Alphaviruses suppress host immunity by preventing myeloid cell replication and antagonizing innate immune responses

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ABSTRACT

Alphaviruses are medically important mosquito-borne viruses that cause a range of diseases in humans from febrile illness to arthritis or encephalitis. The innate immune response functions to suppress virus replication through upregulation of antiviral molecules and contributes to development of the adaptive immune response. Myeloid cells act as master regulators of virus infection by initiating both the innate and adaptive immune responses. Alphaviruses are capable of antagonizing individual components of these responses to increase replicative fitness *in vivo*. However, recently, studies have demonstrated that some alphaviruses avoid myeloid cell replication altogether to achieve a similar effect. In this review, we summarize how alphaviruses evade myeloid cell infection and individual inductive mechanisms, thereby limiting the activation of the innate immune response.

Introduction

Myeloid cells, such as macrophages and dendritic cells, function as key regulators of both the innate and adaptive immune response during viral infection. Myeloid cells respond to infection through pattern recognition receptors (PRRs) located in the cell membrane or cytosol recognizing viral pathogen associated molecular patterns (PAMPs) leading to secretion of the primary antiviral molecule, interferon (IFN)- α/β [1]. These cells also prime the adaptive immune response in lymphoid tissues through antigen presentation and secretion of cytokines and chemokines that provide polarization and homing signals to naïve T and B cells [2].

Alphaviruses are mosquito-borne viruses that cause two major types of human disease. The Old World alphaviruses (e.g., Sindbis (SINV), Chikungunya (CHIKV), and Ross River viruses (RRV)) cause arthritis and arthralgia while the New World alphaviruses (e.g., Eastern (EEEV), Western (WEEV) and Venezuelan equine encephalitis virus (VEEV)) cause encephalitides [3]. Alphaviruses have evolved specific mechanisms to evade myeloid cells and the antiviral immune responses to establish infection (Figure 1) [4]. Two such mechanisms include 1) limiting myeloid cell tropism and replication and/or 2) antagonizing myeloid cell responses to virus infection. This review will summarize recent advancements in our understanding of mechanisms through which alphaviruses evade innate and adaptive immune response by sculpting their interactions with myeloid cells.

Heparan sulfate binding prevents virus access to myeloid cells in lymphoid tissues

Eastern equine encephalitis virus (EEEV) is unique among the alphaviruses in that virus access to peripheral lymphoid tissues and infection of myeloid cells is restricted by efficient binding to heparan sulfate (HS) [5,6]. HS is one of four types of sulfated glycosaminoglycan (GAG) long hexose chain molecules that can be attached to core proteins via o-linkages forming proteoglycans [7]. GAGs can function as tissue structural components, cell surface receptors (e.g. for concentration of cytokines on cell surfaces) and cell adhesion molecules [8].

Many reports have been published that demonstrate a role for GAGs, particularly HS receptors, for cellular attachment of both DNA and RNA viruses [5,6,9,10]. Use of HS as a receptor is controversial, however, as this phenotype can be rapidly acquired during amplification of virus isolates *in vitro*, especially RNA viruses which possess error-prone polymerases and highly adaptable genomes [11-13]. In general, until the sequence of the attachment protein of a laboratory strain is validated by comparison with unpassaged, primary field isolates as we have done with EEEV [5], HS binding as a phenotype of naturally circulating viruses is in question. Viruses that bind to HS typically have positively charged amino acid motifs in attachment proteins that may be repeating and may alter its surface charge leading to an increased interaction with structures other than specific cell entry receptors in the intact host [5,6,12,14]. The HS binding domain of EEEV involves lysine residues 71, 74 and 77 (71 and 74 most critical) of the E2 attachment protein [6]. In a mouse model, we observed that wild-type (WT) EEEV virus exhibited significantly less replication and numbers of infected cells in the draining lymph node (DLN) after subcutaneous inoculation than an E2 71-77 charge-neutralization mutant that no longer bound HS efficiently [5]. Elimination of the HS

binding domain increased levels of serum IFN- α/β and survival times due to decreased virus replication and dissemination within the central nervous system (CNS) [5,6]. This is similar to reports with both Theiler's murine encephalomyelitis virus [15] and neuroadapted SINV [16]. Together, these data suggest that the HS binding domain of WT EEEV suppresses virus access to permissive cells in DLNs while simultaneously increasing virus infection of neurons and spread in the CNS contributing to the severe manifestations of EEEV.

Other studies have demonstrated that the *in vivo* dissemination of HS binding alphaviruses is restricted generally. In comparisons of the serum retention time of radiolabeled HS-binding and non-binding VEEV strains, the efficient HS-binding viruses were cleared from mouse serum within minutes of intravenous inoculation, while non-binding viruses could be detected for over an hour [17]. Similarly, viruses isolated from mice later during infection with HS-binding SINV had frequently lost the HS binding phenotype, suggesting that it was an inhibitor of virus spread and serum retention [18].

