

Defensin driven viral evolution

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Abstract

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Enteric α -defensins are potent peptide effectors of innate immunity that are abundantly expressed in the small intestine. Certain enteric bacteria and viruses are resistant to defensin-mediated killing and can even appropriate defensins to enhance infection, even though infection by closely related microbes is strongly neutralized by defensins. We therefore hypothesized that enteric α -defensins impose selective pressure during fecal-oral transmission. To test this hypothesis for non-enveloped viruses, we passaged a defensin-sensitive serotype of human adenovirus in the presence of a human defensin. Over many replication cycles, we observed the accumulation of mutations in two external hypervariable loops of the major capsid protein hexon. In contrast, prior studies identified the vertex proteins, fiber and penton base, as important determinants of defensin antiviral activity. Through infection and biochemical assays, we found that although all three capsid proteins serve a critical role in defensin-mediated neutralization,

hexon is the primary determinant of enhancement in adenovirus as no virus without homologous HVRs was enhanced. Importantly, we established human enteric α -defensins as mediators of selection during an infection. These results extensively revise our understanding of the interplay between defensins and non-enveloped viruses, providing a rationale for the difference in infection phenotypes of closely related adenoviruses. In addition, they establish the feasibility of defensin-mediated neutralization shaping viral evolution within the gastrointestinal system.

We then expanded our studies of defensins as an evolutionary pressure to a different non-enveloped virus, rotavirus. Rotavirus is fecal-orally transmitted, as such virions encounter enteric α -defensins during a natural infection and likely also undergo selective pressure to escape neutralization. However, the interaction between rotavirus and defensins had yet to be studied. Initial experiments established the strong rotavirus enhancing potential of enteric α -defensins. To understand the evolutionary effects of defensins on rotavirus, we then tested the infection levels of rotavirus in the presence of both myeloid and enteric α -defensins from multiple species. Preliminary data focusing on mouse, human, and rhesus rotaviruses and α -defensins identified patterns of enhanced infection in the presence of enteric α -defensins and neutralization in the presence of myeloid α -defensins. These preliminary studies further support the potential of defensin as a selection pressure and provide a new system to understand the interaction between α -defensins and non-enveloped viruses.

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DEDICATION

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Chapter 1: Introduction

Human Adenovirus

Pathogenesis

Human adenovirus (HAdV) was first discovered in 1953 in the adenoidal tissue of children, hence the origin of the name [1]. HAdV is widespread globally and can cause a range of diseases including fever, upper and lower respiratory infections, diarrhea, and epidemic keratoconjunctivitis, all of which are usually self-limiting and usually resolve within 2 weeks [2-6]. However, HAdV infection in immunocompromised patients can cause more severe and at times fatal symptoms including dissemination of the virus, highlighting the importance of understanding HAdV pathogenicity [7-9].

Transmission and tropism of HAdV is highly diverse, as there are over sixty serotypes of HAdV divided into seven species (A-G) based in part on serology, hemmagglutination, and more recently genome sequencing [10]. Generally the virus can be acquired through the mouth, nasopharynx, or the conjunctiva of the eye [2, 3, 11, 12]. Of the seven species, HAdVs in species A, F, and G are often associated with gastrointestinal pathogenesis while species C and E more commonly cause respiratory tract infections. Certain species D serotypes cause infection in the ocular conjunctiva and the gastrointestinal tract [5, 13]. Species B viruses have been further subdivided into B1 and B2 based on their restriction enzyme digestion patterns and genomic similarities which correlate with the use of different host receptors and their differing tropisms [14, 15]. Subspecies B1 viruses cause respiratory tract infections, while subspecies B2 viruses cause infections in the kidney and urinary tract [16]. Although infection with HAdV will lead to serotype-specific immunity, certain HAdV serotypes can maintain a persistent infection, with shedding of virions in the stool for months after initial infection and often asymptotically [11].

Reactivation of HAdV while in an immunocompromised state, for example when receiving a stem cell transplant, solid organ transplant, or in combination with congenital or acquired immunodeficiency, can endanger the success of the transplant and overall patient survival [7, 17, 18].

In addition to the severe HAdV infections in immunocompromised patients, HAdV epidemics have occurred frequently throughout history. For example, keratoconjunctivitis epidemics have occurred through contact with contaminated pool water as well as through contaminated equipment used in medical settings [13, 19-21]. Of note, acute respiratory disease within new military recruits transmitted by airborne HAdV and fomites was also a large problem requiring hospitalization of up to 25% of cases until the establishment of a live oral vaccine specific to serotypes 4 and 7 [2-4, 9, 22-25]. Similar epidemics to this have been seen in other areas where large numbers of people are in close contact including university dormitories [24, 25]. Overall, HAdV continues to be a global public health concern.

Structure

HAdV is a non-enveloped virus with a linear double-stranded DNA genome contained within a T=25 icosahedral capsid. A virion is comprised of a total of eleven different proteins. The capsid is formed by three major capsid proteins: hexon, penton base (PB) and fiber [26, 27]. Additional minor capsid proteins IIIa, VI, VIII, and XI stabilize hexon-hexon interactions [27]. Within the capsid, proteins VII, V, and μ coat the viral DNA while terminal protein is covalently bound to the ends of the genome [27]. Twelve trimeric hexons form each facet of the icosahedron for a total of 240 hexon trimers (720 hexon monomers) making it the most abundant capsid protein. Hexon is composed of a conserved core that faces internally and a highly variable external face. The hyper variable regions (HVRs) or loops on the external aspect of hexon are so named because

they vary widely across serotypes and, more broadly, across human and non-human AdVs [28]. Neutralizing antibodies are produced in response to these HVRs, hence they were a major determinant of HAdV classification before genome sequencing [28-31]. Each of the twelve vertices is comprised of a pentameric penton base (PB) complex non-covalently associated with a trimeric fiber protein [26]. Fiber can be further divided into two domains, a shaft of variable length depending on serotype and a distal knob. PB contains an RGD motif on a discrete, variable loop that allows for recognition and interaction with host integrins. The RGD motif facilitates efficient internalization, while the viral attachment protein fiber determines receptor utilization [32, 33].

Cell Binding and Entry

HAdV fiber attachment to the host cell is mediated by different receptors. In general, serotypes in species A, C, E, and F use coxsackievirus and adenovirus receptor (CAR). Other known receptors are CD46 which is used by viruses from species B1, desmoglein 2 that is used by species B2 viruses, and sialic acid which is used by viruses from species D [34-39]. More recently, hexon has also been shown to act similarly to a viral attachment protein. Specifically, hexon HVRs have been shown to bind coagulation factors XI (FXI) and X (FX) as well as lactoferrin. FXI and FX then mediate binding of the virus to heparan sulfate and uptake into epithelial cells including hepatocytes [31, 40-44]. Although a specific receptor has not been identified, lactoferrin also mediates viral interaction with the host cell surface increasing HAdV levels of infection [45, 46]. Furthermore, interaction with a primary receptor is not always required for a successful infection, and interactions with host integrins (α_v) alone are enough to mediate entry albeit at a lower rate than in the presence of a primary receptor [47, 48].

Once bound, multimeric PB binding to integrins triggers downstream signaling pathways leading to actin cytoskeleton rearrangement and endocytosis [49-51]. Removal of the vertices releases protein VI to lyse the host membrane [52]. It is unknown exactly how removal of the vertices from the capsid is triggered; however, a few different potential mechanisms have been described. One school of thought is that the acidification of the endosome triggers release of PB from the capsid [52-55]. Another is that movement of the primary receptor on the cell along the cell membrane relative to integrins causes local destabilization of the viral capsid, removal of the receptor-bound PB and fiber, and release of the lytic protein VI [51, 52]. Once in the cytosol, the protected genome travels on microtubules towards the nucleus, where it's genome is imported to begin replication [56].

Human Defensins¹

Before a virion reaches a host cell, it encounters multiple levels of resistance from the host. One such host defense is antimicrobial peptides. Defensins are one of the most abundantly expressed classes of human antimicrobial peptides. Defensins are small (18-45 residues), cationic (+2 to +11 net charge), and amphipathic peptides that form a conserved three-dimensional structure comprised predominantly of β -sheets stabilized by three disulfide bonds. Human defensins can be further subdivided on the basis of structure and disulfide connectivity into α - and β -defensins.

¹ Adapted in part from 57. Holly MK, Diaz K, & Smith JG (2017) Defensins in Viral Infection and Pathogenesis. *Annu Rev Virol* 4(1):369-391.

There are two major classes of α -defensins, myeloid and enteric, based on gene organization and expression patterns [58, 59]. Myeloid α -defensins were first identified and are abundantly expressed in neutrophils [59]. Human neutrophils contain three abundant α -defensins called human neutrophil peptides 1, 2, and 3 (HNP1-3). HNP4 is also found in human neutrophils at <2% of the levels of HNP1-3 [60]. Localization of HNP1-4 principally to the azurophilic or primary granules of neutrophils is tied to the timing of their expression during neutrophil differentiation [61]. Like many proteins stored in secretory vesicles, these peptides are produced as pre-propeptides. The signal peptide is removed by signal peptidases. The inactive propeptides are then packaged in azurophilic granules where they are fully processed into mature defensins by neutrophil elastase and proteinase 3, which are all co-packaged with HNPs [62]. Azurophilic granules undergo restricted secretion and are most commonly directed for fusion with the phagolysosome, where their contents would interact at high concentration with phagocytosed microbes [63, 64]. Upon holocrine secretion (e.g., during formation of neutrophil extracellular traps), HNP1-4 would be released into the extracellular milieu, although at much lower concentrations [61]. This may be significant during inflammation (e.g., in the Fallopian tube [65]), where infiltrating neutrophils may leave a residue of α -defensins on surrounding cells but does not result in appreciable concentrations of HNP1-4 in plasma [66].

Although they share certain features, including nearly identical structures, enteric α -defensins are distinct from their myeloid counterparts. Humans express two enteric α -defensins called human defensin 5 and 6 (HD5 and HD6). They were first discovered and are most abundantly expressed in specialized epithelial cells of the small intestine called Paneth cells, which are located at the base of the crypts of Lieberkühn [67]. Like the myeloid α -defensins, HD5 and HD6 are synthesized as inactive pre-propeptides. HD5 and HD6 are sorted via a regulated

secretory pathway to the prominent cytoplasmic granules that are the hallmarks of Paneth cells. Since Paneth cells are capable of repeated rounds of defensin synthesis and merocrine secretion over their ~30 day life span, this process is distinct from the “targeting by timing of biosynthesis” mechanism of HNP sorting by neutrophils [61]. HD5 and HD6 are packaged intracellularly in the pro-defensin form and co-packaged with a trypsin zymogen. Upon secretion of the granule contents into the bowel lumen, trypsin is activated and subsequently cleaves pro-HD5 and pro-HD6 to form the mature, active peptides [68, 69]. Thus, unlike myeloid α -defensins, enteric α -defensins act extracellularly and accumulate at high concentration in the lumen of the crypt and in the intestinal mucus. Enteric α -defensins are generally constitutively expressed and regulated at the level of secretion. Enteric α -defensin expression is clearly regulated by and tied to Paneth cell differentiation, and transgenic expression of HD5 in mice using the native HD5 promoter results in restricted expression in Paneth cells [67, 70]. Therefore, increased α -defensin expression can occur due to expansion of the Paneth cell population [71, 72].

Interestingly, HD5 is also expressed in the male and female genitourinary tract, including the kidney, and expression increases with inflammation at these sites [65, 73]. Elevated HD6 expression upon *Neisseria gonorrhoeae* infection has also been reported in immortalized vaginal and cervical epithelial cells, although production of HD6 protein was not verified [74]. HD5 mRNA and peptide are found in the vagina, ectocervix, and in inflamed fallopian tubes [65]. Although variably expressed across individuals, HD5 is localized to cytoplasmic granules of a subset of columnar epithelial cells in the fallopian tube, endometrium, and endocervix. Active HD5 is found in cervicovaginal lavage [65], while pro-HD5 is detectable in urine and may be processed to active forms by neutrophil proteases during inflammation [73, 75].

The mechanism of Paneth cell degranulation leading to defensin secretion is contentious. Ayabe *et al.* proposed merocrine secretion in response to purified bacterial ligands using mouse intestinal crypts cultured *ex vivo* [76]. Efforts to repeat these results using enteroids (primary, untransformed, intestinal epithelial cells) have been unsuccessful, and a holocrine secretion mechanism in response to IFN- γ treatment was put forth [77-79]. Paneth cell regeneration from holocrine secretion requires time for new Paneth cells to differentiate from intestinal stem cells. In contrast, merocrine secretion allows for rapid regranulation of the existing Paneth cells, which remain intact and viable during granule exocytosis. While IFN- γ -dependent Paneth cell extrusion is likely a physiologically relevant event [80], it does not account for the long-lived nature of Paneth cells *in vivo* [81]. Merocrine secretion in response to cholinergic agents can be reproduced in enteroids and occurs *in vivo* [76, 77], but the role of bacterial and possibly viral ligands in this process needs clarification.

β -defensin genes in most species are more numerous than α -defensin genes and many have clear orthologs across species, reflecting evolution of β -defensin gene lineages in an early common ancestor. The human genome encodes ~ 37 β -defensin genes some of which have high copy number variation among individuals [82, 83]. Only a fraction of the human β -defensins (HBDs) has been examined at the protein or transcriptional level [84, 85]. Although there are many reports of HBD transcripts in various tissues, in general, expression is restricted to keratinocytes of skin and epithelial cells of the genitourinary, gastrointestinal, and respiratory tracts [66, 85, 86]. Furthermore, a significant subset of β -defensins is only expressed in the male reproductive tract and involved in fertility independent of microbial infection [82, 87, 88].

Unlike α -defensins that are commonly regulated at the level of secretion, β -defensin expression is most often transcriptionally regulated. For example, HBD-2 expression is induced

and only detectable during infection or inflammation of the gut [66, 86]. However, HBD1 is an exception as it is constitutively expressed by most epithelial cells in the small intestine and colon. Because the induction of HBD expression is driven by signaling pathways including innate immune sensors (e.g., TLRs), the differential expression of these sensors in tissues (e.g., lung vs. gut) results in tissue-specific upregulation by particular ligands [86]. Unlike α -defensins, β -defensins are processed solely by signal peptidases to their mature form and are most likely secreted through the constitutive rather than regulated secretory pathway [89]. Thus, most cells have the machinery to secrete functional β -defensins. However, there may be additional regulation post-secretion. For example, studies have demonstrated a requirement for a reducing environment, like that in the colon, to uncover potent anti-bacterial activity of HBD1 [90]. Moreover, HBD1 and HBD2 antibacterial activities are inhibited by physiologic salt concentrations, potentially restricting their function to certain extracellular environments [85].

