

Host-defense peptides enhance enteric viral infection in a small intestinal organoid
model and *in vivo*

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A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2015

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Program Authorized to Offer Degree:

Microbiology

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Abstract

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The human small intestinal epithelium is a physical barrier to microbes, but it also produces numerous proteins and peptides that form a chemical barrier to infection. The most abundant of these peptides are the enteric α -defensins, which are produced by specialized epithelial cells called Paneth cells. Studies examining the role of α -defensins in enteric infection have been hampered by an inability to grow Paneth cells in culture. Using a recently described method for growing primary stem cell-derived small intestinal organoids, we established an infection model to examine the impact of naturally secreted α -defensins on enteric pathogens *in vitro*. Small intestinal organoids cultured from wild type and functional α -defensin-deficient mice (*Mmp7*^{-/-}) were microinjected with enteric pathogens to mimic apical infection. Wild type organoids were shown to produce functional α -defensins, which were capable of restricting the luminal growth of *Salmonella enterica* serovar Typhimurium (STM). Growth restriction was demonstrated for multiple strains of STM by both microscopy and viable bacteria counts, but growth of STM was not inhibited in *Mmp7*^{-/-} organoids. This model was then validated in a separate study demonstrating that mucoid *Klebsiella pneumoniae* variants are more resistant to

mouse enteric α -defensin killing than non-mucoid variants of *K. pneumoniae*, which lack a capsule. The small intestinal organoid infection model was next used to examine the impact of naturally secreted α -defensins on enteric viral infection. Consistent with results using purified enteric α -defensins, infection by mouse adenovirus-2 (MAdV-2), a natural enteric pathogen of mice, was enhanced after microinjection into wild type organoids. Enhancement was not observed upon infection of colonic or *Mmp7*^{-/-} organoids, which lack mature α -defensins. Establishing that results observed in small intestinal organoids are predictive of *in vivo* effects, wild type mice orally infected with MAdV-2 shed more virus in their feces than *Mmp7*^{-/-} mice. In summary, we have created and validated a model to investigate interactions between enteric pathogens and small intestinal epithelial cells. These studies are the first to examine the impact of direct prolonged *ex vivo* co-culture of enteric pathogens with viable primary epithelial cells capable of naturally producing enteric α -defensins.

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List of Abbreviations

<u>Abbreviation</u>	<u>Definition</u>
Abu.....	α -amino-n-butyric acid
AMP.....	antimicrobial peptides
ANOVA.....	analysis of variance
AU-PAGE.....	acid-urea PAGE
BK.....	BK virus
CFU.....	colony forming units
CMV.....	cytomegalovirus
CPS.....	capsular polysaccharide
Crp.....	cryptdin
CRS.....	cryptdin related sequence
DC.....	dendritic cell
gB.....	glycoprotein B from herpes simplex virus
gD.....	glycoprotein D from herpes simplex virus
GFP.....	green fluorescent protein
HAdV.....	human adenovirus
HBD.....	human β -defensin
HD.....	human defensin
HIV.....	human immunodeficiency virus
HNP.....	human neutrophil peptide
hpi.....	hours post-infection
HPIV.....	human parainfluenza virus
HPV.....	human papillomavirus
HSV.....	herpes simplex virus
IAV.....	influenza A virus
iPSC.....	inducible pluripotent stem cells
JCV.....	JC virus
LPS.....	lipopolysaccharide
LTB4.....	leukotriene b4
MAdV.....	mouse adenovirus
MBD.....	mouse β -defensin
MDP.....	muramyl di-peptide
MMP7.....	matrix metalloproteinase 7, matrilysin
MMTV.....	mouse mammary tumor virus
MUC.....	mucoïd
NHE3.....	Na ⁺ /H ⁺ -exchange isoform 3
NK.....	natural killer
NMV.....	non-mucoïd variant

NOD2.....nucleotide-binding oligomerization domain containing 2
OVA.....ovalbumin
PKC.....protein kinase C
RC-2.....retrocyclin 2
RSV.....respiratory syncytial virus
SFB.....segmented filamentous bacteria
SFM.....serum free media
SP-D.....surfactant protein D
STM.....*Salmonella enterica* serovar Typhimurium
T_H17 cells.....IL-17 producing T helper cells
VSV.....vesicular stomatitis virus

Acknowledgements

I would like to thank numerous people for their contribution to this work.

First and foremost, I would like to thank Jason G. Smith for his mentorship and guidance. You have been incredibly supportive throughout my time in graduate school, and I am thankful to have had the experience of being your first student.

Members of my thesis committee: Michael Lagunoff, Daniel Stetson, Denise Galloway and Jaisri Lingappa for their advice and insight. I would like to especially thank Michael and Dan for agreeing to be on my reading committee.

Mayim Wiens and Michele LeRoux for helpful conversations, support, and occasionally convincing me to do something not work related.

Beth A. Bromme for performing experiments in Chapter 4, and for being such a great undergraduate to mentor.

Mayumi K. Holly for performing experiments in Chapter 2 and Chapter 4, and for being convinced to join the Smith lab and continue the organoid research.

Other members of the Smith laboratory, past and present, for constant encouragement, and for making the lab an exceptional place to do research.

Bobbie Thomasson and Joe Maxwell for helpful discussion about organoids as well as for NOD2 organoid isolation.

Members of the Cookson lab for STM strains and plasmids. Especially Mary Stewart for help in STM strain construction.

Joseph Mougous and members of the Mougous lab for providing access and technical expertise for microcopy.

Dana Miller and Joe Horsman for helping to establish a microinjection system.

Robert Edwards and Kehui Wang provided guidance on establishing organoid cultures.

Wuyuan Lu, Andre Ouellete, Mark J van Raaij, Steven Larsen, Katherine Spindler, Silvio Hemmi and Susan Compton for reagents.

The University of Washington Department of Microbiology for the Stanley Falkow Award and the Helen Riaboff Whiteley Fellowship.

This work was supported in part by T32 GM07270 and T32 AI083203

Dedication

I dedicate this dissertation to my parents, Susan and Dexter Wilson, who helped foster my love of learning and to Jason Tracy Smith, for his support and love.

Chapter 1: Introduction

1.1 Mammalian enteric defenses and Paneth cells

The gastrointestinal tract is both home to a diverse repertoire of commensal bacteria and serves as an entry point for a large number of pathogenic viruses and bacteria. Intestinal epithelial cells help to define and defend this critical interface through a variety of mechanisms including (1) physical processes such as cell turnover, (2) chemical barriers provided by secreted proteins and mucus, and (3) signaling cascades that can augment and shape downstream adaptive immune responses¹. Epithelial cell derived antimicrobial peptides (AMPs) are one of the most ancient and well-conserved forms of chemical defense. Nearly all multicellular organisms, including plants, insects and mammals, produce AMPs². In the gastrointestinal tract, AMPs have a dual role, in that they both protect from pathogen invasion but also maintain homeostasis of the commensal microbiota³. Therefore, the production of epithelial AMPs is critical in defining the ecology of the gastrointestinal tract.

The small intestine, a major source of enteric AMPs, is made up of finger-like protrusions called villi and invaginations known as the crypts of Lieberkuhn. The small intestine is the most rapidly self-renewing tissue in the body⁴. Intestinal stem cells in the crypt base can undergo asymmetric division, with one daughter cell maintained as a stem cell in the crypt base and one daughter cell immediately differentiating while moving upward toward the villi⁵. These upwardly mobile cells differentiate into three main epithelial cell types; mucus-producing goblet cells, hormone producing enteroendocrine cells, and enterocytes (Figure 1.1). The entire process, from crypt base to villus tip takes 4-5 days. Cells that reach that villus tip are either sloughed off or undergo apoptosis and are detached⁴.