One study with HS binding variants of CHIKV may suggest that the host innate immune response contributes to the restriction of HS binding viruses. The CHIKV live-attenuated vaccine strain, 181/25, encodes two attenuating amino acid mutations in the E2 glycoprotein [19,20]. In particular, an arginine mutation at amino acid position 82 of E2 singly confers high efficiency HS binding [19] and also inhibits virus dissemination into peripheral tissues and reduced serum viremia in normal mice [19,20]. However, in the absence of IFN responsiveness 181/25 DLN and spleen titers were comparable to the non-HS binding WT. This may suggest that localized IFN responses contribute to inhibition of HS-binding viruses. However, in this study, systemic viremia and

dissemination to peripheral tissues was still reduced compared to WT CHIKV [20]. This higher levels of mutant virus replication could reflect increased virus replication in migratory DCs entering the DLN and spleen in the absence of IFN [20], differences in GAG binding characteristics as seen in strain differences with EEEV [6], or effects of overwhelming serum viremia on the accuracy of plaque titration in lymphoid tissues.

We propose that promiscuous interaction of HS binding domains in virus particles with ubiquitously expressed HS results in sequestration of virus particles to non-productive structures *in vivo*. This may lead to limitations of virus spread from initial sites of inoculation (e.g., footpad to DLN in murine models) and, therefore, may represent an adaptation of some viruses to limit immune responses by suppressing myeloid cell replication. We are attempting to derive definitive conclusions regarding the access of HS-binding viruses to DLNs and subsequent immune responses by labeling virus particles with fluorescent dyes or nanoLuciferase [21] and tracking virion spread after subcutaneous inoculation of animals. While the use of HS receptors by naturally circulating viruses will remain controversial until sequencing of unpassaged isolates becomes commonplace, the role of HS binding in suppressing virus spread *in vivo* could be important in the pathogenesis of a number of viruses.

Myeloid cell-specific inhibition of viral replication by the microRNA, miR-142-3p

If EEEV is not sequestered by HS receptors and gains access to the DLN, a second, unrelated mechanism prevents translation of the EEEV RNA genome and virus replication within these cells. Hematopoietic cells (e.g. monocytes, macrophages, dendritic cells, granulocytes) express miR-142-3p, a microRNA (miRNA) required for

maintaining dendritic cell homeostasis [22], regulating IL-6 production [23], and regulating macrophage differentiation [24,25]. After virus entry, miR-142-3p prevents translation of the EEEV genome through binding to complementary miR-142-3p binding sites in the virus 3' non-translated region (NTR). Restriction of EEEV translation in myeloid cells suppresses the activation of the innate immune response and production of systemic IFN- α/β leading to increased virulence *in vivo*. Mutation of the miR-142-3p binding sites or ablation of miR-142-3p from myeloid cells relieves the translation and replication blockade leading to the IFN- α/β production and attenuation *in vivo*. This increase in systemic IFN- α/β in serum is associated with a higher number of infected CD11b⁺ macrophages and CD11b⁺ CD11c⁺ dendritic cells in the DLN; however, the specific source of the IFN- α/β requires confirmation [26].

Coordinate elimination of HS binding and miR-142-3p restriction in a single mutant virus leads to an additive effect *in vivo*, as higher levels of virus replication within DLNs and serum IFN- α/β are observed. This demonstrates a requirement for virus access to lymphoid tissues and myeloid cell infection/replication for serum IFN- α/β production [26]. Suppression by EEEV of additional cytokine and chemokine responses in myeloid cells [5] could adversely affect the generation of an adaptive immune response or trafficking of immune cells to peripheral tissues. Further work is needed to understand how myeloid cell restriction changes the cytokine milieu inside the lymphoid compartment and how this influences the generation of an adaptive immune response to EEEV. It is worth noting that CHIKV, which does not appear to bind to HS efficiently [27] and does not possess miR-142-3p binding sites [26] also does not replicate well in

murine myeloid cells [20] and induces low levels of IFN- α/β in mice [28]. The mechanism underlying this phenomenon remains to be investigated.

Viral antagonism of the innate immune response

During the host response to infection, secretion of IFN- β or IFN- α engages the type I IFN receptor complex activating the JAK/STAT signaling cascade leading to upregulation of antiviral interferon stimulation genes (ISGs) that inhibit alphavirus replication [29-33]. Alphaviruses are known to suppress the induction of the innate immune response during infection through different mechanisms. VEEV, SINV and CHIKV are known to inhibit STAT phosphorylation and translocation to the nucleus to prevent IFN- α/β upregulation and ISG synthesis [34-36]. As described above, EEEV prevents IFN- α/β induction through miR-142-3p restriction of EEEV replication in myeloid cells [26]. Finally, alphaviruses can inhibit ISGs through disguising viral PAMPs or direct antagonism of ISG activity. Several studies have concluded that the activation of PRRs is delayed in some alphavirus-infected cells likely due to sequestration of virus replicative structures into membrane-bound vesicles [37,38] thus blocking their detection. The activity of the translation-inhibiting ISG, IFIT-1, is limited by the lack of free nucleotides in the 5' NTR of VEEV and the presence of a rigid stem-loop structure. This prevents the recognition of the virus type 0 cap structure by IFIT-1 and inhibition of virus translation [31,33]. Mutations in the 5' NTR secondary structure present in the attenuated TC-83 vaccine strain lead to IFIT-1 recognition of VEEV RNA and *in vivo* attenuation [31]. While this line of research has primarily been developed in non-myeloid cells, myeloid cells in culture respond rapidly to alphavirus infection and

produce high levels of IFN- α/β [4]. Therefore, deciphering the mechanism alphaviruses use to inhibit myeloid cell-specific IFN- α/β induction will provide insight into how myeloid cells contribute to alphavirus pathogenesis.

