimately leads to inhibition of the spread of viral infection. But inhibition of PKR activation causes the viral infection to spread and thus helps in evasion of the immune system. Secreted cytokine receptors or binding proteins are mainly encoded by Poxviruses which actually encode soluble versions of receptors for IFN- α and - β (IFN- α /bR) and IFN- γ (IFN- γ R), which also block the immune functions of IFNs 6. The IFN- α /bR secreted by Vaccinia virus (VV) is also localized at the cell surface to protect cells from IFN [21, 22]. Additionally, several viruses inhibit the activity of IFN- γ , a key activator of cellular immunity, by blocking the synthesis or activity of factors required for its production, such as interleukin (IL)-18 or IL-12. CPV cytokine response modifier (Crm) A inhibits caspase-1, which processes the mature forms of IL-1b and IL-18 [23]; various poxviruses encode soluble IL-18-binding proteins (IL-18BPs) [24]; measles virus (MeV) binds CD46 in macrophages and inhibits IL-12 production [15]; herpes viruses and poxviruses express IL-10 homologs that diminish the Th1 response by downregulating the production of IL-12 [25, 26].

Cytokines play a key role in the initiation and regulation of the innate and adaptive immune responses, and viruses have learned how to block cytokine production, activity and signal transduction. African swine fever virus (ASFV) replicates in macrophages and encodes an I κ B homolog that blocks cytokine expression mediated by nuclear factor (NF)- κ B and the nuclear factor activated T cell (NFAT) transcription factors 13. Many viruses block signal transduction by ligands of the tumor necrosis factor (TNF) family, whereas others deliberately induce some cytokine pathways; For example, the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) recruits components of the TNF receptor (TNFR) and CD40 transduction machinery to mimic cytokine responses that could be beneficial for the

virus, such as cell proliferation [27]. One of the most interesting mechanisms identified in recent years is the mimicry of cytokines (virokines) and cytokine receptors (viroceptors) by large DNA viruses like herpesviruses and poxviruses [28, 29]. The functions of these molecules in the animal host are diverse. Soluble viral cytokine receptors might neutralize cytokine activity and cytokine homologs might redirect the immune response for the benefit of the virus. Alternatively, viruses that infect immune cells might use these homologs to induce signalling pathways in the infected cell that promote virus replication. The herpesvirus cytokine homologs vIL-6 and vIL-17 might have immunomodulatory activity but might also increase proliferation of cells that are targets for viral replication [28]. Viral semaphoring homologs have uncovered a role for the semaphorin family, previously known as chemoattractants or chemorepellents involved in axonal guidance during development in the immune system, and have identified a semaphorin receptor in macrophages that mediates cytokine production [30, 31].

Apoptosis, or programmed cell death, can be triggered by a variety of inducers, including ligands of the TNF family, irradiation, cell cycle inhibitors or infectious agents such as viruses. The cellular proteins implicated in the control of apoptosis are targeted by viral anti-apoptotic mechanisms [32, 33]. Viruses inhibit activation of caspases: encode homologs of the anti-apoptotic protein Bcl-2, block apoptotic signals triggered by activation of TNFR family members by encoding death-effector-domain-containing proteins and inactivate IFN-induced PKR and the tumor suppressor p53, both of which promote apoptosis. Epstein-Barr virus and oncogenic human herpes viruses use Bcl-2 orthologs like BHRF1 and BALF-1 to block mitochondrial release of cytochrome c [34, 35]. Mouse γ -herpesvirus (MHV) -68 encodes a Bcl-2 ortholog (MHVBcl-2) that protects the infected cell against TNF-mediated apoptosis [36]. An alternative mechanism is provided by the glutathione peroxidase of mollusum contagiosum virus (MCV), which provides protection from peroxide or UV induced apoptosis and perhaps from peroxides induced by TNF, macrophages or neutrophils.

Infection with the human and simian immunodeficiency viruses are unique in that the infections give rise to prolonged, continuous viral replication in the infected host. Destruction of virus-specific T helper cells, the emergence of antigenic escape variants and the expression of an envelope complex that structurally minimizes antibody escape to conserved epitopes contribute to persistence. Moreover, the virus encoded protein Nef prevents the viral antigen presentation [37].