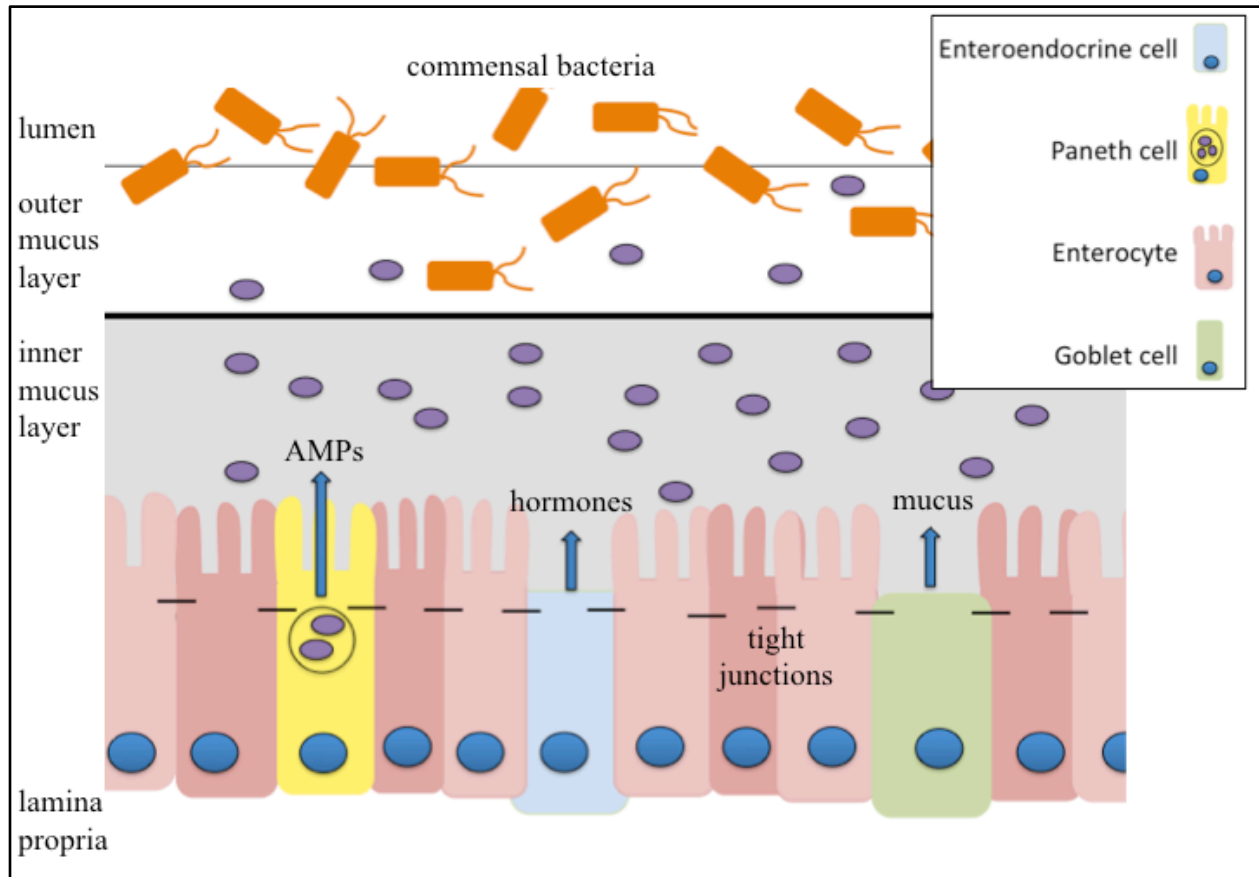


Figure 1.1 Differentiated epithelial cell types of the small intestine. The small intestinal epithelium consists of mucus producing goblet cells, hormone producing enteroendocrine cells, AMP producing Paneth cells and enterocytes. AMPs are retained in the inner mucus layer, where they restrict they growth of bacteria under homeostatic conditions. Commensal bacteria are generally found in the outer mucus layer.

In some cases, the daughter cell of an asymmetric division migrates downward into the crypt base, where it differentiates into a Paneth cell. In contrast to the other differentiated epithelial cells of the small intestine, Paneth cells are long-lived, and can persist in the crypt base for weeks to months⁴. As the primary producers of AMPs in the small intestine, Paneth cells are defined by their large secretory granules. AMPs packaged in Paneth cell granules include secretory phospholipases, lysozyme, the antibacterial lectin REG3 α (REG3 γ in mice), and α -defensins². However, α -defensins are the major products of Paneth cells and account for the majority of the antibacterial activity^{4,6}.

Paneth cells are restricted to the small intestine, except in cases of disease where ectopic expression in the colon or esophagus has been described⁴. In the small intestine, AMPs released by Paneth cells are trapped in the inner mucus layer, creating a gradient where the highest concentrations of AMPs are found in closest proximity to the epithelial cells⁷ (Figure 1.1). Due to the presence of AMPs, commensal bacteria are virtually absent from the inner mucus layer, and are most abundant in the lumen⁷.

1.2. Defensins

a. Classification and structure[#]

Defensins are small (~29-42 amino acid), cationic, amphipathic peptides with a predominantly β -sheet structure stabilized by 3 disulfide bonds (Figure 1.2). They can be broadly classified on the basis of structure and disulfide bond organization into three groups: α -, β -, and θ -defensins. As humans and mice lack θ -defensins, they will not be discussed further⁸. Humans

[#] Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

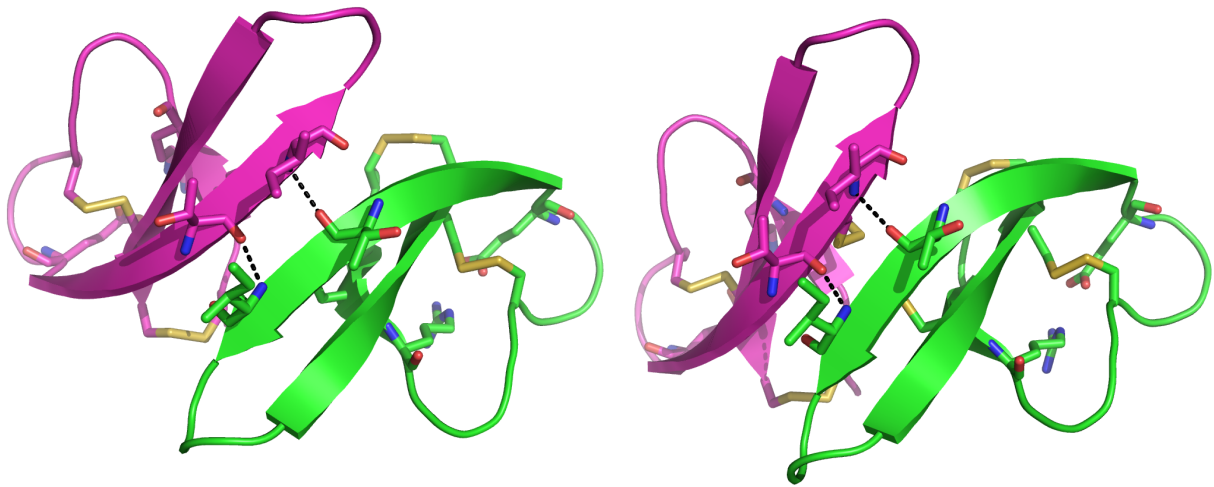


Figure 1.2 Structure of α -defensins. A representative structure of HNP3 as a dimer is shown from two different angles. Disulfides bonds are highlighted in yellow. Residues contributing to the salt bridge and the dimer interface are shown as ball and stick models. Dotted lines indicate hydrogen bonds at the dimer interface.

express six α -defensins and up to 31 β -defensins⁹. The α -defensins can be further subdivided into myeloid human neutrophil peptide (HNP) 1-4 and enteric [human defensin (HD) 5 and 6] peptides on the basis of both expression patterns and genetic organization¹⁰. Mice lack neutrophil α -defensins, but have undergone an expansion of their enteric α -defensin locus, and encode upwards of 20 enteric α -defensins, termed cryptdins, for crypt defensins^{11,12}. Mice also express over 40 β -defensins, though few have been characterized at the protein level¹³. Another distinct feature of mice is the expression of the antimicrobial cryptdin related sequence peptides (CRS), which are co-expressed with cryptdins in mouse Paneth cells¹⁴. CRSs are cationic and cysteine rich, similar to cryptdins, but unlike the six cysteine residues that define α - and β -defensins, they have nine or eleven cysteine residues.