Antiviral Mechanisms

Enveloped Viruses

Herpesviridae, human immunodeficiency virus (HIV-1), and to a lesser extent influenza A virus (IAV) are the most studied enveloped-viruses in regard to defensins. Herpes simplex virus 1 (HSV-1) has the distinction of being the first virus tested for defensin antiviral activity [91], and most work has continued to focus on HSV-1 and -2 within the *herpesviridae* family. Furthermore, defensin in the context of HIV-1 infection is the most extensively studied viral-defensin interaction.

Interest in defensin activity against HIV-1 was piqued by an early study identifying myeloid α -defensins as the antiviral factor secreted by CD8+ T cells (CAF) [92]. Although the

cellular origin of the defensins in these cultures and the identity of CAF as α -defensins were subsequently cast into doubt, the antiviral activity of α -defensins was not disputed [59, 93, 94]. There have since been a number of papers describing anti-HIV activity of α - and β -defensins. Myeloid α -defensins (HNP1-4) are potent inhibitors of laboratory and clinical isolates of HIV-1. Inhibition is reversible, except under certain conditions, and appears to be through multiple mechanisms [95, 96].

HNP1-3 are lectins capable of binding to gp120 and CD4, although glycosylation-independent binding has also been described [95, 97]. HNP4 is 2- to 4-fold more potent than HNP1-3 and binds to Env independently of glycosylation [97, 98]. While capable of inhibiting Env-CD4 interactions and Env-coreceptor interactions that result in fusion, defensins do not measurably alter virus binding to the cell and still inhibit infection of viruses that are bound to cells prior to HNP addition [95, 99]. In addition, these peptides rapidly and transiently downregulate CD4 and, to a lesser extent, CXCR4 but not CCR5 in multiple cell types, although these effects have not been observed uniformly [95, 99, 100].

HNP binding to Env and CD4 blocks Env-mediated fusion by interacting with Env fusion intermediates. Fusion inhibition is exacerbated by receptor downregulation and selective inhibition of viral uptake without causing pleiotropic effects on endocytosis [95, 99]. The antiviral activity of these peptides is strongly abrogated by serum, which may restrict their activity to mucosal surfaces or other serum-free anatomical locations [95-97]. How serum attenuates HNP activity is not clearly defined. There is data both for and against serum attenuating HNP1-3 binding to viral and cellular targets, and it has been suggested that HNP self-association may be the required serum-sensitive step [95, 97]. In addition to effects on fusion, HNP1 antiviral activity is associated with inhibition of protein kinase C (PKC) signaling, which is required for productive infection

[96]. And, by interacting with fusion intermediates and altering the kinetics of fusion, even sub-inhibitory concentrations of HNP sensitize HIV to inhibition by anti-gp41 antibody and peptide inhibitors of fusion [101].

β -defensins are expressed at sites like the oral and vaginal mucosa that are important for HIV transmission [102, 103]. *In vitro* studies have shown antiviral activity of HBD2 and HBD3, solely against X4 tropic HIV-1, at defensin concentrations equal to or lower than those found in the oral mucosa; however, the mechanism for this antiviral activity is unclear [104]. HBD2 and HBD3 binds to both target cells (T cell lines) and X4 tropic viruses and cannot be removed by extensive washing in PBS. Although virions were not visibly damaged in the presence of defensins when viewed by EM, it was suggested that inhibition is irreversible. However, this premise was not evaluated by testing infection in the presence of serum, which abrogates defensin inhibition. HBD2 and HBD3 also downregulate CXCR4 but not CCR5 expression to about 50% of basal levels in peripheral blood mononuclear cells, and even greater downregulation was observed in T cell lines [104]. Furthermore, although HBD3 competes with chemokine binding to CXCR4, HBD3-dependent downregulation of CXCR4 does not activate downstream signaling in lymphoid or myeloid cells [105, 106]. Thus, the best supported mechanism for HBD2 and HBD3 inhibition of HIV-1 infection is through downregulation of the CXCR4 co-receptor.

Additional effects of HBD2 and HBD3 on X4 and R5 tropic viruses have been reported, including inhibition when defensins are added post-entry, but the basis for these findings is unclear [100, 107]. Interestingly, adult tonsil epithelial cells express more HBD2 and HBD3 than infant tonsils [108], and HBD2 and HBD3 expression correlates with inactivation of HIV that traverses an adult but not fetal polarized epithelial layer [109]. Thus, differential expression of these host

defense peptides may contribute to the lack of oral transmission of HIV in adults compared to infants and neonates.

Both retrocyclins and rhesus θ -defensins are capable of almost complete inhibition of R5 and X4 isolates of HIV-1 from multiple subtypes, with low micromolar IC₅₀'s [97]. Retrocyclin-2 was particularly potent, exceeding the antiviral activity of HNP1 [97]. As is true for the myeloid α -defensins, the main mechanism of HIV-1 inhibition by retrocyclins is at the entry step of infection by blocking HIV-1 Env-mediated fusion [110, 111]. Binding to gp41 heptad repeat 2 prevented the formation of the six-helix bundle, thus inhibiting viral entry into the host cell [111].

CD4 and gp120 are both bound by retrocyclins, without crosslinking membrane proteins and without fully inhibiting host-virus binding [110, 111]. Consistent with this finding, patch-like aggregates of retrocyclins have been seen on the surface of CD4⁺ cells and form around CD4, CXCR4, and CCR5 [110, 112]. De-glycosylation of CD4 and gp120 attenuates inhibition of HIV-1 infection by retrocyclins, thus implicating the importance of defensin's lectin properties [113]. The binding of retrocyclin 2 to CD4 and gp120 is also sensitive to, but not completely inhibited by, serum [97]. Retrocyclin 1 is effective against fusion inhibitor-resistant mutants of HIV-1 [114]. Moreover, mutations in HIV-1 heptad repeat 2 that arise to overcome retrocyclin often render virions incapable of successful infection [114-116]. As such, retrocyclins have become a target for therapeutic development as an antiviral agent [114].

Of the eight human herpesviruses, four have been studied in the context of human defensins: cytomegalovirus (CMV), herpes simplex virus-1 and -2 (HSV-1 and -2), and varicella-zoster virus (VZV). Like for HIV-1, there is no visible disruption of the viral particles in the presence of inhibitory concentrations of defensins [117], inhibition is greatly reduced in the presence of serum [117], and neutralization requires the disulfide-stabilized structure of the

defensins [117, 118]. In contrast to HIV-1, both myeloid and enteric α -defensins neutralize HSV-1 and HSV-2 infection. HNP1-3 block infection by HSV-1 and HSV-2 with similar potencies [117, 118]; however, CMV is only weakly inhibited by high HNP1 concentrations ($>50 \mu\text{M}$) [117]. Of the enteric α -defensins, HD5 is a potent inhibitor of HSV-2, while HD6 has only a moderate effect [118]. Among the HBDs that have been tested, HBD3 but not HBD1 or HBD2 potently inhibits HSV-2 [118]. In addition, HBD2 is active against VZV, but other HBDs have not been tested [119]. Finally, both retrocyclin 1 and 2 have been shown to inhibit HSV-1 and HSV-2 infections [120, 121].

Time of addition experiments indicate that HSV-2 binding to cells is inhibited to varying degrees by all α -defensins, HBD3, and retrocyclin-2 [118, 120, 122]. The antiviral activity of HNP1 against HSV-1 is correlated with binding to viral particles, as both antiviral activity and defensin binding to virus are greatly decreased at temperatures below 20°C [117]. Defensin binding to the gB and gD viral glycoproteins of HSV-1 or HSV-2 and to cell surface receptors for HSV-2 (heparan sulfate) correlates with inhibition [118, 120, 122]. Thus, HBD1 and HBD2, which do not bind to viral glycoproteins or to the host cell receptor, have no effect on HSV-1 infection.

Our understanding of α -defensin anti-viral mechanisms against HSV-2 is complicated by the observation that HNP1 and HD5 also inhibit infection when added as late as 8 h post-infection [118]. These defensins are known to bind to viral DNA, but the extent to which this contributes to antiviral activity is unclear. Moreover, HNP1 and HD5 are both taken up by epithelial cells via an unknown mechanism, where they may interact with incoming viral particles. However, the post-entry activities of HNP1 and HD5 have not been uniformly observed [120, 122]. A post-entry mechanism was also recently proposed for *in vitro* inhibition of Dengue virus infection of Vero cells by retrocyclin 1, possibly by interfering with protease activity [123]. Additional studies

are needed to clarify effects of defensins on cellular and viral targets that become accessible post-entry or that manifest at later stages of infection.

Influenza A virus (IAV) also interacts with defensins, but the effect of defensins on IAV infection is understudied. All α -defensins, HBD3, and retrocyclins 1 and 2 inhibit IAV infection [95, 124-128]. In contrast, HBD1 and HBD2 have little direct effect on the virus [127]. For myeloid α -defensins, inhibition of PKC activity, as mentioned previously for HIV-1, has been suggested as an antiviral mechanism [124]. In addition, although HNP1 does not block hemagglutination or affect endocytosis of IAV, it has been shown to inhibit fusion [95, 126]. HBD3 and retrocyclin 2 also inhibit hemagglutinin-mediated fusion by cross-linking host glycoproteins through their lectin activity, thereby decreasing the mobility of these host proteins in the membrane [128]. However, unlike HIV-1 inhibition by myeloid α -defensins, there was no effect of HBD3 or retrocyclin-2 on conformational changes in hemagglutinin that initiate fusion. Due to their direct interactions with viral particles, HNP1, HNP2, and retrocyclin-2 are able to aggregate IAV [129]. Although aggregation alone is not inhibitory, aggregation and opsonization increase uptake of IAV by neutrophils, which may impact viral infection and pathogenesis *in vivo*.

In summary, enveloped viruses are susceptible to α -, β -, and θ -defensins. Antiviral mechanisms of defensins include the inhibition of interactions between viral glycoproteins and cellular receptors resulting in disruption of fusion. However, this concept has not been revealed mechanistically for enveloped viruses to the same extent as HIV-1 inhibition.

Non-Enveloped Viruses

Early experiments with α -defensins found no effects on non-enveloped viral infection, reinforcing the assumption that defensin antiviral activity was a consequence of envelope

disruption as is seen in bacteria [117]. Consequently, there are fewer studies of the effects of defensins on infection by non-enveloped viruses. Once clear antiviral activity was demonstrated for α -defensins against human papillomavirus (HPV) infection [130], a series of papers have converged on a common neutralization mechanism. These studies have shown that non-enveloped viruses from several families (*Adenoviridae*, *Papillomaviridae*, and *Polyomaviridae*) are inhibited by α -defensins [130-139]. However, when tested, β -defensins have had little to no effect.

For each of these viral families, binding studies, indicate that α -defensins target the virus rather than the cell to exert their antiviral effect [130, 132, 133, 135, 137-139]. Interactions between the viral capsids and purified α -defensins can lead to aggregation *in vitro*; however, with the exception of BK polyomavirus [139], aggregation is not a significant antiviral mechanism [132, 134]. These viruses are successfully internalized in the presence of inhibitory concentrations of α -defensins. Furthermore, when tested, the defensins inhibit viruses that have been pre-bound to their normal receptors on the cell surface [137, 138]. For human AdVs (HAdVs), more virus binds to cells in the presence of α -defensins, and some of this binding is receptor-independent [135]. The mechanism of this enhanced binding has not been resolved. α -defensin binding to non-enveloped viruses makes the particles resistant to thermal denaturation, mechanical force, and proteolysis [131, 137, 138, 140]. This stabilization prevents uncoating during cell entry. A failure to uncoat precludes interactions between viral proteins and host factors that are required for productive infection.

Despite the diversity of non-enveloped capsids, each of these viruses is bound and inhibited by α -defensins. For HAdV, release of the internal and membrane lytic capsid protein VI is blocked [136, 137]. In the absence of protein VI activity, the incoming virus cannot escape the endosome. For HPV, α -defensin binding has at least two effects. First, cleavage of the minor capsid protein

L2 by host furin at the cell surface is inhibited [132]. Lack of this cleavage in other contexts (e.g., in the presence of furin inhibitor) is associated with retention in the endosomal system and a failure to infect. Second, the association of L1, L2, and the genome is stabilized in the presence of HD5, preventing the usual separation of L1 from an L2 and genome complex that occurs during productive infection [131]. This stabilization happens even for viruses that are furin-independent and prevents L2 and the genome from reaching the *trans*-Golgi network.

As a consequence to structure stability, trafficking through the endosomal system is altered. HAdV and HPV genomes accumulate in the lysosome and fail to reach the nucleus [131, 137]. For JC polyomavirus, α -defensins reduce viral trafficking to the ER and block uncoating of capsids that are still able to reach the ER, consistent with capsid stabilization [138]. Infection by the parvovirus adeno-associated virus is also inhibited at HNP concentrations found in bronchoalveolar lavage fluid [141]. Although detailed studies of the antiviral mechanism of α -defensins against adeno-associated virus are lacking, we predict that they will demonstrate a similar effect of α -defensins on entry. Thus, all of the families of non-enveloped viruses that have been extensively studied conform to this general mechanism.

A major outstanding question is to identify common features of these disparate viral capsids that mediate defensin binding. Unlike glycoproteins from enveloped viruses, a common biochemical feature (e.g., glycosylation) that is recognized by a known function of α -defensins (e.g., as lectins) has not been identified for non-enveloped viruses. However, additional evidence for a conserved mechanism is provided by the identification of a common interface on the α -defensin HD5 that mediates neutralization of HAdV and HPV [133]. Similar studies of the defensin features required for antiviral activity, beyond the need for disulfide bonds to stabilize the defensin tertiary structure and a role for defensin dimerization [142], have not been published

for other viruses. Also, information regarding the location of defensin binding is only available for HAdV [33, 135]. Principles guiding the recognition of protein components of non-enveloped viruses may also apply to certain enveloped viruses such as HIV, where glycosylation-independent binding and neutralization have been reported, and to the interaction of defensins with cellular receptors that mediate their chemotactic activities.