3. Recognition of CSFV by immune system

Amidst the diversified mechanisms evolved by different viruses to evade the host immunity (innate or adaptive), CSFV plays a unique role in evading the host defense and maintain the infection. The virus expresses two major PAMPs: the ssRNA genome and the dsRNA replication intermediates. The TLR's sensing such patterns are located in the endosomal compartment [38] or in the cytoplasm in case of the cellular helicases Retinoic acid-Inducible Gene 1 (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA-5) [39]. TLR3 binds dsRNA [40, 41], whereas TLR7 recognizes ssRNA [42, 43]. Conventional DC mainly

expresses TLR3 [44] while plasmacytoid DC (pDC) express TLR7 [45]. RIG-I and MDA-5 both bind dsRNA. Recently however it was shown that RIG-I can sense uncapped viral single stranded RNA bearing a 5'-triphosphate [46, 47]. The stimulation of TLR3 leads to the activation of NFkB (early NFkB response) or to the activation of IRF3, which in turn upregulates type I IFN transcription and subsequently transcription of NFkB (late NFkB response) [48]. TLR7 stimulation leads to the activation of IRF7 but not of IRF3 [49]. Thus there is induction of type I interferons and various pro-inflammatory cytokines which play crucial role in antiviral host immune responses. Understimulation of any of these two TLR's (*i.e.*, either TLR 3 or 7) leads to down regulation of host immune response and over stimulation leads to exaggerated immune response.

4. Few salient features about the disease Classical Swine Fever

Classical swine fever (CSF) is a disease of domestic pigs and wild boar caused by CSF virus (CSFV). CSFV, first reported in the United States in 1833 causes important economical losses worldwide. Besides the United States of America, only Australia, Canada, Ireland, New Zealand, the Scandinavian countries and Switzerland are currently considered free of CSFV. In Europe the recent outbreaks occurred in Bulgaria Croatia and Germany in the year 2006 [50].

The natural reservoir for CSFV is the wild boar, which remains the major threat for new outbreaks. The virus is endemic in most of the Eastern European countries but the domestic pig population of Western Europe can be considered free from the disease. The control measures for CSFV include stamping out with a non-vaccination policy. Consequently pigs have to be free of virus and antibody against CSFV. Whether seroconversion results from vaccination or disease, pigs seropositive for CSFV must be eliminated. Acute or endemic CSF in domestic pigs has large economic impact on general restriction on pig meat trade [51]. The outbreak of CSF, and occurrences of CSFV in the tissues of pigs were reported from India as well [52].

There are three distinct genogroups of the virus (*viz.*, 1, 2 and 3) with three or four subgroups [53, 54]. Even though group 1 viruses are predominant in India, group 2 viruses are also rapidly spreading and may form a major threat in future [55].

Early stage of the disease (CSF) is characterized by fever and diarrhoea. The gradual progression of the disease results in a severe wasting syndrome. The terminal stage is signified by a blue discoloration of the skin and weakness of the hind legs along with neurological symptoms. Autopsy finding includes disseminated intravascular coagulopathy, extensive tissue hemorrhages and thymus atrophy [56].

5. A few salient features of the structure, composition and function of the CSFV genome

Classical swine fever virus (CSFV) is a member of the family *Flaviviridae*, genus *Pestivirus* [57]. The species consist of small, spherical enveloped viruses with an approximate diameter

of 40-60 nm based around an electron-dense inner core structure of about 30 nm [58]. The virus bears a single stranded positive sense RNA molecule spanning approximately 12.5 kbp and is made up of a single open reading frame (ORF) flanked by a 3' and 5' nontranslated region (NTR), the latter contains conserved regions implicated in the translational events [59, 60]. Notwithstanding the fact that the virus has a RNA genome, it is reported to be relatively stable [61]. Nevertheless, a recent study [62] indicated that recombination between strains is possible. The ORF is translated into a single polypeptide of about 3900 amino acids which is co- and post-translationally processed into mature peptide by a number of virus and host encoded proteases [63, 64, 65, 66]. The virion is made up of 4 structural proteins *viz.*, C, Erns, E1 and E2 which are encoded at the 5' end of the genome. The spherical nucleocapsid coat of the virus is composed of numerous proteins while the surface is made out of Erns, E1 and E2 in homodimeric (Erns, E2) or heterodimeric (E1E2) form [67, 68]. E1 and E2 consist of transmembrane domains whereas Erns has no transmembrane spanning domain and its attachment to the virion is rather tenuous. In addition to the structural proteins, the CSFV viral genome encodes further 8 non-structural proteins, including an N-terminal protease (Npro), p7, the non-structural proteins (NS) 2, 3, 4A, 4B, 5A and finally 5B [64, 69].