All of the human α -defensins have been shown to multimerize into at least dimers either in solution or in crystal structures¹⁵⁻¹⁸. In contrast, rabbit neutrophil α -defensins and the mouse enteric α -defensin Cryptdin-4 (Crp4), are monomeric in solution^{8,19}. HNP1-4 are predominantly expressed by neutrophils but can also be found in or expressed by monocyte/macrophages, natural killer (NK) cells, some T cells, B cells, and immature dendritic cells (DCs)^{10,20}. HD5, HD6 and cryptdins are expressed by epithelial Paneth cells of the small intestine^{14,21,22}. HD5 is also expressed by epithelial cells in the male and female genitourinary tract²³⁻²⁶.

Quantification of physiologic defensin concentrations *in vivo* is complex, as defensins are present at high local concentrations within specific cell types or upon release from cells into confined anatomical niches (e.g., crypts of the small intestine) but can become diluted in extracellular fluids. For the myeloid α -defensins, Daher et al. estimated ~ 3 mM (10 mg/ml) HNPs in neutrophils, with even higher local concentrations in the azurophilic granules in which they are stored²⁷. For the enteric α -defensins, Ayabe et al. estimated concentrations of ≥ 3.5 mM

(15-100 mg/ml) in the crypt lumen, the site of Paneth cell degranulation⁶. These concentrations are likely similar in the human small intestine, where HD5 expression exceeds that of HD6 by 6-fold²⁸. In healthy patients, epithelial lining fluid of the lung contains 31-79 nM HNP1-3, nasal fluid contains ~2.7 μ M HNP1-3, saliva contains 0.3-3 μ M HNP1-3, and vaginal secretions contain ~1.5 μ M HNPs and 0.3-14 μ M HD5²⁴. However, in certain diseased states defensin levels can be highly elevated. For example, 57 μ M to 2.4 mM concentrations of HNP1 have been found in epithelial lining fluid of cystic fibrosis patients²⁹. Overall, the concentrations of α -defensins present *in vivo* are generally within the range that is needed for direct antimicrobial activity.

b. Processing and expression of enteric α -defensins

All α -defensins are produced as pre-pro-peptides, and undergo proteolytic processing to their mature forms. Only the mature forms of α -defensins have antimicrobial activity in cell culture³⁰⁻³². In mice, the protease matrix metalloproteinase 7 (MMP7, or matrilysin) is expressed in Paneth cell granules and cleaves pro-cryptdins to their mature forms²². As the creation of a complete α -defensin knock-out mouse is complicated by the large number of α -defensins mice encode, *Mmp7*^{-/-} mice, which lack mature α -defensins, have been used to study the role of α -defensins *in vivo*²². Human Paneth cells lack MMP7, and instead trypsin is responsible for processing human enteric α -defensins³³. While mouse enteric α -defensins are stored in their mature form in Paneth cells granules, human enteric α -defensins are stored in pro-forms, and cleavage to mature forms is thought to happen either during secretion or immediately upon release of Paneth cell granules into the intestinal crypt³³.

Unlike some AMPs, enteric α -defensins are constitutively transcribed independent of microbial signaling. This is supported by the fact that mature enteric α -defensins are produced both in germ-free mice and prenatally in humans^{34,35}. Instead, expression of α -defensins is linked to cellular differentiation and is under the control of TCF4, a WNT pathway transcription factor^{36,37}. Constitutive secretion of α -defensins is detected under baseline conditions³⁸, although higher levels of secretion can be induced in a calcium dependent manner by acetylcholinergic agents^{39,40}, bacteria and bacterial derived products^{6,41}, and other TLR agonists, possibly through IFN- γ signaling^{4,42-44}. As discussed previously, Paneth cells can live for upwards of one month and are thought to constantly release AMPs into the intestinal lumen⁴. Therefore, unlike some granulocytes, Paneth cells must be able to survive degranulation, although the precise mechanism that controls degranulation and regranulation is not clear.

1.3 Interactions between microbes and purified α -defensins in cell culture

a. Mechanisms of antibacterial activity

Membrane disruption

The major mechanism through which defensins kill bacteria is non-enzymatic membrane disruption. A lipid disruption model of killing is supported by both experiments using model microbial membranes of various lipid compositions as well as studies using whole bacteria⁴⁵⁻⁴⁷. In this model, cationic α -defensins first bind to negatively charged bacterial surface components, and then hydrophobic α -defensin regions facilitate membrane insertion⁴⁸. The specific membrane disruptive mechanism observed is dependent on defensin quaternary structure. Dimer forming defensins, such as HD5 and HNP1-3, are predicted to form multimeric pores after insertion, as has been shown for HNP2^{2,49}. These pores can conduct ions and are highly stable,

as the α -defensins do not undergo secondary structural rearrangements when they enter membranes^{2,50,51}. In contrast, defensins such as rabbit NP-1 and Crp4 that are monomers in solution induce graded leakage from model membranes that is highly dependent on peptide concentration, indicating that they do not form stable pores^{18,30}. However, in both cases defensin binding can lead to permeabilization of the outer and then inner membrane, an efflux of metabolites and ions, a reduction in membrane potential, and eventual osmotic lysis^{45,52}.

Binding to Lipid II

For some organisms binding and subsequent permeabilization alone is not sufficient to explain activity. Consistent with this idea, some have hypothesized that pore formation is instead a means to allow α -defensins to gain access to the bacterial cytosol⁵⁰. In this model, the major antibacterial targets of α -defensins are intracellular, and the concomitant membrane damage is a transient side effect. Diverse classes of antibacterial compounds, such as β -lactam antibiotics (e.g., penicillin, carbapenem, etc.) and glycopeptides (vancomycin, daptomycin, etc.) target the prokaryotic cell wall and its synthetic machinery. Recently a related mechanism has been posited for defensins, as HNP1 and human β -defensin (HBD) 3 have been shown to bind to and sequester the cell wall building block lipid II, which is highly conserved among bacteria⁵³. Lipid II sequestration leads to bacterial death, an outcome that can be avoided when bacteria with altered levels of lipid II are used⁵³. More work is needed to elucidate other intracellular targets of defensins and to understand the relative contributions of membrane disruption and intracellular targeting to the killing of Gram-positive versus Gram-negative organisms.

Mechanisms of bacterial resistance to defensins

As AMPs represent a major barrier to productive infection, bacteria have evolved mechanisms to circumvent their effects. These can be divided into 3 main categories (1) inactivation or degradation of AMPs, (2) repulsion of AMPs through bacterial surface and cell membrane modifications, and (3) expulsion of AMPs via efflux pumps⁵⁴. A number of bacterial species secrete proteins that can inactivate or degrade AMPs. Streptococcal inhibitor of complement (SIC) and related proteins are produced by several strains of group A streptococci and have been shown to bind and inhibit the activity of HNP1 and HBD1-3 *in vitro*⁵⁵. Highlighting the importance of this antagonism for productive infection, bacteria with mutations in SIC display impaired colonization in a mouse model⁵⁶. *Staphylococcus aureus* and *Staphylococcus epidermidis* both secrete proteases that have been shown to degrade extracellular AMPs^{57,58}. A related mechanism of action is secretion of cytolytic toxins that can promote the degranulation of neutrophils⁵⁹. *In vivo* this may allow bacteria to disarm neutrophils at a distance, thereby enabling escape from α -defensin killing.