Although very few non-enveloped viruses have been studied in the context of defensins, the mechanisms identified within the above three families share common properties. Most notably, α -defensins block infection through direct interactions with the viral capsid that prevent uncoating and result in misdirection of the internalized virus to the lysosome for degradation [131]. Further studies are necessary to identify the viral determinants of defensin neutralization in these and other non-enveloped viruses.

Pro-Viral Mechanisms

Despite the strong antimicrobial properties described above for defensins, there has been increasing evidence of certain microbes that can escape or even take advantage of the presence of defensins to enhance their infection. Namely, HIV, shigella, and certain HAdV serotypes in addition to bacteria of the gastrointestinal microbiome [74, 135, 143-150]. How these microbes developed resistance is unknown, but we hypothesize defensins can act as an evolutionary pressure.

Enveloped Viruses

In contrast to the myeloid α -defensins, the enteric α -defensins HD5 and HD6 specifically enhance rather than inhibit HIV-1 infection [74, 151-153]. This effect is observed upon infection

of both primary T cells and epithelial cells expressing HIV receptors, occurs with both synthetic and naturally secreted α -defensins, and can reach up to two orders of magnitude more infection in the presence of HD5 and HD6 than in their absence. Although enhancement is independent of CD4 and co-receptors, R5 viruses are more enhanced than X4 viruses [74]. Like the inhibitory activity of the myeloid α -defensins, enhancement is dependent upon the disulfide-stabilized conformation of HD5. HD5 is a lectin and binds to gp120 [154]. This interaction increases HIV-1 attachment to target cells, promoting infection and overcoming the effects of entry and fusion inhibitors [152, 153]. HD6 enhancement is intriguing given the unique structure and multimerization capability of this defensin [155]. Increased attachment to cells independent of Env interactions with CD4 and co-receptors could expand the tropism of HIV-1 for other cell types, although this concept has not been explored. In addition, increased HD5 and possibly HD6 expression in the genitourinary tract due to bacterial infection could potentially generate sufficiently high concentrations of these molecules to enhance HIV-1 infection *in vivo* [74].

Efforts to identify correlates for the *in vitro* effects of myeloid and enteric α -defensins in clinical HIV transmission, replication, or pathogenesis have often led to conflicting interpretations (reviewed in [156]). Many of these efforts are limited by small sample sizes. For example, an initial study found that myeloid α -defensins in breast milk protected against vertical transmission through breastfeeding [157]. When samples with equivalent viral loads were compared, higher α -defensin levels were protective. Nonetheless, a subsequent study found no protective effect [158]. Additional studies have found antiviral activity in cervicovaginal secretions due to the presence of α -defensins and other cationic antimicrobial peptides, but a clear role for α -defensins in protection from HIV transmission has not been demonstrated [159, 160]. In addition, increased transmission of HIV-1 is paradoxically correlated with higher foreskin and cervicovaginal secretion levels of

HNP1-3 [159, 161]. Expression of α -defensins is part of an inflammatory process to sexually transmitted infections, which in turn increases the risk for HIV acquisition. Thus, it is difficult to determine from these studies whether or not the antiviral activity of α -defensins that is observed in cell culture has a functional role in either promoting or blocking HIV-1 transmission.

Non-Enveloped Viruses

We have observed modest HNP1- and HD5-dependent increases in infection of certain serotypes of HAdV, similar to the enhancement of HIV infection by enteric α -defensins described above. Receptor-dependent and -independent attachment to cells is increased for both defensin-neutralized and defensin-enhanced serotypes [135]. However, entry by neutralized serotypes is blocked at the level of endosome escape [137]. How enhanced serotypes evade neutralization has not yet been determined. Like for HIV, enhancement could alter viral tropism; however, it has not yet been demonstrated *in vivo*. Moreover, although HAdV serotypes appear to be more uniformly resistant or sensitive to α -defensins in general rather than differentially sensitive to myeloid and enteric α -defensins [135], this may not be true for all non-enveloped viruses. In this regard, cutaneous and mucosal HPV serotypes differ in their sensitivity to the two classes of α -defensins [130]. Although we and others have not observed papillomaviruses that are resistant to or enhanced by α -defensins, it would not be surprising if this were true for other viruses and warrants investigation.

Chapter 2: Adenovirus determinants of defensin neutralization and enhancement

Introduction

Despite their broad antimicrobial activity, enteric α -defensins are not able to inhibit all non-enveloped viruses. Echovirus, reovirus, and enteric AdVs from both humans and mice are resistant to enteric α -defensin inhibition [117, 135, 147, 162]. One hypothesis to explain these observations is that resistance stems from evolutionary pressure imposed by enteric α -defensins during fecal-oral transmission. Consistent with this hypothesis, rather than kill the enteric bacterial pathogen shigella, HD5 was recently found to promote its cell binding and infection [145, 146]. And, enteric α -defensins play a role in shaping the microbial communities of the gastrointestinal tract through differential susceptibility of commensal bacteria to defensin killing [163-165]. Collectively, these observations suggest that defensin-driven evolution of enteric microbes is a common cross-kingdom occurrence.

To directly test the ability of enteric α -defensins to drive viral evolution, we used human AdV (HAdV). We previously demonstrated that HAdV infection can either be neutralized, resistant to, or enhanced by HD5, depending on serotype [135]. Thus, the naturally occurring diversity of HAdVs is an appealing substrate to identify viral determinants for neutralization and enhancement by defensins. The icosahedral AdV capsid consists of three major proteins: hexon, PB, and fiber. In previous studies, we used a rational design approach to identify the vertex proteins, fiber and PB, as determinants of HD5 neutralization [135]. Here, we evolved HD5 resistance in a defensin-sensitive serotype. From this, we identified a hypervariable loop in hexon as a novel determinant that both demonstrates the capability of enteric α -defensins to drive viral evolution and substantially revises our understanding of the mechanism of HD5 neutralization.

Results

Hexon hypervariable region 1 is a novel determinant of HD5 sensitivity

To determine whether HD5 could impose a selective force for HAdV evolution, we utilized a previously described HAdV-5-based “mutator” vector encoding a polymerase with reduced fidelity to facilitate *de novo* mutagenesis [30]. Over 50 passages, we found that the HD5 IC₉₀ used for selection increased 7-fold from ~2.5 μM to ~17.5 μM (Fig. 1A). We expanded the viral pool from every 5th round of HD5 selection and the 10th and 20th rounds of passaging control virus. As expected, there was a significant positive correlation between the round of selection and the HD5 IC₅₀ of the population (Fig. 1C). Whole genome sequencing yielded an average of 6000 mapped reads per base [166]. Both the initial inoculum and the passaged control samples contained only low frequency (<1%) mutations. In contrast, we observed numerous mutations across the viral genome that exceeded 1% frequency in the HD5 selected samples (Table 1). Mutations in the DNA binding protein (V340A), protein VI (A225T), protein VII (G50D), 52K protein (H193Y), and IIIa (A39) reached a frequency of >50%; however, none of these mutations stayed above this threshold (Fig. 1B). In contrast, mutations in polymerase (R149H, P132, and C228), the U Exon (G5D), hexon (I114K and E154K), and L4-100K (G745D) became fixed in the population as early as the 15th round of selection (Figs. 1A and 1B). In addition, a third mutation in hexon (E424K) was trending towards fixation (Fig. 1A).

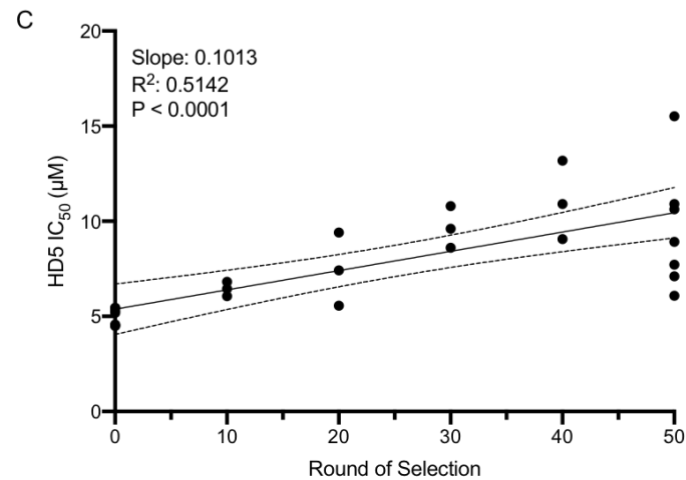
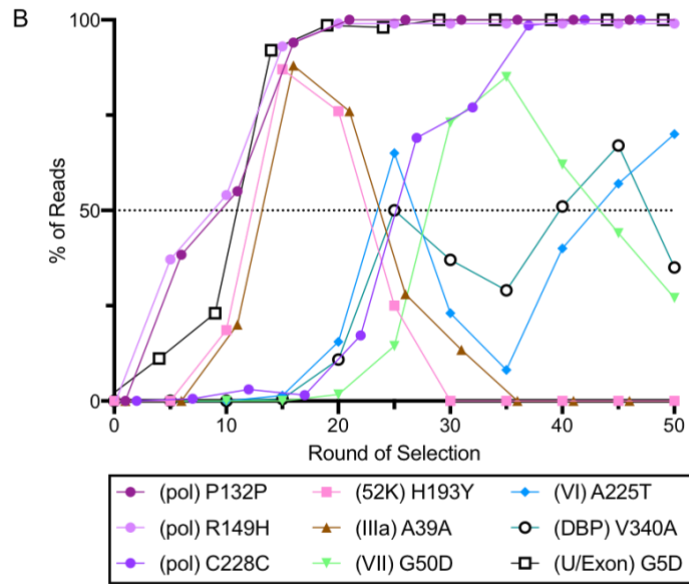
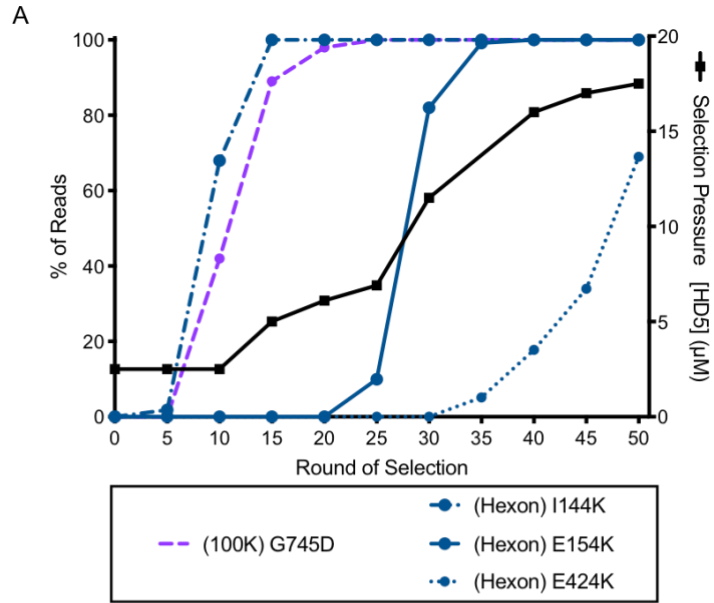


Figure 1 Selection of HD5-resistant HAdV-5.

HAdV-5 “mutator” virus was pre-bound to 293 β 5 cells and then incubated with HD5 to select for resistant viruses. (A) The right y-axis indicates the concentration of HD5 used for selection (solid black line). The left y-axis indicates the percentage of reads containing the denoted mutations in hexon (blue) and L4-100K (purple) in pools of virus expanded from the bulk selected population. (B) All mutations in other proteins that were found in at least 50% of the population during selection. (C) HD5 IC₅₀s of pools of virus expanded from the bulk selected population was determined on 293 β 5 cells. Each data point is an independent experiment, and linear regression with 95% confidence bands is graphed.

Table 1: Summary of Mutations

Protein	S	NS	Tv	Ts
eGFP	1	5	2	4
Hexon	2	3	1	4
Fiber		2		2
VI	1	1		2
IX	1	1		2
TP	1	2		3
VII	1	2		3
DBP		2		2
100K	1	2		3
33K		1		1
52K	1	1		2
U/Exon		1		1
Pol	2	3	1	4
TOTAL	11	26	4	33

Mutations found at >1% frequency at any point during selection with HD5

S: Synonymous Mutation,

NS: Non-Synonymous Mutation,

Tv: Transversion, Ts: Transition

Each of the hexon mutations introduced positive charge in one of two hypervariable regions, HVR1 (I144K and E154K) or HVR7 (E424K), located on the outer face of hexon (Fig. 2C); therefore, we focused on their contribution to HD5 resistance. We plaque purified viruses with one, two, or three of these hexon mutations in combination with the L4-100K mutation. We were unable to isolate viruses that contained only hexon mutations in the absence of the L4-100K mutation, perhaps due to the role of this protein as a chaperone for the proper folding, trimerization, and transport of hexon [167, 168]. Thus, we engineered a virus containing both HVR1 mutations in the absence of the L4-100K mutation. Viruses that contained only the mutation in L4-100K had an IC_{50} equivalent to that of HAdV-5 (salmon in Fig 2A). Viruses with the L4-100K mutation and only one (pink in Fig. 2A) or both (blue in Fig. 2A) of the hexon mutations in HVR1 had a significantly higher IC_{50} compared to the starting population but were equivalent to each other. The presence of all three hexon mutations (green in Fig. 2A) resulted in a ~3-fold increase in IC_{50} from the starting population. Interestingly, the virus engineered with the two mutations in HVR1 but without the mutation in L4-100K (purple in Fig. 2A) was just as resistant to HD5 as viruses with all three hexon mutations and L4-100K. Taken together, these results indicate an important role for HVR1 and HVR7 in sensitivity of HAdV-5 to neutralization by HD5.

To further explore the potential importance of HVR1 and HVR7 for HD5 interactions, we created chimeric viruses at these regions between HAdV-5 and HAdV-64, an HD5-enhanced serotype [135]. Although we recovered virus from genomic constructs containing HAdV-64 HVR1 and HVR7 in the HAdV-5 background, we failed to recover the reverse chimeras. Placing HAdV-64 HVR1 into HAdV-5 increased the HD5 IC_{50} of the virus ~5-fold over HAdV-5, which was also significantly higher than the HD5 IC_{50} of the pool of viruses from round 50 of the selection (Fig. 2B). In contrast, placing the HAdV-64 HVR7 into HAdV-5 had no effect. And, replacing

both HVR1 and HVR7 in HAdV-5 resulted in an intermediate phenotype compared to the single HVR changes. Overall, these studies identify HVR1 as a novel determinant for HAdV-5 neutralization by HD5.