CSFV is normally a noncytopathogenic (ncp) virus. A rare cytopathogenic (cp) form can occur spontaneously in cell culture [70] and has also been found in wild boar [71]. Its significance in CSFV pathogenesis is unknown. The CSFV genome consists of single stranded positive sense RNA. This RNA carries a single large open reading frame (ORF) flanked by a 5' and a 3' non-translated region (NTR). The NTR at the 5' end harbours an internal ribosome entry site [72, 73, 74]. Therefore the RNA can directly undergo cap independent translation upon uncoating. The large ORF encodes a single polyprotein which is co and post-translationally cleaved into altogether 12 structural and non-structural proteins including N^{pro} (the first protein encoded by the ORF) by either cellular signalases or viral proteases [75]. It exhibits auto protease activity and cleaves itself from the nascent polyprotein [76]. N^{pro} is the only viral gene that can be deleted without altering virus replication [77]. There is also report of counteraction of the type I IFN induction pathway by N^{pro} [78, 79, 80] by down-regulating the expression levels of the interferon regulatory factor 3 (IRF3) [81, 82]. IRF3 is the rate limiting component of the INF- β promotor enhanceosome and thus regulates the transcriptional activity of this gene [83, 84]. The second protein translated by the ORF is the capsid protein C (Core). It contains the E^{ns} signal sequence [85] and a signalase recognition site [86]. The C gene is followed by the other three structural genes Erns, E1 and E2, the three envelope proteins of CSFV. All these proteins are cleaved by signalases [86]. E^{ns} exists in secreted form [87]. It exhibits RNase activity *in vitro* [85]. It remains unclear whether this RNase activity has a specific role in the life cycle of CSFV. A lymphotoxic function of the secreted Erns has been reported [88]. More recently it has been shown that Erns of BVDV is involved in the inhibition of dsRNA-mediated type I IFN induction [89]. A very recent report proposed a cooperative effect of N^{pro} and Erns of BVDV on transplacental infection in cattle [90]. Encoded downstream of envelope protein gene E1, the glycoprotein E2 harbours the major immunogenic epitopes. The antigenic region of E2 was divided in the three domains A, B and C based on analysis using monoclonal antibodies (mAb) [91, 92]. E1 and E2 form either homodimers or heterodimers. They both contain

transmembrane regions that anchor the glycoproteins in the viral envelope. Erns has no transmembrane region and is associated with the envelope by interaction with E1 and/or E2 or by hydrophobic interactions with the membrane. The p7 protein is not part of the virion but was found to be essential for virus assembly [93]. Protein p7 of the closely related hepatitis C virus (HCV) forms an ion channel [94]. It is not clear yet whether p7 of CSFV has the same function. The non-structural gene products are cleaved by the NS2 autoprotease between NS2 and NS3 [95] and by the NS3 protease at the downstream cleavage sites [96, 97, 98]. NS2 is an inducible autoprotease that is activated by four cellular proteins [99, 100]. Enhanced cleavage between NS2 and NS3 correlates with the appearance of the cp biotype [101]. The uncleaved NS2-3 protein is essential for the formation of viral particles [101, 102]. The cleaved NS3 protein is produced essentially during the first few hours post-infection. Besides being a protease the NS3 protein has also helicase [103, 104] and NTPase activity [105, 106]. The NS4A protein is an essential co-factor of the NS3 protease [107]. NS4B is assumed to be a co-factor of the RNA-dependent RNA-polymerase encoded by the NS5B gene. This RNA-polymerase contains a GDD (Glycine-D-aspartate-D-aspartate) active site motif, otherwise known as the motif c [108]. The binding and entry of pestiviruses is a multistep process involving initial attachment of virions, interaction with specific receptor(s), internalization, and membrane fusion [109, 110, 111, 112]. The surface protein CD46 was proposed as receptor for BVDV [104]. Specific cell surface receptors for CSFV have not yet been identified. It has been shown that recombinant E2, E1 and Erns can independently bind to the cell surface [113, 114]. E2 adsorption competitively inhibits infection with homotypic and heterotypic pestiviruses [115]. After capsid uncoating, RNA replication and translation takes place in so-called replication complexes. These complexes have been well characterized for the closely related hepatitis C virus [116] and for some members of the genus *Flavivirus* [117]. The assembly pathway of pestiviruses is poorly understood. As mentioned above the uncleaved NS2-3 precursor protein in association with NS4A are essential for particles formation [101, 118]. Several studies on different pestiviruses have revealed that NS4B is an endoplasmic reticulum (ER)-associated integral membrane protein that contains four putative transmembrane domains flanked by cytoplasmic N- and C-terminal regions [119, 120, 121, 122]. Interaction of CSFV NS4B with molecular components of the immune system has also been reported [123].

6. N^{pro} and its role in induction of poly (IC) induced antiviral activities

The first protein encoded is the non-structural protein N^{pro}. The gene coding for this protein is the only non-essential gene in the pestivirus life cycle [124]. It exhibits autoproteolytical activity and cleaves itself off the downstream nucleocapsid protein C [125, 126, 127]. When CSFV, BVDV and BDV are compared, the amino acid sequence identity of N^{pro} is found to be higher than 70 per cent [128] and the residues Glu22, His49, and Cys69 are essential for the proteolytic activity of N^{pro} [125]. Moreover, the residues Cys168 and Ser 169 surrounding the cleavage sites are also conserved [126]. Resistance to poly(IC)-induced cell death and control of IFN induction are dependent on the presence of the N^{pro} gene, indicating a function of N^{pro} in innate immune evasion of CSFV [129]. The characterisation of N^{pro} gene is also found to be beneficial for the development of inactivated vaccine [130].