The bacterial surface and cell membrane are highly anionic, which plays a crucial role in α -defensin mediated killing of bacteria as charge drives interactions between these components and cationic α -defensins. Gram-negative bacteria have a thin layer of peptidoglycan sandwiched between an inner and outer membrane. The outer membrane has an inner phospholipid layer, while lipopolysaccharide (LPS) forms the outer leaflet. In contrast, Gram-positive bacteria only have an inner membrane but are surrounded by a thick, multi-layer peptidoglycan cell wall, which includes various types of teichoic acid polymers. Both LPS and teichoic acid are highly anionic and are the major contributors to the net negative charge of the bacterial surface^{60,61}. Therefore, a major defense mechanism employed by bacteria against α -defensins is to reduce the negative charge of LPS and teichoic acid, which leads to a direct reduction in α -defensin

accumulation and subsequent killing^{54,62,63}.

The gram-negative pathogen *Salmonella enterica* serovar Typhimurium (STM) has been well studied in this regard, and it has been shown that specific modifications to the lipid A portion of LPS can increase resistance to AMPs⁶⁴. Remodeling of lipid A is transcriptionally regulated via activation of the PhoP-PhoQ two component regulatory system, which has been shown to directly sense and respond to the presence of AMPs⁶⁵. Once phosphorylated, the transcriptional regulator PhoP activates the PmrA-PmrB two component regulatory system. Activation of PmrA-PmrB is necessary for the production of charged-neutralized lipid A due to the incorporation of aminoarabinose and ethanolamine moieties⁶². To further defend against AMPs, PhoP also activates the alternative sigma factor RpoS, which increases resistance to oxidative stress⁵⁴. Finally, PhoP-PhoQ activation leads to a decrease in membrane fluidity and permeability due to changes in the acetylation of lipid A^{66,67}. STM mutants unable to alter lipid A, such as those lacking a functional PhoP-PhoQ regulon, are sensitive to AMPs *in vitro* and are avirulent in mouse infection models^{62,68}. Furthermore, as PhoP-PhoQ homologs are found in a variety of other Gram-negative organisms, it is likely that similar mechanisms are employed by these bacteria to defend against AMP-mediated killing⁵⁴.

In Gram-positive bacteria, the *dltABCD* operon is responsible for the charge neutralization of teichoic acids via the incorporation of D-alanine^{54,69}. Mutation of this operon reduces the survival of *S. aureus* after *in vitro* exposure to AMPs and has been shown in mouse models to reduce the virulence and colonization of multiple Gram-positive organisms including *S. aureus* and *Listeria monocytogenes*⁷⁰⁻⁷². A final cell surface modification that can protect from AMP killing is the production of capsular polysaccharides, such as those made by *Klebsiella pneumoniae*, *Neisseria meningitidis*, and *S. epidermidis*⁵⁴. In all cases tested, disruption of

capsular biosynthesis sensitized bacteria to AMPs and reduced virulence in relevant mouse models⁵⁴.

In addition to having negative surface components, bacterial cell membranes in general are composed of a high percentage of acidic phospholipids such as phosphatidylglycerol, and therefore, have a net negative charge². As eukaryotic membranes are asymmetric and have a tendency to place negatively charged phospholipid head groups on the inner leaflet, this also helps explain the lack of toxicity observed to eukaryotic cells in spite of exposure to high local α -defensins concentrations². Both Gram-negative and Gram-positive organisms are able to modify phosphatidylglycerol with lysine in a process dependent on membrane protein MprF^{70,73}. MprF activity decreases the net negative charge of the bacterial cell membrane, and mutations in MprF have been shown to increase susceptibility to AMP killing *in vitro* and *in vivo*^{70,73}.

Finally, although efflux pumps have been reported to be important for the bacterial resistance to some AMPs, including cathelicidins, their importance for defensin resistance is less clear⁵⁴. *K. pneumoniae* AcrAB efflux pump mutants are more susceptible to HNP1, HBD1 and HBD2 killing than wild type bacteria⁷⁴. However, a separate study found no role for efflux pumps in AMP resistance, as overexpression of AcrAB, MexAB, and NorA in *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* did not confer resistance to HD5, HNP1, HBD2 or HBD3⁷⁵. Therefore, the role of efflux pumps is likely both AMP and bacterium specific and warrants further investigation.

b. Mechanisms of antiviral activity[#]

Antiviral mechanisms through direct interactions between defensins and virus

[#] Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

Modes and determinants of defensin binding to viruses

Although lipid membrane disruption is the major mechanism of α -defensin antibacterial activity, it is insufficient to explain the antiviral activity of α -defensins. A growing literature has demonstrated that α -defensins inhibit many species of viruses, including numerous non-enveloped viruses, which by definition lack a lipid target. Therefore, there are likely multiple modes of defensin binding to viral particles. First, defensins can interact with lipid bilayers of enveloped viruses, which is facilitated by the presence of negatively charged phospholipids^{10,20,49}. Second, four of the α -defensins (HNP1-3 and HD5) and HBD3 are lectins capable of binding to glycoproteins and glycolipids⁷⁶⁻⁷⁹. Third, defensins can potentially engage in protein-protein or protein-DNA interactions. As they are both cationic and amphipathic, defensins can interact with ligands through charge-charge and hydrophobic interactions. Defensin oligomerization, particularly for α -defensins, and conformational stability imparted by disulfide bonds may further influence binding. Each of these interactions contributes to the antiviral activity of defensins, and their relative importance depends on the specific virus/defensin pair under investigation.

The property of defensins that has been most widely investigated for its contribution to antiviral activity is stabilization of the 3D structural fold through the formation of disulfide bonds. Generally, destabilized or “linear” defensins are generated by substituting the conserved cysteine residues either in toto, individually, or in pairs to natural or non-natural residues that cannot form disulfide bonds such as serine or α -amino-n-butyric acid (Abu). Alternatively, wild type defensins are reduced and chemically modified (alkylated) to prevent disulfide bond formation. All reported studies have shown that the disulfide-stabilized forms of α -defensins are required to either inhibit [herpes simplex virus-1 (HSV-1), human adenovirus serotype 5

(HAdV-5), influenza A virus (IAV), and human immunodeficiency virus-1 (HIV-1)] or enhance (HIV-1) virus infection^{27,80-84}. Together, these studies suggest that the effects of α -defensins on virus infection are more likely to be due to their amphipathicity or ability to multimerize, which are structurally dependent, rather than the net positive charge of the molecule that is common to both native and “linearized” forms.

The capacity of defensins to function as lectins and bind selectively to sugars contributes to their antiviral properties; however, defensins also bind to host cellular and serum proteins^{27,76}. The relative affinity for viral targets versus serum components may explain why some defensins are only antiviral against particular viruses in the absence of serum. Although it has been shown that HD5 binds natural viral glycoproteins, notably HSV-1 glycoprotein D (gD) and HIV-1 gp120, with a higher affinity than bovine serum albumin or fetuin, serum substantially attenuates the antiviral activity of HNP1 against HSV-1, even at low concentrations^{27,76,85}. Nonetheless, this effect can be overcome at higher HNP1 concentrations or by pre-incubating the virus with HNP1 before it is added to cells. The addition of serum also abrogates the antiviral activity of HNP1-3 against both X4 and R5 tropic HIV-1^{86,87}. Although it has been reported that HD5 enhances HIV attachment and infection of primary T cells in the presence of 5-10% serum, a recent study suggests that HIV-1 infection of these cells is blocked in 0-2% serum^{82,88-91}. Upon further investigation, it was revealed that the apparent antiviral activity of HD5 against HIV-1 infection of primary CD4⁺ T cells in the absence of serum is due to increased HD5-mediated cell toxicity, which is not observed with other cell types⁹². Serum also competes for HD5 binding to and inhibition of HAdV, which lacks viral glycoproteins⁹³. Two notable exceptions to the abrogating effects of serum are inhibition of human papillomavirus (HPV) and human BK virus (BK) infection by α -defensins, which are not blocked by 10% or 5% serum respectively^{94,95}.