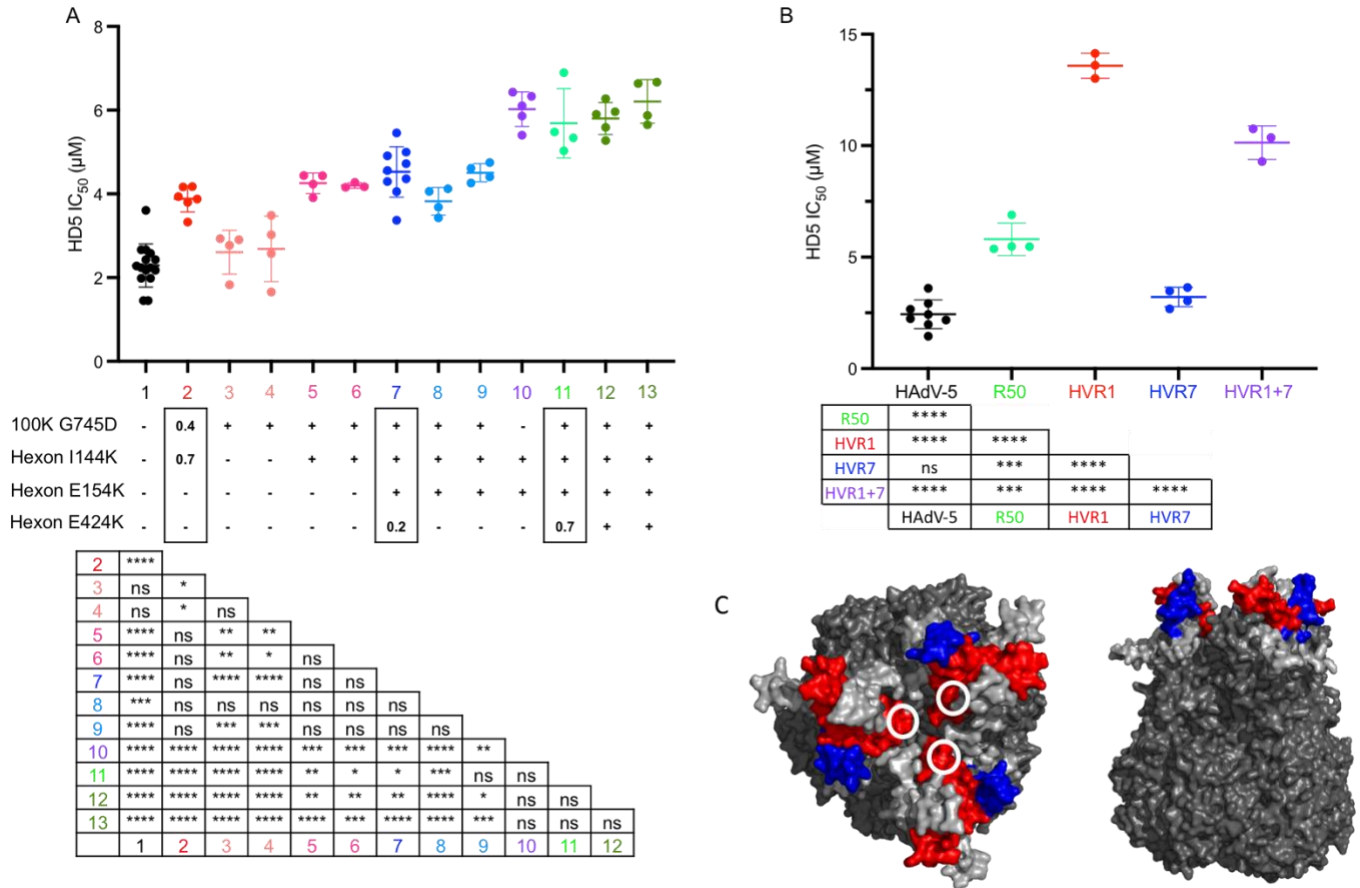


Figure 2 Hexon HVR1 is a determinant of HD5-mediated neutralization.

(A) The HD5 IC₅₀s of HAdV-5 “mutator” virus (column 1, black); pools of virus expanded from rounds 10 (column 2, red), 40 (column 7, dark blue), and 50 (column 11, light green); plaque purified viruses from rounds 10 (columns 3-4, salmon; columns 5-6, pinks), 40 (columns 8 and 9, blues), and 50 (columns 12 and 13, greens) of the selection; and an engineered virus containing only the indicated hexon mutations (column 10, purple) were determined on 293β5 cells. The presence of each hexon and L4-100K mutation is denoted below the graph. For the pooled viruses, the fraction of reads containing each mutation is indicated within the rectangles. Each data point is an independent experiment, and bars are the mean ± SD. The results of one-way ANOVA with Tukey’s multiple comparison test are indicated by asterisks below. (B) The HD5 IC₅₀s of the

indicated viruses were determined on A549 cells. Each data point is an independent experiment, and bars are the mean \pm SD. Virus expanded from the 50th round of selection (R50) is equivalent to sample 11 in panel A. Results of one-way ANOVA with Tukey's multiple comparisons test are shown by asterisks below. (C) Space-filling model of the structure of a HAdV-5 hexon trimer (PDBID:6CGV [169]) from top and side views. HVR1 (blue) is modeled using the structure of the shorter HVR1 from species D HAdV-26 (PDBID:5TX1 [170]). White circles indicate the position of E424 in HVR7 (red). All other HVRs are light gray.

Penton base and fiber contribute to HD5 sensitivity

The absence of mutations in fiber and PB in the evolved viral populations was unexpected based on our previous studies [135]. To substantiate our prior findings and more narrowly delineate neutralization determinants in PB, we created a series of HAdV-5-based chimeric viruses in which portions of PB were swapped with the corresponding residues from HAdV-64. These constructs also contain a DTET to GYAR mutation in fiber, which acts in concert with changes in PB to ablate HD5-dependent neutralization [135]. Initially, we chose highly conserved regions spaced evenly within the HAdV-5 PB sequence as junction points for our chimera designs. As in our prior studies, HAdV-5 infection was potently neutralized, HAdV-64 infection was enhanced 4- to 5-fold, and infection by a chimera (C1) containing the entire HAdV-64 PB was moderately enhanced 2- to 3-fold when incubated with 5 μ M or 10 μ M HD5 (Fig. 3A). If C-terminal residues 288 to 571 of PB are from HAdV-5 (C5), then the virus is neutralized by HD5. If they are from HAdV-64 (C4), then infection is not neutralized but enhanced. An intermediate phenotype of partial HD5 sensitivity was observed when residues 288 to 434 were derived from HAdV-64 and residues 435 to 571 were derived from HAdV-5 (C2), while the opposite construct (C3) completely recapitulated the HD5-sensitivity of HAdV-5. Thus, residues 288 to 434 from HAdV-5 are necessary for complete neutralization, while residues 435-571 also contribute.

Although not exhaustive, additional chimeras were created to probe the contribution of specific variable sequences within the C-terminal half of PB to HD5-dependent neutralization (Figs. 3B and 3D). In the description that follows and in Fig 3B-D, colors indicate where the equivalent residues from HAdV-64 are substituted into HAdV-5 PB. The highly variable RGD loop (black) from HAdV-5 is necessary for HD5 neutralization. A moderately conserved motif C-terminal to the RGD loop (cyan) also contributes to HD5 sensitivity, while one N-terminal to the

RGD loop (yellow) does not (compare C6, C7, C8, and C9). And, the four variable positions between residues 533 and 562 (purple) contribute to neutralization (compare C4 and C10), while the 22 variable residues between 395 and 472 (orange) do not (compare C7 and C13). Although no definitive conclusions can be drawn, a comparison of C10 and C11 suggests that the variable positions between residues 479 and 506 (red and green) also contribute modestly to neutralization. Collectively, this analysis suggests that complete neutralization of HAdV-5 by HD5 reflects the additive effects of multiple residues in PB.

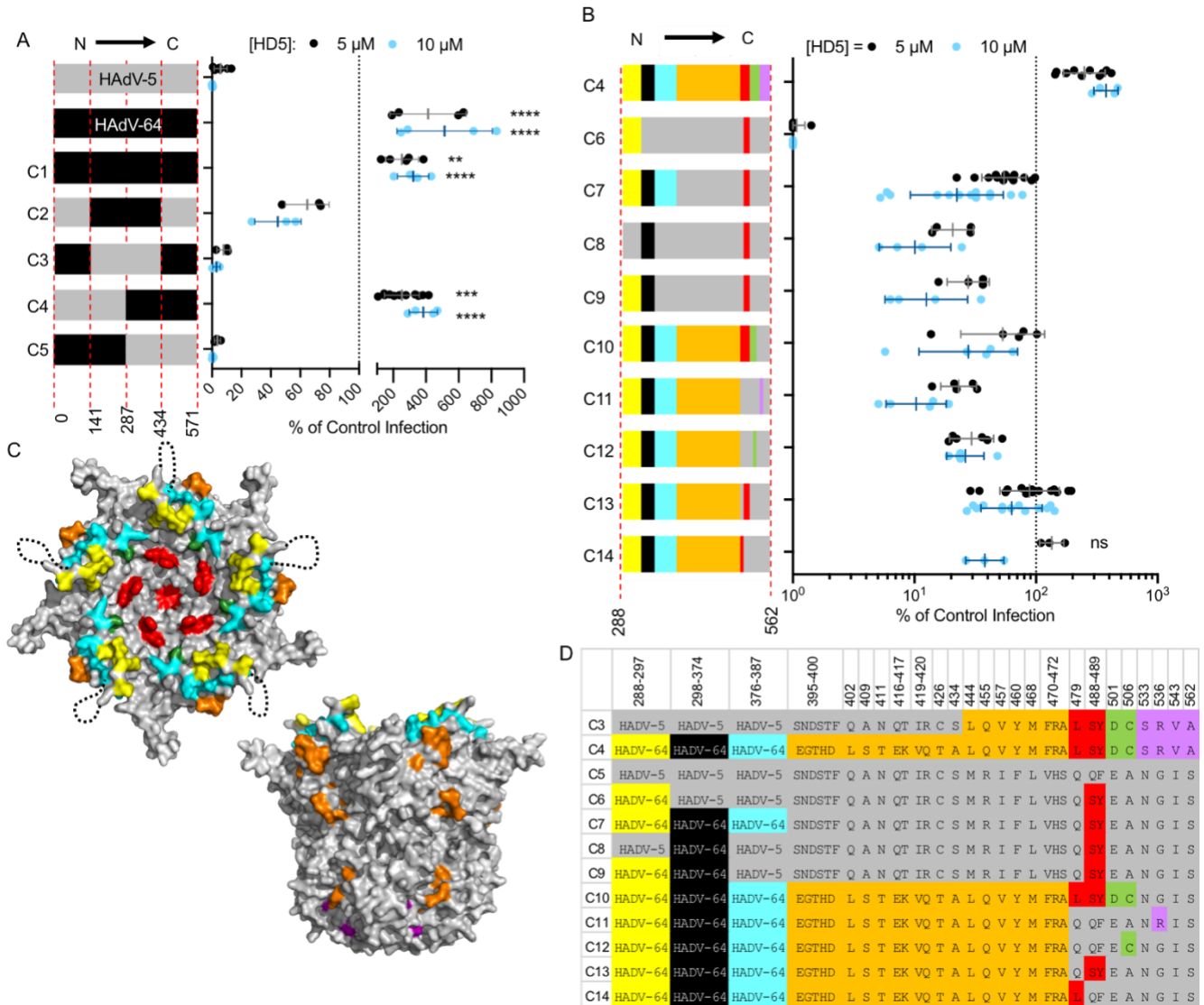


Figure 3 Determinants of HD5-mediated neutralization are located within the C-terminal half of penton base.

(A and B) HAdV-5, HAdV-64, and chimeric viruses were incubated with 5 μ M (black) or 10 μ M (blue) HD5 and then assessed for infectivity on A549 cells. In (A), colors denote sequences derived from HAdV-5 (grey) or HAdV-64 (black), and amino acid residue numbers refer to HAdV-5 PB. In (B), colors correspond to the chart in (D) of the differential amino acid residues between HAdV-5 and HAdV-64 in the C-terminal half of PB, numbered according to HAdV-5. Each data point is

an independent experiment, and bars are the mean \pm SD of the percent infectivity compared to control cells infected with each virus in the absence of HD5. Note that the same data for C4 is plotted on both graphs. For (A), results of two-way ANOVA with Dunnett's multiple comparison to HAdV-5 are indicated by asterisks. For (B), all comparisons to chimera C4 are significant ($P \leq 0.01$) except where indicated as not significant (ns). (C) Space-filling model of the crystal structure of a pentamer of HAdV-5 PB (PDBID:6CGV³⁹) in top and side views, with differential residues colored as in (D). The unresolved RGD loop (residues 298-374) is denoted by a dotted black line.

The role of capsid proteins in determining the outcome of HD5-virus interactions depends on the timing of exposure to HD5 relative to cell binding

Rational design and directed evolution implicated different major capsid proteins as HD5 neutralization determinants. We suspected that the protocol used to assess the PB chimeric viruses versus that used to select for resistant viruses caused this discrepancy. We therefore determined the HD5 IC₅₀ for key viruses by either pre-incubating virus with HD5 and then adding this mixture to cells (protocol 1) or by adding the defensin to virus pre-bound to cells (protocol 2). The phenotypes of HAdV-5 (Fig. 4A), the round 50 pool (Fig. 4B), and hexon HVR1 chimera (Fig. 4C) were largely protocol-independent. The only difference in the phenotype of HAdV-64 (Fig. 4D) was enhancement in protocol 1 compared to protocol 2. In contrast, the phenotype of the vertex C4 chimera was dramatically protocol-dependent (Fig 4E), demonstrating that cell binding can alter HD5 sensitivity.