7. Immune evasion and immunopathogenesis of CSF

CSF virus (CSFV) has high affinity for vascular endothelial cells and lymphoreticular cells including T cells, B cells and monocytes [122]. Severe depletion of B cells and T cells in Peripheral Blood Mononuclear Cells (PBMC) and virus persistence in lymphoid tissues is thought to be the most important characteristics of CSFV infection that leads to the acquired immunosuppressive state [131, 132].

Recently it has been observed that ncp BVDV induces translocation of IRF-3 into the nucleus without subsequent binding to DNA [133]. Furthermore, ncp BVDV was able to block Semliki Forest virus-induced IFN production through a block in the formation of IRF-3 – DNA complexes [134]. Whether this is also true for CSFV and whether N^{pro} is involved in this process remain to be investigated. But we can not ignore the fact that the presence of N^{pro} permits efficient infection of monocytic cells, including monocytes, macrophages, and even dendritic cells. These cells are among the main targets for CSFV allowing high-level replication and permit cell-associated spreading and colonization of immunological tissue by CSFV. Furthermore, they appear to play a central role in virus-induced immunomodulation [135].

Dendritic cells (DCs) are one of the primary immunological sentinels of the immune system [136, 137]. Their strategic localization at mucosal surfaces and dermal layers makes them an early target for virus contact [138]. Functional disruption of DCs is an important strategy for viral pathogens to evade host defences [139, 140]. Monocytotropic viruses such as CSFV can employ such a mechanism as the virus can suppress immune responses and induce apoptosis without infecting lymphocytes. The virus infects both conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) [141, 142, 143]. The infected DCs display neither modulated MHC nor CD80/86 expression. Interestingly, similar to macrophages, CSFV do not induce IFN- α responses in the cDCs as N^{pro} protein promotes proteosomal degradation of interferon regulatory factor (IRF) 3 [144, 145]. So, it can be said that CSFV can replicate in cDCs and control type I IFN responses, without interfering with the immune reactivity [146]. However, in pDCs, IRF 7 is more prominent and there is lack of interference of N^{pro} with IRF 3 which results in augmented IFN α response by pDCs. This is the reason for an exaggerated pDC response, relating to the immunopathological characteristics of the disease [147, 148, 149].

Regulation of CSFV RNA turnover with minimal accumulation of dsRNA is an important factor governing the evasion of host defense by the virus [144]. The temporal modulation of NS2-3 processing by the NS2 autoprotease is crucial in RNA replication control and the intracellular level of NS3 strictly correlates with the efficiency of RNA replication [150]. But, whether these proteins regulate the dsRNA levels remains to be established. The viral structural protein E^{ms} is also actively involved in the dsRNA-mediated induction of IFN β [151].

IL-6 is an important cytokine in providing protection during early part of CSFV infection. The synthesis of NS4B protein during viral replication in the tonsil down regulates the expression of IL-6 and this is especially true with CSFV strain Brescia [123]. Swine

Leukocyte Antigen I (SLA I) molecules present the endogenous peptides to activate the CD8⁺T cells that control viral replication within cells. CSFV interferes with the expression of SLA I molecules by the monocytic cells, thereby, inhibiting apoptosis of the cells. This strategy seems to be quite helpful for the virus to escape the host immuno-surveillance and establishment of persistence in tissues [152]. Antibodies may be temporarily detected in serum sample. But these antibodies can not eliminate the virus from the host system. Consequently, the antibodies are neutralized by the virus and cease to be detectable [153].

Blocking B-lymphocyte maturation by infection and destruction of germinal centers is a key event in the pathogenesis of acute, lethal CSF before the development of generalized infection [154]. Immature B lymphocytes (*i.e.*, centroblasts, centrocytes and B blasts) can themselves be the cellular targets of the virus in any stage of maturation within follicles [155] or they may lack critical cytokines because of an infection of the supporting follicular dendritic cell network [154]. However, it is clear that depletion of B lymphocytes can not account for all the pleiotropic symptoms of this disease. But, as it is generally held that antibodies against CSF can be protective and as recovery from acute infection is known to be associated with seroconversion [156, 157] it appears justified that B-follicle tropism of an HCV isolate is an important determinant for the course of disease [154].

8. Conclusion

The understanding of the virus-host interaction network is important to design antiviral strategies and to formulate antiviral drugs. In this context, the ability of the viruses to evade the host immune system plays a key role. The understanding of the complex mechanisms of host immune system manipulation will ultimately result in undertaking suitable immunoprophylactic measures.

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