Rapid inactivation by binding to serum components may protect host cells from damage by defensins; however, defensins likely remain potently antiviral *in vivo* at high local concentrations upon initial secretion and in serum-free anatomical locations (e.g., phagocytic vacuoles or the bowel lumen).

Although much of the antibacterial activity of defensins is attenuated at physiologic salt concentrations¹⁰, this is not generally true for their antiviral activity. We have shown that super-physiological concentrations of salt inhibit HD5 binding to HAdV, which implicates charge-charge interactions in HD5 binding to the viral capsid^{93,96}. In general, differential salt sensitivity may reflect variation in both the molecular interactions and the mechanisms of defensin-mediated killing or neutralization of viruses versus bacteria.

A limited number of structure-function studies have evaluated the roles of additional features of α -defensins in modulating viral infection. We have shown that the conserved salt bridge stabilizing a loop between two beta strands of HD5 is dispensable for HAdV-5 inhibition⁸¹. Similarly, mutation of an invariant glycine residue (Gly17) of HNP2 to glutamate severely attenuates the antibacterial activity of HNP2 but results in only a minor loss of antiviral activity against HPV-16^{94,97}. In contrast, specific arginine residues are critical for HD5 binding to HAdV-5 and HPV-16, and this activity is not purely charge-dependent, as lysine substitutions for selected arginine residues did not preserve antiviral activity⁹⁶. Likewise, a double R9H/R13H mutant of HD5 was attenuated for enhancement of HIV-1 infection in the presence of serum, indicating that this property is also not simply charge-dependent⁹⁸. Furthermore, the capacity of HD5 to self-associate is critical for antiviral activity against both HAdV-5 and HPV-16 and for HIV-1 gp120 and HSV-1 gD binding^{76,96,99,100}. These properties were revealed through mutations that disrupt defensin activity. In the converse approach, residues in HD5 under

positive selection were mutated in an effort to augment the antiviral activity of HD5 against HSV-2⁹⁰. One mutant, (HD5 E21R) demonstrated improved anti-HSV-2 and anti-HIV-1 activity in cell culture and was both prophylactically (1 hour before infection) and therapeutically (24 hours after infection) protective against lethal HSV-2 challenge in a mouse model. Collectively, these studies suggest that specific features of viruses are selectively bound by defensins, and that the binding interface of the defensin is sequence-specific and not merely charge-dependent.

The basis for selective recognition of diverse viruses by defensins is largely unknown. Defensins may interact with either the lipid bilayer or envelope glycoproteins of enveloped viruses; however, protein-protein interactions must be critical for binding to non-enveloped viruses (e.g., HPV and HAdV) and likely contribute to binding to enveloped viruses as well. For HIV-1, competition with site-specific antibodies was used to map HNP2 binding sites on gp120¹⁰¹. For species C HAdV (HAdV-C), we have used structural studies and a chimeric approach to identify capsid determinants of HD5 binding^{81,102}. Other than for these two viruses, almost nothing is known about specific determinants of the viral particle that dictate defensin binding. Nonetheless, the fact that closely related defensins have differential antiviral (or infection-enhancing) effects indicates selectivity and may inform the design of future studies to elucidate the basis for recognition.

Direct virus inactivation by affecting envelope

Direct interactions between defensins and structural components of the virion, particularly the lipid bilayer of enveloped viruses, could destroy or destabilize the virus and render it non-infectious. This mechanism was proposed in the first studies of the antiviral activity of human and rabbit neutrophil α -defensins^{27,80,85,103}. In support of this hypothesis,

HSV-1 inactivation by rabbit NP-2 or HNP1 was impaired at temperatures below 20°C and was more effective at 40°C than 37°C, implicating the fluidity of the membrane rather than binding as an important parameter^{27,80}. HNP1 has also been shown to bind directly to HSV-1 and to model membranes containing phosphatidylserine²⁷. Nonetheless, the morphology of HSV-1 by electron microscopy was not altered by incubation with a neutralizing concentration of HNP1²⁷. Similarly, treatment of HIV-1 with HNP1 in the absence of serum irreversibly decreases infectivity, although a physical change in particle integrity was not determined^{88,104}. More recently, the lipid bilayer of respiratory syncytial virus (RSV) exposed to neutralizing HBD2 but not non-neutralizing HBD1 was visibly damaged when assessed by electron microscopy¹⁰⁵. Although the universality of this phenotype was not quantified, the proteins of the bulk population of the virus were more buoyant in a density gradient, suggesting that most of the viral particles were disrupted. The altered morphology was correlated with a 70% reduction in virus attachment to the cell. This is perhaps the most convincing data for direct viral envelope disruption by a defensin and likely extends to human parainfluenza virus 3 (HPIV-3), which has a profile of defensin sensitivity similar to that of RSV¹⁰⁵.

The broad neutralization of many enveloped viruses supports the hypothesis that the lipid bilayer is the target; however, enveloped viruses are not universally susceptible and their sensitivity to α -defensins can be highly variable. For example, in one study rabbit NP-1 and NP-2 inhibited HSV-1 up to 1000-fold, HSV-2 10-fold, vesicular stomatitis virus (VSV) 100-fold, and IAV 56-fold but had no effect on cytomegalovirus (CMV)⁸⁰. Similarly, HNP1 potently inhibited HSV-1 (1000-fold) and HSV-2 (100-fold) but only weakly inhibited CMV (6-fold), VSV (7-fold), and IAV (6-fold)^{27,85}. Defensin perturbation of lipid bilayers is dependent upon their composition. It is favored by negatively charged phospholipids; whereas, neutral bilayers

are largely inert to defensin^{99,103}. The lipid content of viral envelopes is dependent upon the subcellular location and membrane microdomains from which they bud and likely varies among viral families¹⁰⁶. If direct membrane perturbation contributes to the antiviral effect of defensins, then differences in the lipid composition of the envelope may in part explain the differential susceptibility of viruses to defensins.

In contrast to disruption of enveloped viruses, increased capsid resistance to mechanical force or heat has been observed upon HD5 binding to non-enveloped HAdV-C^{81,93,107}. This effect correlates with the inability of the HD5-bound particle to uncoat, similar to a genetic mutant of HAdV-C that is stabilized by the presence of unprocessed precursor capsid proteins^{93,108,109}. A failure to uncoat precludes release of an internal, membrane-permeabilizing capsid protein, thereby blocking HAdV-C escape from the endosome and introduction of the viral genome into the nucleus, its replication niche^{93,109-111}. Thus, unlike enveloped viruses where destabilization of the virion impairs infectivity, an opposite, stabilizing interaction with the non-enveloped HAdV-C capsid produces a similar outcome.

Extracellular aggregation

As many defensins form multimeric structures, which have been demonstrated in both crystal structures and in solution^{15-18,76,112}, there is the potential that interactions between defensin peptides bound to neighboring viruses will cause virions to aggregate. This has been shown directly for IAV by HNP1 or HNP2 and for HAdV-5 and BK virus by HD5^{95,96,113}. For IAV, it is unclear if defensin-mediated aggregation is required to block infection. Similarly, aggregation of HAdV by HD5 is not sufficient to inhibit infection, as a mutant of HD5 that is able to induce aggregation is non-neutralizing⁹⁶. In contrast, aggregation and a concomitant

inability to bind host cells have been shown to be the dominant mechanisms of neutralization for BK virus ⁹⁵. In addition to multivalent binding due to defensin oligomerization, neutralization of capsid charge by defensin binding may reduce repulsion between virions. We have shown this directly for HAdV-C ⁹⁶, and this effect may facilitate the aggregation of other viruses. These effects are likely interrelated, as mutants of HD5 that are impaired in self-association are incapable of both aggregating HAdV-C and fully neutralizing the capsid charge. Aggregation can impact viral infectivity by directly impeding cell binding or by causing viruses in clumps to enter fewer cells.