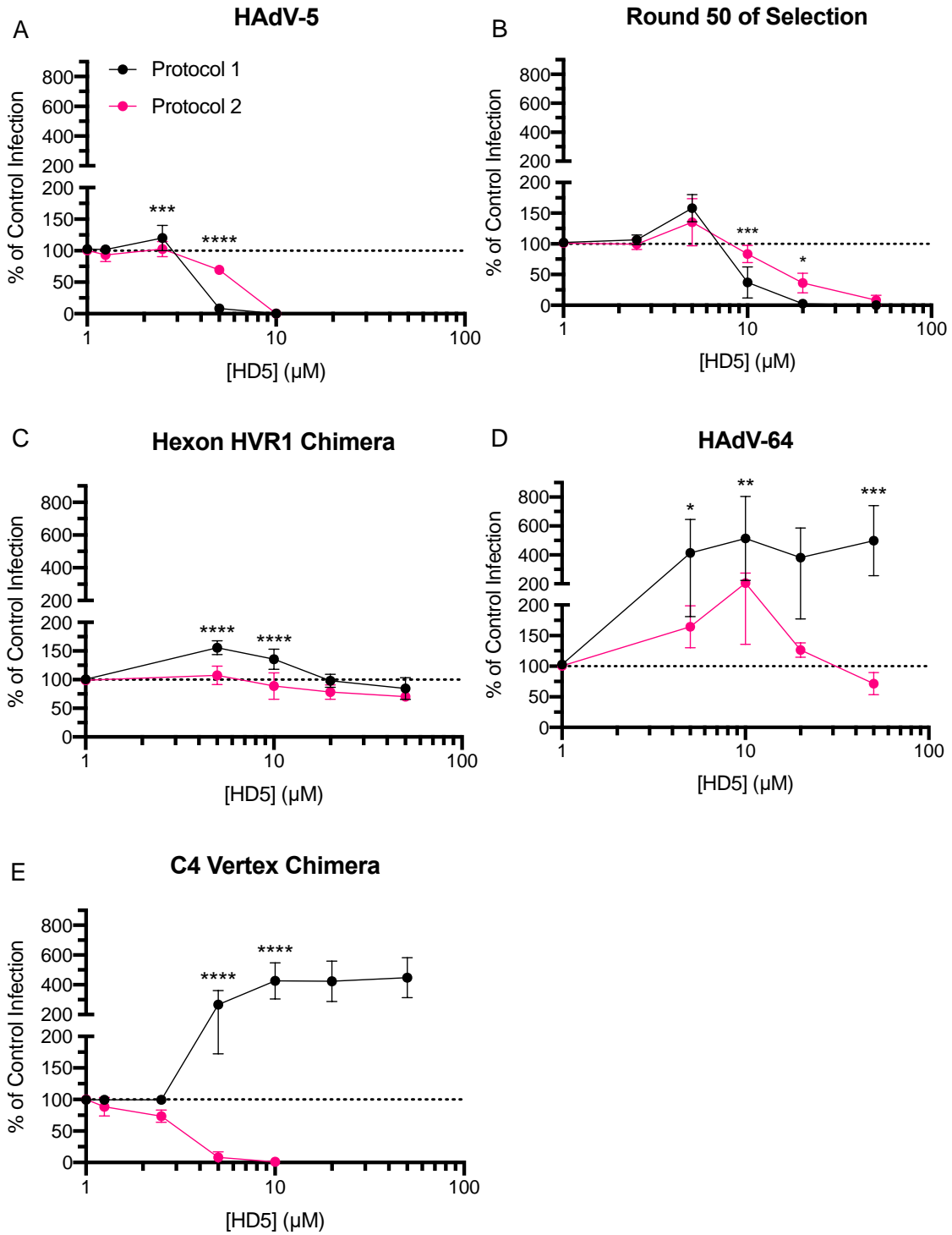


Figure 4 Hexon and vertex play different roles during virus-defensin interactions.

Purified (A) HAdV-5, (B) virus expanded from round 50 of selection, (C) HAdV-64, (D) hexon HVR1 chimera, and (E) C4 vertex chimera were either incubated with HD5 and then added to A549 cells (protocol 1 – black) or bound to A549 cells prior to HD5 addition (protocol 2 – pink). Data is the mean of at least 3 independent experiments \pm SD. Results of two-way ANOVA with Sidak multiple comparisons at each HD5 concentration are indicated by asterisks.

We then created and tested an additional construct combining the changes from the C4 vertex and hexon HVR1 chimeras. In protocol 1, the hexon HVR1/C4 vertex chimera had the same phenotype as the hexon HVR1 chimera rather than the C4 vertex chimera: it was resistant to neutralization by HD5 at all concentrations but not enhanced (Fig 5A). In protocol 2, the hexon HVR1/C4 vertex chimera exhibited an intermediate phenotype: it was ~3-fold more HD5-resistant than the C4 vertex chimera but ~2-fold more HD5-sensitive than the hexon HVR1 chimera (Fig 5B). Thus, both hexon HVR1 and the vertex are important determinants of viral infectivity in the presence of HD5, but they do not act synergistically. Rather, the phenotype of HVR1 is predominant in protocol 1, while HVR1 and the vertex appear to have additive effects in protocol 2.

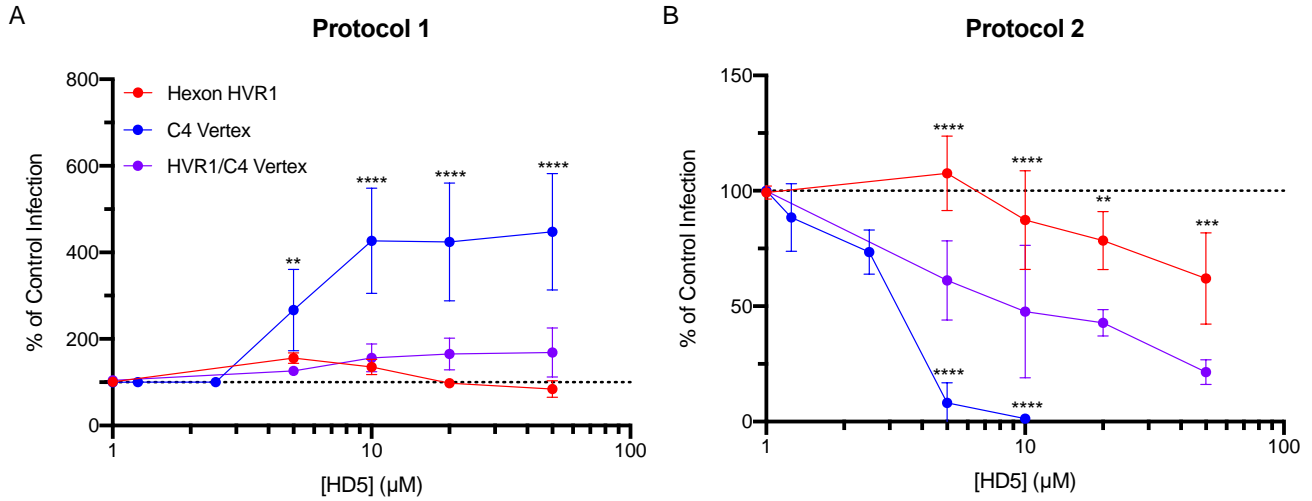


Figure 5 The roles of hexon HVR1 and vertex are not synergistic for virus-defensin interactions.

Hexon HVR1 (red), vertex C4 (blue), and the combined hexon HVR1/C4 vertex (purple) chimeric viruses were either (A) incubated with HD5 before being added to A549 cells (protocol 1) or (B) bound to A549 cells before the addition of HD5 (protocol 2) and then assessed for infectivity. Note that the data for hexon HVR1 and vertex C4 chimeras are reproduced from Fig. 4 (D) and (E). Data is the mean of at least 3 independent experiments \pm SD. Results of two-way ANOVA with Dunnett multiple comparisons to hexon HVR1/vertex chimera are indicated by asterisks.

Vertex and hexon proteins dictate HD5 binding to the viral capsid

Based on previous studies demonstrating a direct interaction between HD5 and HAdV [133-135, 137], we quantified the average number of HD5 molecules bound to the capsid of each virus. At a concentration of 20 μM , HD5 binds at a high molar ratio to HAdV-5 (7090 \pm 1550 molecules of HD5 per HAdV-5 virion), as shown previously [135], and \sim 83-fold less to HAdV-64 (Fig 6A). Interestingly, the C4 vertex chimera had a \sim 2.3-fold increase in binding compared to HAdV-5, while the hexon HVR1 chimera showed a \sim 5-fold decrease. Finally, HD5 bound to the combined hexon HVR1/C4 vertex chimera at the same levels as HAdV-5. At lower concentrations (5 μM and 10 μM), HD5 binding to HAdV-5, C4 vertex chimera, and hexon HVR1/C4 vertex chimera was equivalent (Fig 6B). Thus, changes in both hexon and the vertex proteins alter the stoichiometry of the HD5-capsid interaction.

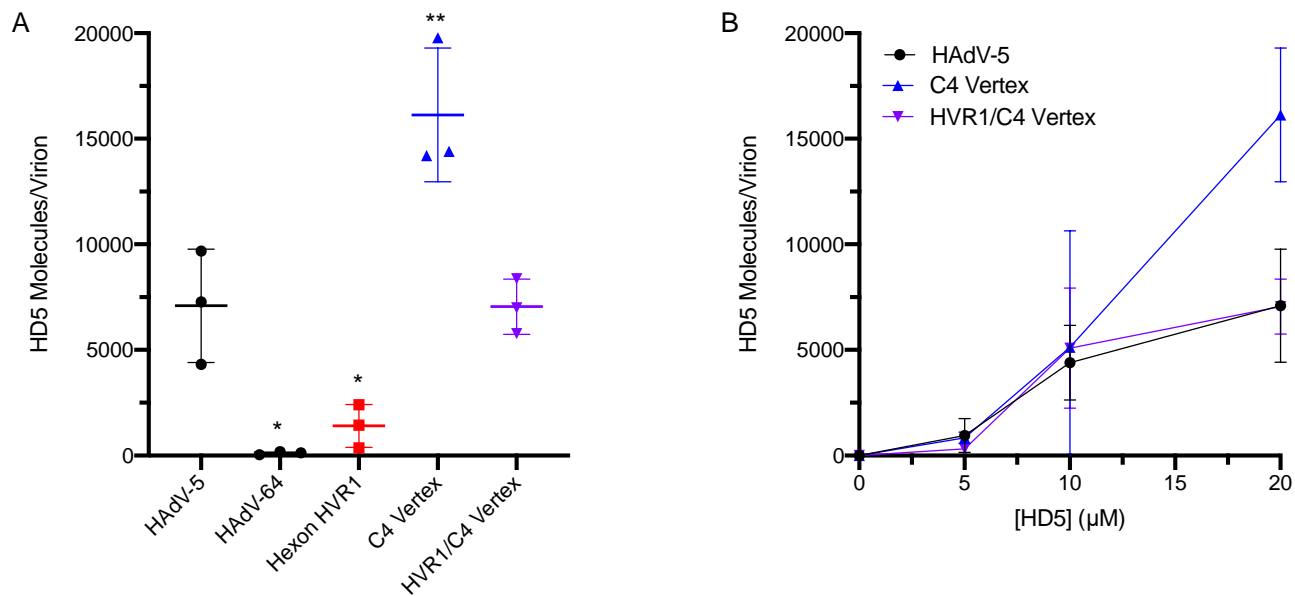


Figure 6 Both hexon HVR1 and vertex contribute to HD5 binding.

(A) HAdV-5, HAdV-64, and chimeric viruses were incubated in the presence of 20 μ M HD5. The amount of HD5 bound per virion was quantified from 3 independent experiments. (B) HD5 molecules bound per virion of HAdV-5 and chimeras C4 vertex and hexon HVR1/C4 vertex were also determined in the presence of 5 μ M and 10 μ M HD5. Note that the data for 20 μ M HD5 in (A) for these viruses is reproduced in (B). Lines are the mean \pm SD. The results of ordinary one-way ANOVA with Dunnett's multiple comparisons to HAdV-5 is denoted by asterisks.

Defensin binding and stabilization of capsid proteins

Our prior studies are consistent with a mechanism in which HD5 neutralizes HAdV-5 by stabilizing the capsid and preventing shedding of the vertex proteins [135, 137]. Thus, capsid changes could impact the thermostability of the virus in the presence or absence of HD5. We first determined the temperature at which 50% of fiber dissociates from the viral capsid (T_m) in the absence of HD5. As expected, all viruses remained intact at 44°C, and fiber was completely dissociated at 50°C (Fig 7A and B). The T_m of the hexon HVR1 chimera was identical to that of HAdV-5 (46°C), while the T_m s of the C4 vertex and hexon HVR1/C4 vertex chimeras were similar to each other and warmer than that of HAdV-5 by ~2°C. Thus, amino acid changes in the C4 vertex stabilized the fiber-capsid interaction, while amino acid changes in hexon HVR1 had no effect. We then tested the effect of HD5 on fiber dissociation, when samples were heated to 2°C above the T_m to assure full fiber dissociation in the absence of HD5. Despite their different infection phenotypes and HD5 binding capacities, the fiber of each of the viruses was fully capsid-associated upon incubation with 20 μ M HD5 (Fig 7C). HAdV-5 and C4 vertex chimera fibers were 50% capsid-associated at 5 μ M HD5 and had identical HD5-dependent dissociation profiles. The hexon HVR1/C4 vertex chimera required a 2-fold lower HD5 concentration than HAdV-5 to be 50% capsid-associated and was significantly more stabilized than HAdV-5 at both 2.5 μ M and 5 μ M HD5. In contrast, the hexon HVR1 chimera required at least a 2-fold higher HD5 concentration than HAdV-5 to be 50% stabilized. Overall, the composition of both HVR1 and the vertex both influence HD5-mediated fiber stabilization; however, the phenotype of the combination does not mirror the individual contributions of each capsid component.