While not yet confirmed in myeloid cells, a more global mechanism that alphaviruses use to inhibit synthesis of IFNs and ISGs is through inhibition of host macromolecular synthesis involving both transcription and translation activities [39,40]. Identification of a role for individual alphavirus proteins in macromolecular synthesis inhibition has been complicated due to the redundant roles of some virus proteins, their essential functions in virus replication, and the close relationship between transcription and subsequent translation. However, it is clear that several proteins can inhibit these essential host activities leading to inhibition of innate stress responses in some cell types. Multiple studies have implicated nonstructural protein 2 (nsP2) in transcriptional and translation inhibition with Old World arthritogenic viruses and the capsid protein with transcriptional shutoff in New World encephalitic viruses [35,41-45]. Recent work in our laboratory has confirmed the role of the alphavirus proteins in host macromolecular inhibition through a virus-independent system utilizing plasmid expression of virus proteins [39]. The VEEV and EEEV capsid protein, and the CHIKV and SINV nsP2 proteins inhibit host transcription. Interestingly, the VEEV, CHIKV, and SINV nsP2 proteins also inhibit host translation, independent of transcriptional suppression, while the EEEV nsP2 protein does not. The VEEV and EEEV capsid do ultimately inhibit host translation but this may be a by-product of transcription shutoff. Interestingly, IFN- α/β responses as measured in sera of mice are unaffected by virus mutants lacking these inhibitory capabilities [39]¹ suggesting that there may be important cell-specific

differences in macromolecular synthesis inhibition capabilities or cell responses to infection.

Finally, the natural transmission cycle of an alphavirus may have an impact upon its propensity to replicate in and activate myeloid cells. Host cell-directed, post-translational glycosylation of the virion surface glycoproteins occurs with all alphaviruses studied to date [3]. The structure of these is affected by the extent and nature of carbohydrate addition and processing, which differs dramatically between arthropod and mammalian cells [46]. Therefore, virus carbohydrate moieties may interact with cell surfaces differently dependent upon the organism of origin and this has potential for unique effects on myeloid cells due to the specialized receptors they express. Mosquito cell-derived RRV is a poor inducer of IFN- α/β in dendritic cells in comparison to mammalian-derived RRV, likely reflecting differences in the glycosylation of the E2 glycoprotein [47,48]. The N-linked glycosylation that occurs in mammalian cells appears to be involved in maximal induction of IFN- α/β by dendritic cells independent of subsequent virus replication [48]. In addition, these glycosylation changes can also lead to higher infectivity of myeloid cells due to an increased interaction of mosquito-derived viruses with the C-type lectin, DC-SIGN, which is expressed on myeloid cell subsets [47,49]. The extent to which carbohydrate modifications affect alphavirus pathogenesis *in vivo* has yet to be explored.

Concluding Remarks

Alphaviruses can infect evolutionary divergent hosts, likely requiring both ubiquitous and species- and virus-specific mechanisms to suppress innate and adaptive

immune responses. In vertebrates, myeloid cells are critical mediators of these responses and often the first cell a virus encounters after infection. Initially, suppression of myeloid cell replication and/or the innate immune response prevents induction of IFN- α/β and antiviral ISGs that help to limit viral dissemination within the host. This suppression not only affects the early restriction of virus replication but, since myeloid cells are also important contributors to the generation of an adaptive immune response, the overall ability of the host to combat infection. Future work will be required to understand how myeloid cells contribute to the adaptive immune response during alphavirus infections. A comparison of VEEV, which is highly infectious for these cells and elicits a robust innate response with EEEV, which is essentially non-infectious, will be especially interesting. Furthermore, the modulation of vertebrate myeloid cell infection by the mosquito vector may be an important sculptor of innate and adaptive immune responses and this also needs to be fully explored.

Acknowledgements: We apologize for not being able to cite all our colleagues' work due to space constraints. This work was supported by the National Institutes of Health [R01 AI095436, R21 AI111115, and R21 AI117331] and from the Defense Threat Reduction Agency [HDTRA1-15-1-0047] to W.B.K.

Figure Legends

Figure 1: Coding and non-coding regions of the alphavirus genome that suppress myeloid cell tropism or antagonize innate immune responses. VEEV: Venezuelan equine encephalitis virus, NTR: non-translated region, nsP2: non-structural protein 2, E: envelope protein, EEEV: Eastern equine encephalitis virus

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