Blocking receptor binding

Defensin binding to viral attachment protein(s) could disrupt receptor interactions critical for viral entry into the cell. HNP1-3 and HD5 bind a recombinant viral glycoprotein (gB) of both HSV-1 and HSV-2, which correlates with the ability of these defensins to inhibit HSV-1 and HSV-2 entry and adhesion ^{79,114}. Lectin activity of HNP1 is critical, as deglycosylation of HSV-2 gB abrogates binding ¹¹⁴. The contribution of glycoprotein binding to the antiviral mechanism of HD5 was underscored by a direct correlation between the capacity of HD5 mutants to neutralize HSV-2 and their affinity for recombinant gD ⁹⁰. HNP4 and HD6 also inhibit HSV-1 and HSV-2 infection, but do not bind to viral glycoproteins ⁷⁹. Instead, HNP4 and HD6 have been shown to bind heparan sulfate, the receptor for attachment, as well as other glycosaminoglycans. Overall, blocking host cell receptors and binding to viral glycoproteins is a major mechanism by which defensins inhibit HSV-1 and HSV-2 infection.

Similarly, HNP1-4 bind HIV-1 gp41 and gp120 as well as the cell surface receptor CD4 ^{78,83,115}. The binding sites of HNP1 and HNP2 on gp120 have been mapped in antibody

competition assays to the CD4 and co-receptor binding sites¹⁰¹. Conversely, the HNP1 and HNP2 binding sites on CD4 have been mapped to the gp120 binding site¹⁰¹. The mode of α -defensin binding to gp120 and gp41 may be complex, as both lectin-dependent and -independent binding have been reported. In an early study, deglycosylation of gp120 reduced HNP1 binding and abolished HNP2 and HNP3 binding⁷⁸; however, a more recent paper suggested that deglycosylation of gp120 or gp41 does not affect HNP1 binding⁸³. In addition, HNP4 binding is not abolished by deglycosylation of gp120⁷⁸, consistent with the observation that HNP4 binds more weakly to both polysaccharides and serum proteins¹¹⁵. Nonetheless, HNP4 is a more potent inhibitor of HIV-1 than are HNP1-3¹¹⁵. In summary, like for HSV, defensins directly interfere with HIV-1 binding and attachment.

In contrast to a block in cell binding, we have observed that receptor-dependent and -independent binding of HAdV-C to the cell is enhanced in the presence of a inhibitory concentration of HD5^{81,96}. This effect may be related to neutralization of the net negative capsid charge, which promotes aggregation, in a manner comparable to enhancement of retrovirus infections by polybrene or HAdV infections by poly-cations^{96,116}. Similarly, HIV-1 binding to cells and infection is enhanced by HD5 and HD6^{82,98}. Thus, although in several cases the net effect of defensin binding to the virus is to block cellular attachment, the opposite effect has also been observed. The balance of these activities *in vivo* is unclear.

Inhibition of viral fusion with or penetration of host cell lipid bilayers

To introduce their genomes into host cells, enveloped viruses must fuse their lipid bilayer with that of the host cell¹¹⁷. The fusion protein of the virus mediates this reaction by inserting a hydrophobic stretch of amino acids into the target cell membrane followed by a conformational

change to a less energetic state, termed the six-helix bundle^{118,119}. The energy for lipid fusion is derived from this conformational change. HIV-1 fusion, mediated by gp41, is inhibited by HNP1^{83,101}. Inhibition requires the disulfide-stabilized form of HNP1 and is abrogated by serum⁸³. HNP1 aggregates recombinant peptides of both the carboxyl- and amino-termini of gp41 that comprise the six-helix bundle, suggesting a direct effect on formation of the post-fusion conformation of gp41⁸³. HNP1 also increases the binding and efficacy of neutralizing antibodies specific for the gp41 pre-hairpin conformation, likely due to greater antibody access to hidden neutralizing epitopes as a consequence of slowed refolding kinetics¹²⁰. Thus, HNP1 binding directly alters HIV-1 fusion through interactions with gp41. Whether this mechanism of HIV-1 neutralization extends to other viruses has not been shown.

Rather than fuse with the cell membrane, non-enveloped viruses must penetrate the limiting membrane of the host cell, which is generally mediated by a specific viral protein¹²¹. This step may occur at the plasma membrane, but often follows a conformational change leading to uncoating of the viral capsid triggered by a drop in pH or other host factors in the endosomal pathway¹²². For HAdVs, the internal capsid protein VI is the membrane lytic factor^{110,111}. Upon binding to the viral capsid, α -defensins, including HNP1 and HD5, stabilize HAdV-C and prevent uncoating, release of protein VI, and subsequent disruption of the endosomal membrane^{93,107,109}. To mediate this effect, α -defensins bind directly to the virus, either when mixed together in the absence of cells or when the defensin is added to virus that is pre-bound to receptors on the cell surface^{81,93,96,102,109}. Thus, the defensins can recognize the virus in the complexity of host proteins, carbohydrates, and lipids that could potentially compete with the virus for defensin binding. Neutralization of HAdV by α -defensins is restricted to HAdV-B and -C and to a lesser extent to HAdV-A and -E; whereas, HAdV-D and -F infection is either unaffected or enhanced

by α -defensins^{81,93}. Furthermore, resistance to HD5 neutralization can be conferred to HAdV-C through the replacement of capsid vertex proteins with those from the resistant HAdV-D⁸¹. The basis for enhanced HAdV-D infection is unknown.

Although the molecular mechanism remains unresolved, α -defensins potentially inhibit the membrane penetration of another non-enveloped virus, the polyoma virus JC virus (JCV)¹²³. During infection JCV is trafficked from the early endosome to the endoplasmic reticulum, where the virus uncoats¹²⁴. Although HD5 treatment actually increased the amount of virus entering cells, there was a subsequent reduction in the amount of JCV that reached the endoplasmic reticulum, indicating that HD5 alters intracellular trafficking of JCV¹²³. That the mechanism of inhibition of JCV is distinct from that of BKV, another human polyoma virus discussed previously, highlights the specificity of virus-defensin interactions^{95,123}.

Inhibition of host-cell mediated viral processing

Multiple papillomaviruses, including human cutaneous and mucosal serotypes as well as animal papillomaviruses, have been shown to be sensitive to HNP1-4 and HD5⁹⁴. Initial studies indicated that HD5 inhibited the nuclear localization of the HPV-16 genome, the last step in the virus entry pathway⁹⁴. Unlike other non-enveloped viruses, for HPV the genome is exposed under conditions of HNP1 and HD5 neutralization, implying virus uncoating in the presence of defensins^{93,94}. Consistent with these results, a recent study demonstrated that α -defensins bind to HPV and prevents cleavage of the minor capsid protein L2 by the host protease furin¹²⁵. During HPV entry L2 interacts with and permeabilizes the host cell membrane, a step that is dependent on furin cleavage at the cell surface^{126,127}. Therefore the HD5-mediated block of furin cleavage leads to retention of HPV in a non-infectious pathway, inhibiting productive infection¹²⁵.

Although the mechanisms of inhibition differ, defensins have now been shown to block HPV and HAdV penetration of the cell membrane.

Post Entry Neutralization

Infection is not completed by merely penetrating the host cell membrane. Viral transcription, protein production, assembly, and egress must all occur to complete a replicative cycle. These steps present opportunities for defensins to block viral infection, either by targeting the virus specifically or by targeting the cell. In this regard, HNP1 and HD5 are able to block HSV-1 and HSV-2 when added post-entry⁷⁹. The defensins accumulated intracellularly in the human cervical epithelial cell line used in this study, indicating that they can still come in contact with the viruses in the cell. Hazrati et al. demonstrate that HNP2 and HD5 can bind HSV-2 DNA and speculate that this could contribute to inhibition by blocking gene expression, although a post-transcription block by an unknown mechanism was also suggested by the data⁷⁹. Post-entry neutralization of HIV-1 has also been reported. In one study, HBD2 blocked reverse transcription but had no effect on fusion, although the mechanism of this block was not determined¹²⁸. Additional effects of defensins on HIV-1 infection attributable to disruption of intracellular signaling will be discussed later.