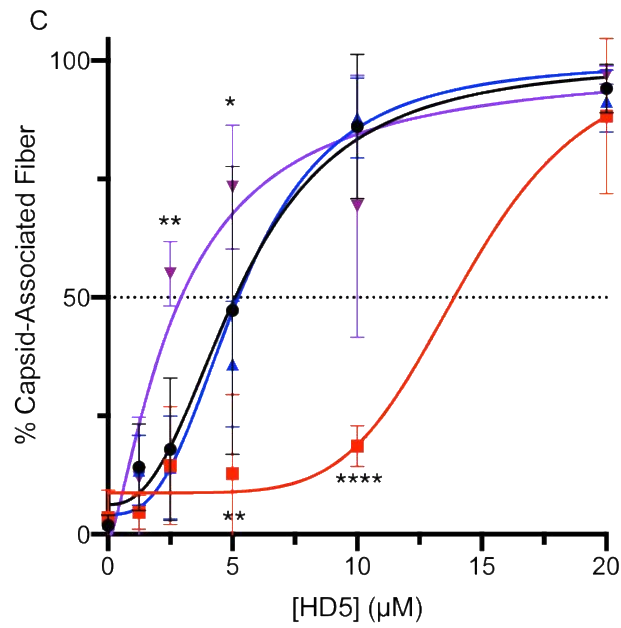
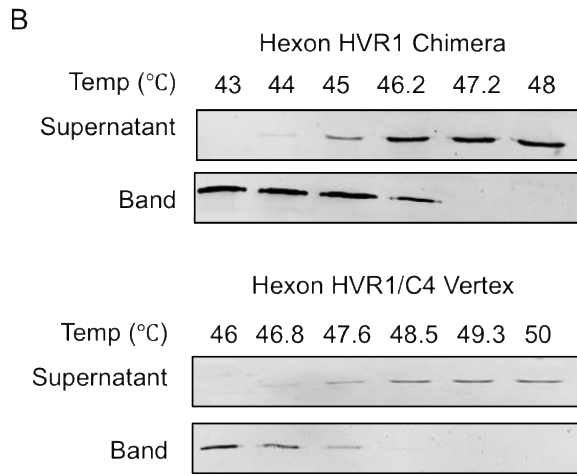
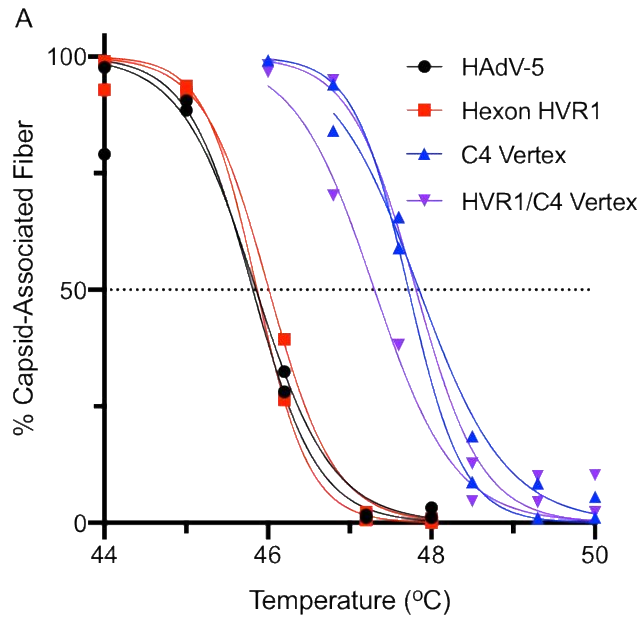


Figure 7 Fiber thermostability does not correlate with infection phenotype.

The percent of fiber that remains capsid associated was determined (A) as a function of temperature in the absence of HD5 or (C) as a function of HD5 concentration for HAdV-5 (black) and hexon HVR1 chimera (red) at 48°C and for C4 vertex chimera (blue) and hexon HVR1/C4 vertex chimera (purple) at 49.3°C. (B) Representative immunoblots from the temperature gradients of hexon HVR1 and hexon HVR1/C4 vertex chimeras are shown. In (A) each point and line is an individual replicate. In (C), each point is the mean \pm SD of 3 independent experiments, and the results of two-way ANOVA with Dunnett's multiple comparisons to HAdV-5 is denoted by asterisks.

Discussion

In this study, we have directly demonstrated that enteric α -defensins can impose selective pressure on non-enveloped viral evolution. We postulated that selection may occur during fecal-oral transmission due to the abundant expression and high concentration of enteric α -defensins in the intestinal lumen [147]. This hypothesis is based on the observation that naturally occurring HAdVs and MAdVs are differentially susceptible to α -defensin antiviral activity and that defensin resistance correlates with AdV species that contain fecal-orally transmitted serotypes [135, 147, 162, 171]. It would also explain the α -defensin resistance of echovirus and reovirus [117]. A prior study used a similar approach to select isolates of HIV-1, an enveloped virus, to retrocyclin, a θ -defensin expressed in non-human primates [116]. The mutations that appeared in both HAdV-5 and HIV-1 during selection increased the positive charge of the viral structural proteins (hexon and gp41, respectively), likely leading to an overall decrease in defensin binding due to the cationicity of defensins. A similar principle of surface charge modulation contributes to the experimental evolution of bacterial resistance to cationic antimicrobial peptides, which also confers resistance to β -defensins [150]. Collectively, there is now direct evidence that defensins can impose selective pressure on the evolution of a wide range of organisms including not only bacteria and enveloped viruses but also non-enveloped viruses.

Although chimera analysis has proven to be a useful tool to understand the principles of defensin-virus interactions, this approach can be confounded by inherent and practical limitations. First, only chimeras resulting in replication-competent virus capable of assembling can be analyzed. In this regard, reverse hexon HVR chimeras in the HAdV-64 background could not be recovered, which has also been reported by others [31, 148, 168]. Second, altering the capsid architecture can create non-natural binding determinants. This possibility may explain the

apparent requirement for a native hexon in enhancement or the extensive HD5 binding to the C4 vertex. Third, chimeras may have capsids with altered stability compared to WT viruses. Thus, one potential explanation for the resistance of a chimera to HD5 is an inherent capsid instability that cannot be overcome by HD5 binding. However, the C4 vertex is more thermostable (fiber T_m 2°C higher than HAdV-5), yet the hexon HVR1/C4 vertex chimera is resistant to HD5 (Fig. 7). Fourth, chimeras have limited resolution, particularly if extensive regions of capsid proteins are involved in HD5 interactions, as may be the case for PB and hexon. For HAdV, this is further confounded by the difficulty in constructing, validating, and amplifying large numbers of chimeras, even with the advantages of recombineering. Accordingly, we have not yet extended our findings more broadly to other serotypes. Finally, chimeras may assemble inefficiently, leading to high particle:pfu ratios. Non-infectious particles may compete for HD5 binding, artificially generating HD5 resistance. To address this concern, our studies have utilized multiple preparations of each chimera with differing particle:pfu ratios. Moreover, our ability to generate multiple PB chimeras with similar phenotypes suggests that this may not be a major concern.

Directed evolution overcame some of these limitations and provided complementary information. A major outcome of our investigation is the identification of a novel determinant of HAdV-5 neutralization by HD5 that fundamentally alters our understanding of the α -defensin antiviral mechanism. Our prior studies supported a model where HD5 binds to the fiber and PB proteins at the vertices of the HAdV-5 capsid and stabilizes their interaction. This action blocks uncoating of the capsid during cell entry and release of the membrane-lytic protein VI, which in turn prevents endosome escape and trafficking of the viral genome to the nucleus. This mechanism was supported by structural, biochemical, biophysical, and genetic studies [133-135, 137, 140];

however, we were unable to account for the extensive HD5 binding to the hexons of HAdV-5 in our cryoEM studies [135]. Directed evolution of HAdV-5 under selection by HD5 has provided new insight into the role of HD5-hexon interactions that substantially revises this model.

The only major capsid protein to contain non-synonymous mutations that were maintained during the selection was hexon, which supports an independent role for this motif. And, unlike antibody neutralization [30], HD5 resistance required the accumulation of multiple mutations. In addition to the three mutations in hexon, non-synonymous mutations became fixed in the 100K protein, polymerase, and U Exon. The 100K protein is known to be a chaperone for the proper folding and transport of hexon [168, 172, 173]. Thus, a temporal association between the appearance of mutations in 100K and hexon indicate a compensatory function. Furthermore, we were unable to isolate viruses that contained only the first hexon HVR1 mutation (I144K) in the absence of the 100K mutation through plaque purification. Despite this, we were able to produce an engineered HAdV-5 containing the two point mutations in HVR1 (Fig 3A) in the absence of the mutation in 100K, which had no apparent replication defects. These results suggest that the mutation in 100K is only necessary when the single hexon mutation is present in the absence of other hexon mutations, although this has not been investigated further. We also did not pursue the importance of the non-synonymous mutations in polymerase or the U exon that became fixed in the population. Notably, none of the mutations identified in the selected viruses were found in the starting population or arose in the control, suggesting that they arose *de novo*. This is further indicated by the accumulation of mutations within the genomes of individual viruses over time.

Directed evolution led us to identify hexon as the primary mediator of HD5-enhanced infection. We define enhancement as ≥ 2 -fold higher infection in the presence of HD5 than in the

absence of HD5. Consistent with prior studies of HAdVs and MAdVs [134, 135, 147], we found that enhancement only occurs when the virus binds HD5 before binding to the cell (protocol 1 in Figs. 3, 4, and 5). Increased cell binding likely occurs in part by neutralizing the repulsive forces of the electronegative capsid in proximity to the cell membrane [134], although HD5 could also bridge interactions between the virus and cellular lipids, glycans, or an unidentified HD5-specific receptor. A similar mechanism has been shown in shigella, another gastrointestinal pathogen that appropriates HD5 to facilitate infection [145, 146]. In addition, each of the viruses that are enhanced contained hexons in which all of the HVRs are from a single serotype, including a recently published HAdV-5 chimera containing all of the HVRs of HAdV-48 hexon [148]. This suggests that homotypic interactions involving hexon HVRs mediate enhancement. A comparable role for hexon in mediating cell binding has been previously described with two other host proteins: 1) coagulation factor X (FX) bridges an interaction between the virus and heparan sulfate proteoglycans on hepatocytes [42, 43]; however, HD5 and FX target distinct hexon HVRs and 2) lactoferrin mediates binding between hexon HVR1 and the cell surface [45]. Thus, our studies have identified a novel role for hexon in cell binding.

In contrast to enhancement, the HVR1 loop of hexon functions in cooperation with the vertex proteins as a previously unidentified determinant of HD5-mediated neutralization. Despite increased cell binding and the potential for enhanced infection, many HAdV serotypes are nonetheless neutralized by HD5 [135]. If either HVR1 or both vertex determinants (four residues near the N-terminus of fiber and the C-terminal half of PB) are derived from a resistant virus, then neutralization does not occur. This suggests that the functions of the capsid determinants are interrelated. However, if the virus is bound to its cellular receptor and co-receptor prior to HD5 addition (protocol 2 in Figs. 4 and 5), HVR1 is the sole determinant of neutralization. Thus, the

virus-cell interaction functionally replaces the vertex in potentiating HD5 neutralization. Mechanistically, this could occur through receptor-induced conformational changes that lead to exposure of HD5-interacting surfaces in the vertex that are buried in the absence of receptor. Alternatively, the receptor-virus interface could provide a novel target for HD5 binding. However, these interpretations suggest that the virus in protocol 1 either doesn't experience the conformation induced at 4°C in protocol 2 or transitions through it too rapidly for HD5 to exert a neutralizing effect. Moreover, it is unknown how many vertices are receptor-engaged under the conditions of protocol 2. If only a subset are bound, then blocking uncoating triggered through these vertices may be the key step impeded by HD5 binding [51]. The nature of the HVR1 loop also dictates the Hill slope of the HD5 inhibition curve in protocol 2 (Fig 5B), suggesting distinct levels of cooperativity and modes of HD5 binding by the two HVR1 loops. And, C4 vertex-containing viruses are neutralized at a lower HD5 concentration than those with the HAdV-5 vertex, which may be due to the inherently higher thermostability of the C4 vertex (Fig. 7A) or to its higher HD5-binding capacity (Fig. 6). Although a minimum amount of HD5 binding to the capsid is required for neutralization, there is not a simple correlation between the degree of neutralization and the amount of HD5 bound. Total HD5 bound appears to reflect additive functions of HVR1 and the vertex. And, neither WT vertex appears to bind HD5 to the same extent as the C4 vertex, suggesting altered binding by the artificial interface in the C4 chimera. We also found that fiber stabilization does not directly correlate with the infection phenotypes of the chimeras and that swapping HVR1 also affected the ability of HD5 to stabilize fiber dissociation. Collectively, these findings suggest that our previous model of vertex stabilization mediated only by HD5 interactions with fiber and PB is incomplete.

A model most consistent with our data is that blocking vertex dissociation through HD5 interactions with fiber/PB is insufficient, and a separate hexon-dependent mechanism, perhaps inter- or intra-hexon “cross-linking” by HVR1-HD5 interactions that prevent hexon dissociation, is also required to prevent uncoating. We cannot formally exclude a model where the capsid determinants act cooperatively to coordinate HD5 binding at the vertex, particularly since the HAdV-5 hexon HVR1 loop is long enough to extend from the peri-pentonal hexons towards fiber. However, in that model it is harder to rationalize a role for the point mutation that arose in hexon HVR7 in the later rounds of selection. Both models are also consistent with the phenotypes of the PB chimeras, where intermediate levels of neutralization result from a subset of the PB changes found in the C4 vertex. Further experimentation will be required to resolve these possibilities.

Chapter 3: Significance and Future Perspectives

Extending defensin studies to rotavirus

Until recently, most defensin studies have focused on understanding their potent antimicrobial properties [57]. Hence, only the pathogens HIV, shigella, and enteric HAdVs have been studied for their ability to exploit the presence of α -defensins to facilitate an infection [74, 143-148, 152, 153, 174]. Interestingly, each of these pathogens relies on the presence of enteric α -defensin HD5 for an enhanced infection [144, 175-177]. As such, HD5 could potentially serve to enhance the infection of other enteric pathogens. Furthermore, this enhancement could extend to other animals that have similar enteric defensins including mammals and reptiles. When we investigated the effect of enteric α -defensin on various non-enveloped viruses, fecal-orally transmitted viruses (norovirus, adenovirus, and rotavirus) were either resistant to, or even enhanced in the presence of enteric α -defensins while non-enteric viruses (papillomavirus and respiratory HAdV) were completely inhibited at the same concentration of defensin [131, 135, 147]. To expand on the above studies and gain insight into the specificity of viral-defensin interactions, we chose to focus on rotavirus (RV).