No mechanism but antiviral activity reported

We lack mechanistic insight for a variety of viruses that have been shown to be sensitive to defensin neutralization. Transduction by recombinant adeno-associated virus is inhibited by relatively high concentrations (100 μ M) of HNPs; however, ≥ 100 μ M HNPs were measured in

epithelial lining fluids from cystic fibrosis patients, which were also inhibitory in the same study²⁹.

In addition to purified peptides, virus inhibition by degranulated neutrophils has been noted. When neutrophils degranulate, for example in response to stimulation by leukotriene b4 (LTB4), they release high concentrations of defensins as well as other antimicrobial factors. CMV infection of human peripheral blood leukocytes containing neutrophils is weakly inhibited by LTB4 treatment¹²⁹. A combination of α -defensins (HNP1-3), the human cathelicidin LL-37, and eosinophil-derived neurotoxin mediates much of the effect. Cell-free supernatants from LTB4-stimulated neutrophils mixed with CMV was similarly inhibitory, and anti-HNP1-3 antibodies reduced the observed activity by half. These results corroborate the data on modest CMV neutralization using purified α -defensins, although the antiviral mechanism remains unknown^{27,129}. In other studies, supernatants from human neutrophils treated with LTB4 or stimulated to synthesize LTB4 strongly inhibited human coronavirus and HSV-1 and more modestly inhibited RSV and influenza B virus^{130,131}. Collectively, these studies demonstrate that naturally secreted defensins retain antiviral activity. In contrast, HNP1-3 have been shown to interact with and reduce the activity of surfactant protein D (SP-D) and bronchoalveolar lavage fluid containing SP-D against IAV^{132,133}. These observations reinforce the importance of studying both purified peptides for their individual biological effects and more complex mixtures in which defensins and other antimicrobial factors are naturally produced, which could demonstrate synergistic or, alternatively, mutually inhibitory effects. Additionally, whether previously described or novel mechanisms contribute to the neutralization of this viruses warrants further study.

Antiviral mechanisms targeting the cell

Blocking fusion by crosslinking host proteins

Rather than directly targeting viral fusion proteins to block enveloped virus fusion with the host cell, HBD3 and a synthetic human θ -defensin called retrocyclin 2 (RC2) have been shown to inhibit IAV fusion by cross-linking host glycoproteins⁷⁷. RC2 and HBD3 binding limit the mobility of host surface proteins in the vicinity of the nascent viral fusion pore, restricting its maturation to full fusion. Inhibition is blocked by serum and by deglycosylation (PNGase treatment) of the cells, indicating that this effect is due to the lectin activity of the defensins. In this regard, a non-defensin mannan-binding lectin had a similar effect. This mechanism may be general, as inhibition is independent of direct binding to the viral hemagglutinin glycoprotein and extends to fusion reactions mediated by unrelated proteins from baculovirus and Sindbis virus. More recently for HIV-1, HNP1 treatment has also been shown to decrease the mobile fraction of CD4, CCR5, and CXCR4 receptors in the cell membrane, reflecting the same phenomenon mediated by the lectin activity of HNP1⁸³.

Modulation of cell surface receptors

HIV-1 has a complex entry pathway that utilizes several cell surface receptors and co-receptors. The relative ability of defensins to directly modulate host surface receptors important for HIV-1 infection has been debated by various groups. In peripheral blood mononuclear cells (PBMCs) and a human T cell line expressing CXCR4 and CCR5, HBD2 and HBD3 but not HBD1 reduced cell surface expression of CXCR4 but not CCR5 in 0-0.5% serum^{87,134}. A subsequent study confirmed CXCR4 downregulation in PBMCs by HBD2 and HNP1 under similar conditions¹⁰⁴. In contrast, the downmodulation of CXCR4 was not observed in PBMCs

treated with HBD2 in the presence of serum^{128,135}. Similarly, in primary CD4⁺ T cells in the presence of 10% serum, HNP1 does not alter CXCR4, CCR5, or CD4 expression on the cell surface⁸⁸; however, this result may be explained by the comparably long (16 hour) period of defensin treatment, which potentially allowed for surface protein turnover. The differences in these studies are likely explained by the use of different cell types and serum concentrations, thus the contribution of changes in cell surface receptor levels to HIV-1 infection remains unresolved.

Changes in intracellular signaling that impact infection

Many viruses regulate protein kinase C (PKC) signaling during entry and infection: HIV-1 requires phosphorylated PKC for viral fusion, transcription, integration, and assembly^{88,136,137}. IAV requires PKC for endosomal escape and nuclear entry^{138,139}. HSV requires PKC for cell entry as well as for nuclear egress of the viral capsid^{140,141}. As HNP1-3 are known to inhibit the activity of PKC *in vitro*¹⁴², altering or inhibiting this cellular signaling pathway may be another defensin-mediated antiviral mechanism, which explains the post-entry block to infection observed for some viruses. HNP1 treatment of cells prior to or during infection with either IAV or HIV-1 reduces the levels of phosphorylated PKC^{84,88}. Treatment of CD4⁺ T cells with a PKC activator, bryostatin-1, partially rescued HIV-1 infection⁸⁸. In addition, HNP1 and the PKC inhibitor Go6976 had similar inhibition kinetics⁸⁸. Inhibition of PKC by HNP1 explains the observed block in nuclear import of the incoming pre-integration complex as well as transcription of the integrated viral genome in HIV-1 infected cells⁸⁸. Although HSV infection is also inhibited by HNP1 through a post entry mechanism, it is not rescued by pretreatment with bryostatin-1⁷⁹. In addition, cellular entry of some HAdV serotypes is also sensitive to PKC inhibition¹⁴³⁻¹⁴⁵; however, differential defensin sensitivity of chimeric HAdVs in which only

certain capsid proteins are variable argues against a role for cellular targets such as PKC in HAdV neutralization by α -defensins⁸¹. Together, these data indicate that the PKC signaling pathway is involved in defensin mediated neutralization of HIV and possibly IAV, but is uninvolved in HSV and HAdV neutralization.

Cell signaling pathways mediated by the chemokine receptor CCR6 also play a role in defensin-mediated HIV inhibition. HBD2 is known to bind CCR6 and has been shown to induce expression of host restriction factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) which has antiviral activity against HIV^{146,147}. Thus, defensins can both inhibit cellular pathways required for viral infection and activate intracellular antiviral mechanisms. The multitude of mechanisms of viral inhibition by α - and β -defensins are summarized in Figure 1.3.

c. Enhancement of viral infection[†]

Although the majority of studies have focused on the antiviral activity of defensins, in some cases α -defensins actually enhance HIV and HAdV infection^{81,148}. For both viruses, enhancement is not observed with linearized defensins, indicating that structure-dependent interactions are required. Treatment of HIV with HD5 or HD6 substantially increases infection, which for some strains can reach >100-fold¹⁴⁸. This enhancement is sufficient to overcome the effects of entry and fusion inhibitors and acts primarily by increasing viral attachment to target cells⁸². Naturally produced HD5 from *N. gonorrhoeae*-infected cells also enhances HIV infection, suggesting that it is likely to occur under physiological conditions *in vivo*¹⁴⁸. Crp3, which is potently antibacterial, has also been shown to increase HIV entry in cell culture⁹¹.