RVs, members of the *Reoviridae* family, cause severe diarrheal disease in nearly every child worldwide by the age of five [178]. They are non-enveloped viruses with 11 segments of double-stranded RNA. The capsid consists of three concentric layers around the genome. VP4 and VP7 form the outermost layer. Notably, cleavage of VP4, the spike protein, to VP5 and VP8 by host trypsin during transit through the digestive tract is important for the production of fully matured virus [179]. VP5 and VP8 are involved in cell binding and entry, neutralization, and virulence [180]. Specifically, VP8 mediates initial binding to the cell [181]. VP5 and VP7 mediate cell entry by interacting with additional co-receptors on the cell surface including

integrins and heat shock cognate protein 70 [182]. Both VP4 and VP7 are targets for neutralization by antibodies, and, like the HAdV hexon protein, could also play a major role in interacting with defensins [183].

To understand the differences and specificity of sensitivity, resistance, and enhancement to defensins, we chose to focus on RVs from three different mammal species (human, rhesus, and mice), as well as enteric α -defensins from the same 3 species, and myeloid defensins from 2 of the 3 species (mice do not produce myeloid defensins). We chose to study rhesus RV (RRV), two human RVs (DS-1 and WA), and one mouse RV (EDIM). All of these viruses are naturally tropic for three different host species with known enteric α -defensins [57]. In addition, DS-1 and WA are the two genotypes that most commonly cause disease in humans [184, 185]. EDIM is used to study RV in a mouse model, which could be used for future *in vivo* studies [186]. Finally, most studies to understand the mechanics of RV infection have been done in RRV [178-182]. The enteric α -defensins that were used are: rhesus enteric defensin (RED) 1 and 3, HD5, and mouse cryptdin 2 (Crp2). The myeloid defensins are HNP1, rhesus myeloid α -defensin (RMAD) 1, and RMAD4. These defensins were chosen to represent the diversity of α -defensins in the three host species [147, 180]. The above peptides are also the most abundantly expressed *in vivo*.

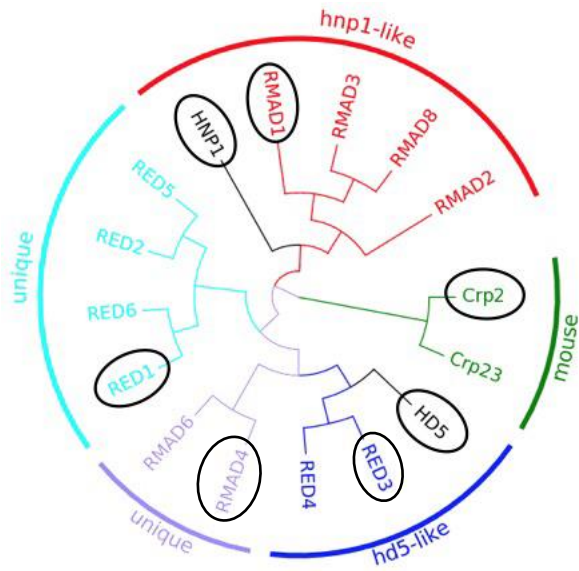


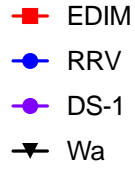
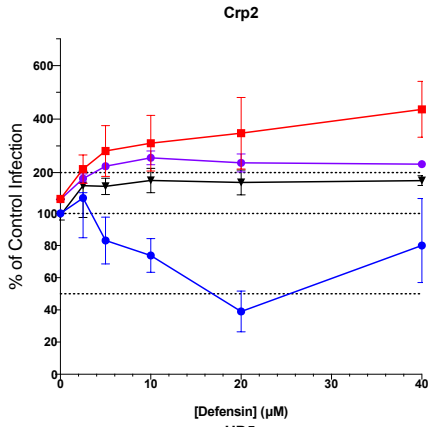
Figure 89 Relatedness of cross species α -defensins¹⁰

The relatedness of α -defensins by ClustalW analysis of peptide sequences is shown. Peptides used in this experiment are circled.

Infection levels of RRV, EDIM, and DS-1 and Wa on MA104 cells in the presence of a range of defensin concentrations (0-20 μ M) was tested (Fig 9). We found that RRV was potently neutralized by HD5 but was resistant to Crp2 and RED3 and unaffected by any concentration of RED1. EDIM and DS-1, however, were potently enhanced with at least a 2-fold increase of infection in the presence of each enteric defensin tested compared to control infection. When incubated in the presence of a myeloid defensin, RRV was strongly neutralized at concentrations above 5 μ M defensin. EDIM was resistant to RMAD1, enhanced by RMAD4, but neutralized by HNP1. DS-1, on the other hand, was resistant to HNP1 and RMAD4, but neutralized by RMAD1. Wa infection is resistant to Crp2, both rhesus enteric defensins, HNP1 and RMAD1. Although still resistant, Wa infection is reduced as much as 30% in the presence of RMAD4 and 70% in the presence of HD5.

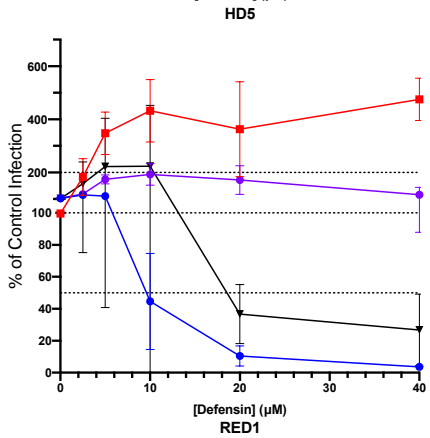
Enteric Defensins

A

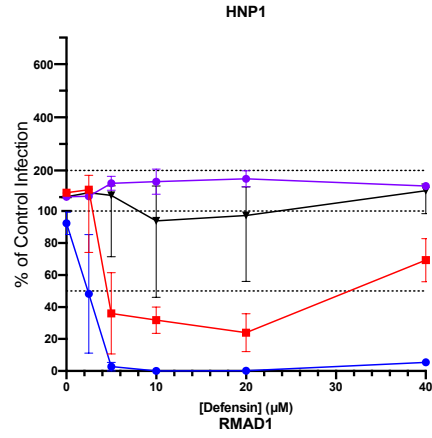


Myeloid Defensins

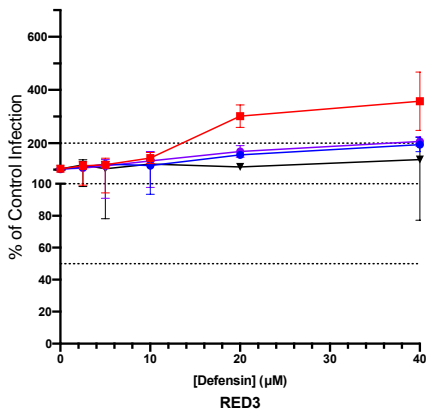
B



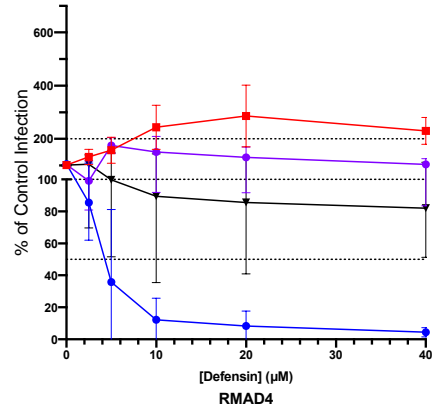
E



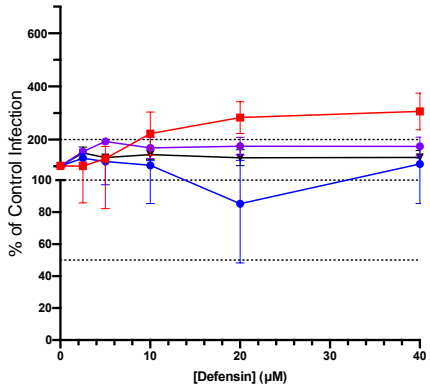
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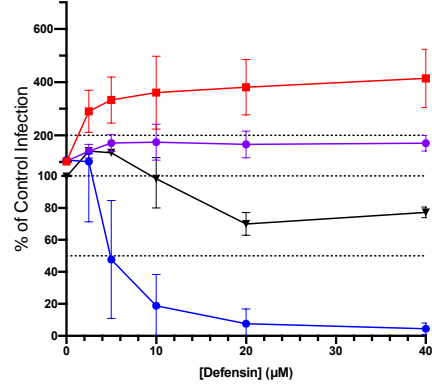


Figure 11 Patterns of RV enhancement, neutralization, and resistance

EDIM (red), RRV (blue), DS-1 (purple), and Wa (black) was incubated with either (A) enteric, Crp2, (B) HD5, (C) RED1 and/or (D) RED3 (gray) and myeloid defensins HNP1 (E), RMAD1 (F), and/or RMAD4 (G) at the indicated defensin concentrations then added to MA104 cells and assessed for infectivity after 24 hours. Data is the mean of at least 2 independent experiments \pm SD except.

Future Perspectives

Defensins are small but potent peptides that are found on human mucosal surfaces and can directly neutralize most viruses while enhancing others [57, 174]. Furthermore, they help bridge the innate and adaptive immune systems as chemokines and as opsonins likely influencing the efficiency of antibody production [57]. Although defensins have been studied extensively, many open questions still remain. These include determining how the *in vitro* activities of defensins translate to and impact viral infection *in vivo*. Because of their direct antimicrobial and immunomodulatory properties, defensins have been considered for therapeutic development as pharmaceuticals or even as vaccine adjuvants, but these avenues continue to be poorly studied [187, 188]. The importance of defensins is highlighted by their presence in the innate immune systems of most mammals, including humans [57, 149, 189]. The challenge now is to adapt the knowledge gained from antiviral studies with defensins to the needs of the world today.

Although characteristics such as their small size and protease resistance are appealing, there are a few challenges to developing defensins for clinical use. Currently, no defensins are mass produced. They all must be synthesized as linear peptides, which then go through a folding and validation process before being ready for use. This process can be expensive and time consuming. In addition, as lectins, defensins bind promiscuously potentially limiting their bioavailability. However, topical applications have been explored, as has been shown in HD5-mediated protection of mice from HSV-2 intravaginal challenge [118, 122]. As such, it may also be possible to formulate defensins for enteric delivery. Another potential use for therapeutics would be to modify existing defensins to make them more effective antivirals [122] or to use defensins from other species, which may enhance their antimicrobial properties, as we saw in

Figure 9 where RMAD1 neutralized DS-1 infection, but HNP1 did not. Finally, because their expression is associated with inflammation in joint replacements and lower levels of defensins are a characteristic of inflammatory bowel disorder, defensins have also been explored as biomarkers [190-192].

Multiple studies have shown α -defensins to be potent adjuvants. Ovalbumin (OVA) co-administered with HNPs led to increased production of OVA-specific IgG [193]. HNP1 or HNP4 co-injected with an HIV p24 peptide induced secretion of β -chemokines from the lamina propria and Peyer's patches to a greater extent than HIV p24 peptide alone [194]. Furthermore, defensin and HIV peptide co-administration resulted in higher levels of IFN- γ and perforin secretion from CD8⁺ T cells. And, HNP1-3 co-administered with antigens adsorbed to aluminum hydroxide increased antigen specific IgG responses and secretion of IL-4 and IFN- γ from spleen cells [193]. Most recently, a paper studying HAdV demonstrated that HD5 can be a potent adjuvant in vaccines and can lead to earlier and stronger antibody expression during an *in vivo* infection [148]. It was also shown that the potential of HD5 as an adjuvant is linked to HD5 enhancing the transduction of the virus *in vitro* [148]. These studies were performed using defensin sensitive HAdV-5 and defensin-enhanced HAdV-48. Interestingly, they identified hexon HVRs as determinants of HD5 adjuvant activity, which we further supported with our studies in chapter 2. This shows that just as microbes have taken advantage of the presence of HD5 during an infection, there are ways that we can apply these advantages to further the advancement of therapies. As such, both their neutralizing and enhancing abilities can be useful in combating diseases. Thus, a better understanding of the relationship between these enhanced viruses and how HD5 mediates enhancement could lead to increased potency of vaccines.

One group of vaccines that could benefit from the adjuvant properties of defensins are those developed against rotavirus. Currently there are two major vaccines available globally: RV1, which is a monovalent, oral, live-attenuated vaccine and RV5, a multivalent, oral, live, human-bovine heterologous vaccine. Interestingly, vaccine efficacy is highly dependent on a country's wealth index. Both RV1 and RV5 have been shown to prevent at least 80% of severe diarrheal rotavirus cases in developed countries, but only 37-57% in developing countries based on a Chochrane review of rotavirus vaccine studies done in 2019 [195]. This known major disparity in RV vaccine efficacy, as well as other enteric vaccines such as those for poliovirus and cholera, has continued to perplex the scientific community [196, 197]. One potential reason behind this disproportionate efficacy could be the increased prevalence of environmental enteropathy (EE) in lower income countries, especially since both RV1 and RV5 are oral vaccines [198, 199]. EE is characterized by villus blunting, crypt hyperplasia, and chronic inflammation of the lamina propria resulting in an overall disruption of the intestinal barrier [198, 200-208]. EE is particularly common in children and is thought to stunt growth, negatively affect the efficacy of oral vaccines, and predispose them to higher incidences of diarrheal infections [204, 208-214]. Furthermore, loss of a functional barrier leads to malabsorption and nutritional deficiency, which further aggravates the already low nutritional intake that precedes the development of EE [198, 215-217]. Interestingly, there is evidence of decreased defensin expression in the Paneth cells of people with EE compared to those in higher-income countries [214, 218]. As such, it is possible that including HD5 as part of the RV vaccine could boost the immune response to increase the efficacy of protection in these lower-income areas. Further studies to explore the ramifications of lower levels of antimicrobial peptides, while in a chronic state of inflammation in EE, are necessary to better understand causation.

In addition to EE, alterations in HD5 expression in the gastrointestinal tract are also connected to other inflammatory bowel diseases such as ileal Crohn's disease [191, 198, 219]. Specifically, there is a decrease in HD5 expression as was seen in EE [220]. This decrease is particularly important, since HD5 is one of the most highly expressed antimicrobial peptides in the small intestine [58, 221]. This decrease in HD5 expression has been shown to negatively affect the microbiome and the overall antimicrobial defenses within the small intestine [81, 191, 192]. For example, mouse studies have shown that the expression of HD5 in their Paneth cells leads to a shift in the proportion of Bacteroidetes in the gut compared to wild type mice and significant loss of segmented filamentous bacteria [222, 223]. The shift in the microbiome in addition to the decreased levels of antimicrobial activity heavily influence the host's innate immunity against the constant barrage of opportunistic pathogens in the gastrointestinal system [224]. This highlights the important role HD5 plays not only in directly neutralizing pathogens but also in modulating the microbiome to further support the maturation of a strong innate immune response. Studies are currently still limited on this aspect of defensin biology, but it shows the importance of understanding both the anti- and pro-microbial properties of defensins.

The goal of our work was to understand the pro-viral effects of defensins and how certain viruses have escaped neutralization. Non-enveloped viruses, such as enteric HAdV, that infect the gastrointestinal system are unaffected by defensins or can even appropriate defensins to enhance their infection [117, 135, 147, 162]. In contrast, respiratory HAdVs are neutralized by the same defensins [137]. How enteric viruses overcome defensin neutralization is not well understood. Our studies are the first to show that defensins can drive the evolution of non-enveloped viruses, providing a reasonable hypothesis for resistance in some, but not all HAdVs. In addition, it is unknown how many enteric viruses use defensins to enhance their infections.

The rotavirus studies we have done are the first to show defensin-mediated enhancement of another enteric non-enveloped virus (Figure 9). The field of study focused on the effects of defensins on non-enveloped viruses is still developing and rife with questions. With our work we identified viral determinants of neutralization and enhancement of infection in the presence of defensin for HAdV. These studies can now be expanded to identify the mechanism of enhancement, identify other non-enveloped viruses that are enhanced by defensins, and then isolate common motifs across these viruses that are determinants of enhancement, starting with rotavirus. To further understand the host innate immune system, we studied homologous defensins across species in an attempt to identify common motifs necessary for neutralization or enhancement. In addition, testing the effect of defensins from multiple species (mouse, human, and rhesus) on viruses from heterologous species could also uncover further evidence of viral evolution to escape HD5 neutralization. A refined and expanded understanding of defensin-viral interactions will continue to inform the development of defensin-based therapeutics.

Chapter 4: Materials and Methods

Cell lines. HEK 293 cells overexpressing human $\beta 5$ integrin (293 $\beta 5$) [135], A549 cells (ATCC), and MA104 cells obtained from Monica McNeal of Cincinnati Children's Hospital Medical Center (Cincinnati, OH) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, and non-essential amino acids (complete media).

Defensins: Partially purified (89%) linear peptides were synthesized (LifeTein, Somerset, NJ), oxidatively folded, and purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) [133]. Fractions containing the correctly folded species were lyophilized, resuspended in deionized water, and quantified by absorbance at 280 nm as described [133]. Purity (>99%) and mass were verified by analytical RP-HPLC and MALDI-TOF mass spectrometry. Defensins were stored at -80°C.

Defensin	Protein Sequence
HD5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
CRP2	LRDLVCYCRTRGCKRRERMNGTCRKGHLMYTLCCR
RED1	TCRCRIRRCRGLESSFGNCILHGQFAKLCCR
RED3	HTCYCRNKRCFTPEFHAGKCKVEGRTYKLCCR
HNP1	ACYCRI PACIAGERRYGT CIYQGRLWAFCC
RMAD1	ACYCRI PACLAGERRYGT CFYLGRVWAFCC
RMAD4	RRTCRCRFGRCFRRESYSGSCNINGRIFSLCCR

Viruses. An E1/E3-deleted, replication-defective HAdV-5 vector containing a CMV promoter-driven enhanced green fluorescent protein (eGFP) reporter gene cassette was used as the parent construct for all of the novel chimeras created for these studies, which were generated by

recombineering in BACmids [135, 225]. The C1 chimera was previously referred to as “PB/GYAR” [135]. Designs of chimeras C2-C14 are described in the Results and depicted in Fig. 3. Hexon chimeras were created by replacing either HVR1 (bp 19247 to 19336 in HAdV-5, NCBI: AC_000008.1), HVR7 (bp 20090 to 20203), or both HVR1 and HVR7 in the HAdV-5 *hexon* ORF with the corresponding sequences from HAdV-64 HVR1 (bp 18196 to 18243, GenBank: EF121005.1) and HVR7 (bp 19027 to 19161). The combined hexon HVR1/C4 vertex chimera was created by replacing the HAdV-5 HVR1 with that of HAdV-64 in the C4 chimera. A previously described E3 deleted, replication-competent HAdV-64 virus containing a CMV-eGFP ORF was also used to study the effects of HD5 on infection of a virus with a WT HAdV-64 capsid [226]. The fidelity of all BACmid constructs was verified by Sanger sequencing of the recombineered region and by restriction digest of the entire BACmid. In addition, the BACmids of HAdV-64, chimera C1, and chimera C4 were sequenced in their entirety by whole genome sequencing.

To produce virus, 293β5 cells were transfected with viral genomes released from the BACmids by *Pac* I endonuclease digestion. Following amplification over several passages in 293β5 cells to generate sufficient inoculum, approximately eight to ten T175 flasks of 293β5 cells were infected at a multiplicity of infection of ~3. Upon development of complete cytopathic effect, virus was precipitated from the supernatant in 8% polyethylene glycol (PEG) [227]. Virus was then purified from cell lysates and the PEG precipitate using a CsCl gradient as previously described [136]. Purified virus was dialyzed against three changes of 150 mM NaCl, 40 mM Tris, 10% glycerol, 2 mM MgCl₂, pH 8.1, snap frozen in liquid nitrogen, and stored at -80°C. The viral particle concentration was determined by Qubit fluorometric quantification

(ThermoFisher) against a DNA standard (1 μg = $2.34\text{E}+10$ virions). Genomic DNA was isolated from purified virus using the GeneJET genomic DNA purification kit (Thermo Fisher), and the fidelity of the changed regions was verified by Sanger sequencing. For biochemical assays, viral protein concentration was determined by Bio-Rad Protein Assay with a bovine serum albumin standard.

Rhesus rotavirus (RRV) was a gift from Monica McNeal. To expand the virus, RRV was trypsin-activated then added to MA104 cells to allow for replication. Once the cells exhibited CPE (cytopathic effect, 3-7 days), the cells were collected and mechanically lysed with three freeze-thaw cycles. Virus lysate was collected, flash frozen, and stored at -80°C .

Infection Assays. As in our previous studies [134], we employed two protocols, which differed in the order of addition of HD5 to the virus relative to cell binding. All adenovirus infection assays were performed in black wall, clear bottom 96-well plates seeded with either A549 or 293 β 5 cells, or MA104 cells for RRV infections. RRV was first activated by trypsin (10 $\mu\text{g}/\text{mL}$) for 1 hour at 37°C before proceeding with infections. Protocol 1 (Figs. 2, 3, 4, 5, and 8): virus and defensin were incubated together on ice in serum free media (SFM) for 45 min. Cells were then washed twice with SFM to remove any residual serum, the virus/HD5 mixture was added to the cells, and the plate was shifted to 37°C . Protocol 2 (Figs. 1C, 4, and 5): cells were incubated on ice for 5 min, washed once with cold SFM, and virus in SFM was then added. After incubation for 45 min on ice, the inoculum was removed, the cells were washed once with SFM, and HD5 in SFM was added. After incubation for 45 min on ice, the plate was shifted to 37°C . For both methods, the cells were washed with SFM after 2 h of incubation at 37°C , and the

media was replaced with complete media made from phenol red free DMEM or, for rotavirus infections, complete media. For RRV infections, infected monolayers were fixed with 2% paraformaldehyde, permeabilized (20 mM glycine, 0.5% Triton X-100), and stained for viral antigen with a primary anti-RV rabbit antibody and fluorescently tagged with a secondary Alexa Fluor 488-conjugated goat anti-rabbit antibody. Total monolayer fluorescence was quantified on a Typhoon (GE Healthcare). For adenovirus infections, cells were imaged 20-28 h post-infection on a Typhoon or Sapphire (Azure) imager. ImageJ was used to quantify background-subtracted total monolayer fluorescence. Data are shown as a percent of control infection in the absence of defensin. Concentrations of each virus were determined in advance that result in 50-80% of maximum signal for inhibition studies or 10-25% of maximum signal for enhancement studies.

HD5 Selection. As previously described, we used purified “mutator” adenovirus (HAdV-5.polF421Y) that was passaged for 10 rounds in 293 β 5 cells to generate diversity in the starting population [30]. We infected 293 β 5 cells in 12-well plates under selection with HD5 at the IC₉₀ following protocol 2 above. We used a ~6-fold lower MOI for control virus passaged in the absence of HD5 to yield comparable infection levels. Upon development of complete cytopathic effect, which typically occurred 3 to 4 days post-infection, the entire culture of cells and media was collected. A clarified freeze/thaw lysate generated from the infected cells was combined with the supernatant and snap frozen in liquid nitrogen for storage at -80°C. An aliquot was used to determine the infectious titer of each sample to ensure a similar level of infection for each round of selection. In addition, the HD5 IC₉₀ was determined periodically on 293 β 5 cells to recalibrate the selective pressure. For subsequent assays, selected viral pools were amplified in

the absence of HD5 over ~5 passages in 293β5 cells to generate sufficient inoculum and then purified from preparations of eight to ten T175 flasks of 293β5 cells as described above.

Plaque Purification. Pooled virus from rounds 10, 40, and 50 of the selection were plaque purified on 293β5 cells in a 6-well plate. For the initial infection, purified virus was used to infect cells at low MOI (0.004 - 0.03), and cultures were overlaid with complete media containing 1% Difco Noble Agar. After 7-14 d, cells and agar plugs from individual plaques were harvested using a pipet tip, resuspended in 100 μL of complete media, and lysed through three freeze-thaw cycles. The plaque-purified isolates were subjected to two additional rounds of plaque purification, expanded, and purified as described above. At intermediate steps, PCR and Sanger sequencing were used to identify plaques containing mutations in the *hexon* and *L4-100K* ORFs.

Whole Genome Sequencing. Genomic DNA was extracted and quantified from purified preparations of the plaque purified viruses and the pooled viruses from every 5th round of HD5 selection and the 10th and 20th round of control selection. The Nextera XT DNA Library preparation kit (Illumina) was used to tagment and barcode the genomic DNA. A MiSeq v3 150 cycle reagent kit (Illumina) was used to sequence the libraries with 75 base paired-end reads. The data was analyzed using the BreSeq pipeline to align the sequences to the parent genome and identify mutations [166].

Virus-Defensin Binding Assay. To measure HD5 binding, 2.5 μg of purified virus was incubated with 5, 10, or 20 μM HD5 in a buffer consisting of 150 mM NaCl, 20 mM Tris, 5%

glycerol, 1 mM MgCl₂ on ice for 45 min. Samples were then layered onto a discontinuous gradient containing 300 µl of 30% Histodenz overlaying 200 µl of 80% Histodenz in 20 mM tris pH 7.4. Gradients were centrifuged using an SW55ti rotor with adaptors (Beckman) at 209,0006 × g (avg.) for 1.5 h at 4°C, and the visible virus band was collected. Samples containing HAdV-5 mixed with HD5 that were not subject to centrifugation were used to generate a standard curve for quantification. All samples were reduced with DTT, heated to 95°C for 5 min, and separated by SDS-PAGE (10-20% tris-tricine gel). The gels were stained with Flamingo fluorescent protein gel stain (Bio-Rad) and imaged on a Sapphire Biomolecular Imager (Azure). Protein bands were quantified using Azure Spot software (Azure). The amount of HD5 in each sample was normalized to protein V and protein VII and quantified against the standard curve using Prism 8.3.0 software (GraphPad).

Thermostability Assay. To measure the capsid association of fiber, 250 ng of purified virus was incubated on ice with or without the indicated concentrations of HD5 for 45 min in serum free DMEM containing 0.05% BSA, 150 mM NaCl, and 10 mM HEPES pH 7.5. Samples were heated in a thermocycler at the indicated temperatures for 10 min and loaded onto a discontinuous gradient containing 400 µl of 30% Histodenz and 200 µl of 80% Histodenz in 20 mM tris pH 7.4. Samples were centrifuged as described above. Fractions were collected as follows: 90 µl from the top (supernatant), 2 middle fractions of 150 µl, and then 90 µl (virus band). The supernatant and band fractions were reduced with DTT, heated to 95°C for 5 min, separated by SDS-PAGE (12% tris-glycine gel), transferred to nitrocellulose, and probed by immunoblot for fiber using the 4D2 monoclonal antibody (ThermoFisher) and an Alexa Fluor 647-conjugated secondary antibody. Blots were imaged and quantified as described above to

determine the fraction of total fiber in each sample that was present in the virus band fraction.

Prism 8.3.0 was used for non-linear regression analysis to calculate T_m and HD5 concentrations resulting in 50% fiber dissociation in Fig. 7.

Statistical analysis and structural rendering. Statistical analysis was performed using Prism 8.3.0. Specific analyses are indicated in the figure legends. For all tests, not significant (ns), $P > 0.05$; *, $P = 0.01$ to 0.05 ; **, $P = 0.001$ to 0.01 ; ***, $P = 0.0001$ to 0.001 ; ****, $P < 0.0001$.

Structural figures were generated using the PyMOL Molecular Graphics System, Version 2.0.7 Schrödinger, LLC.

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- 2) Holly MK, **Diaz K**, & Smith JG (2017) Defensins in Viral Infection and Pathogenesis. *Annu Rev Virol* 4(1):369-391.
- 3) Wyatt EV, **Diaz K**, Griffin AJ, Rasmussen JA, Crane DD, Jones BD, et al. Metabolic Reprogramming of Host Cells by Virulent Francisella tularensis for Optimal Replication and Modulation of Inflammation. *J Immunol.* 2016;196(10):4227-36.
- 4) Dougherty M, Lazar J, Klein JC, **Diaz K**, Gobillot T, Grunblatt E, et al. Genome sequencing in a case of Niemann-Pick type C. *Cold Spring Harb Mol Case Stud.* 2016;2(6):a001222.

Oral Presentations

Diaz K, Smith JG. “Hexon HVR1 is a major determinant of defensin-mediated neutralization”. International Adenovirus Meeting 28 Sep 2018, Hotel Hacienda Vista Hermosa, Cuernavaca, México. Session 9: Virus-Host Interactions II.

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