[†] Adapted from Wiens, M.E., Wilson, S.S., Lucero, C.M. and Smith, J.G. Defensins and Viral Infection: Dispelling Common Misconceptions. *PLoS Pathogens*. 2014. DOI: 10.1371/journal.ppat.1004186

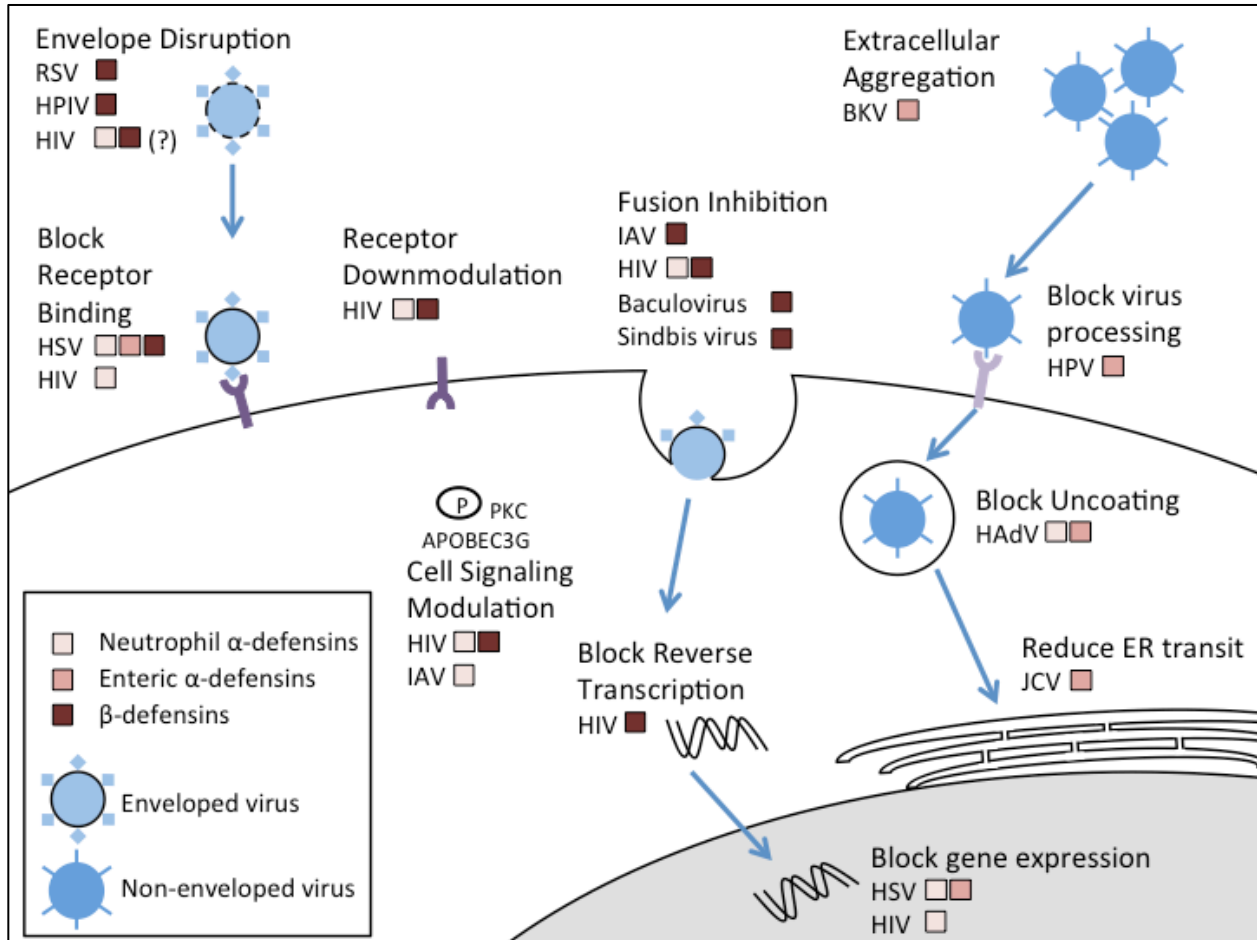


Figure 1.3 Major antiviral mechanisms of defensins. Defensins inhibit many steps in enveloped and non-enveloped viral infection. Mechanistic information is available for respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), human immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza A virus (IAV), BK virus (BK), human adenovirus (HAdV), JC virus (JCV) and human papillomavirus (HPV). Most mechanisms impact viral entry, but post-entry effects have been described. Omitted are defensin effects that have been reported but their contribution to blocking infection is in doubt (e.g., aggregation of HAdV by α -defensins). Note that although the defensins relevant for each virus at each step are indicated in broad classifications (e.g., β -defensins), in most cases not all of the defensins within these groups have been tested or have equivalent activity. Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

The authors speculate that this could be due to stabilization of the virus-cell interaction by the defensin or due to Crp3 working as a lectin to stabilize carbohydrate-carbohydrate interactions between virus and a cell surface receptor. However, as Crp3 is known to induce pores in cultured cells, the authors cannot rule out that these pores are facilitating HIV entry into the cells and increasing the functional infectious dose ¹⁴⁹.

We have observed a similar, albeit much more modest, HNP1- and HD5-dependent increase in infection by certain serotypes of HAdV, which is also correlated with increased receptor-dependent and -independent attachment to cells ^{81,93}. Whereas HIV is sensitive to HNPs but enhanced by HD5, HD6, and Crp3, HAdV serotypes appear to be more uniformly resistant or sensitive to α -defensins in general.

1.4 *In vitro* models to study naturally secreted enteric α -defensins

a. *Ex vivo* stimulation ^{}**

The standard assays for determining the antimicrobial activities of α -defensins described above use purified peptides and are performed under low salt or serum free conditions, as killing of bacteria and some viruses is inhibited in buffers with physiologic levels of salt and viral neutralization can be impacted by serum containing media ^{8,10}. As discussed in previous sections, a limited number of *ex vivo* studies have examined the ability of naturally produced α -defensins or defensin-containing extracellular fluids to inhibit bacterial growth ⁸. For enteric α -defensins, Ayabe et al. purified villi or Paneth cell containing crypts from wild type mice, incubated them with STM and enumerated surviving colony forming units (CFU) ⁶. STM killing was observed after exposure to crypts or crypt secretions but not to villi. In addition, crypts were isolated from

^{**} Adapted from Wilson, S.S., Tocchi, A.T., Holly, M.K., Parks, W.C., and Smith, J.G. A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions. *Mucosal Immunology* **8**, 352-361 (2015).

both wild type and *Mmp7*^{-/-} mice, which lack mature α -defensins. STM killing was markedly reduced in *Mmp7*^{-/-} crypts or by an antibody against mouse α -defensin-1. These data support the notion that secreted α -defensins are capable of directly killing bacteria. However, this study utilized freshly isolated small intestinal crypts, which are unstable and only amenable to short-term experiments. Additionally, bacterial killing was demonstrated under low salt conditions and overall was modest (<10-fold) compared to the level of killing seen using purified peptides (up to a 6-log reduction).

b. Small intestinal organoids

A major hurdle to the study of naturally secreted enteric α -defensins is the lack of cell lines derived from either the small intestine or from Paneth cells specifically. Treatment of intestinally derived cells, such as the colonic Caco-2 cell line, with the Paneth cell ligand FGF9 induces expression of HD5, HD6, and lysozyme RNA¹⁵⁰. However, these cells lack a protease to cleave the pro-defensins, and therefore antimicrobial forms of α -defensins are not produced. In 2009, a new culture system that potentially allows for the study of naturally secreted α -defensins *in vitro* was described¹⁵¹. When either intestinal stem cells or entire crypts were isolated from the gastrointestinal tract, embedded in an extracellular matrix, and overlaid with a completely defined growth media, intestinal organoids were formed¹⁵¹. Organoids form a lumen that is closed off from the extracellular environment, as well as villus and crypt structures (Figure 1.4). Importantly, they recapitulate the intestinal organization and epithelial cell diversity of the section of the gastrointestinal tract from which they are isolated¹⁵². Organoids are comprised of a variety of epithelial cell types including: enterocytes, enteroendocrine cells, goblet cells, stem cells, and Paneth cells, and have successfully been created from both human and mouse tissue⁴. However, only mouse small intestinal organoids are able to continuously grow as a differentiated

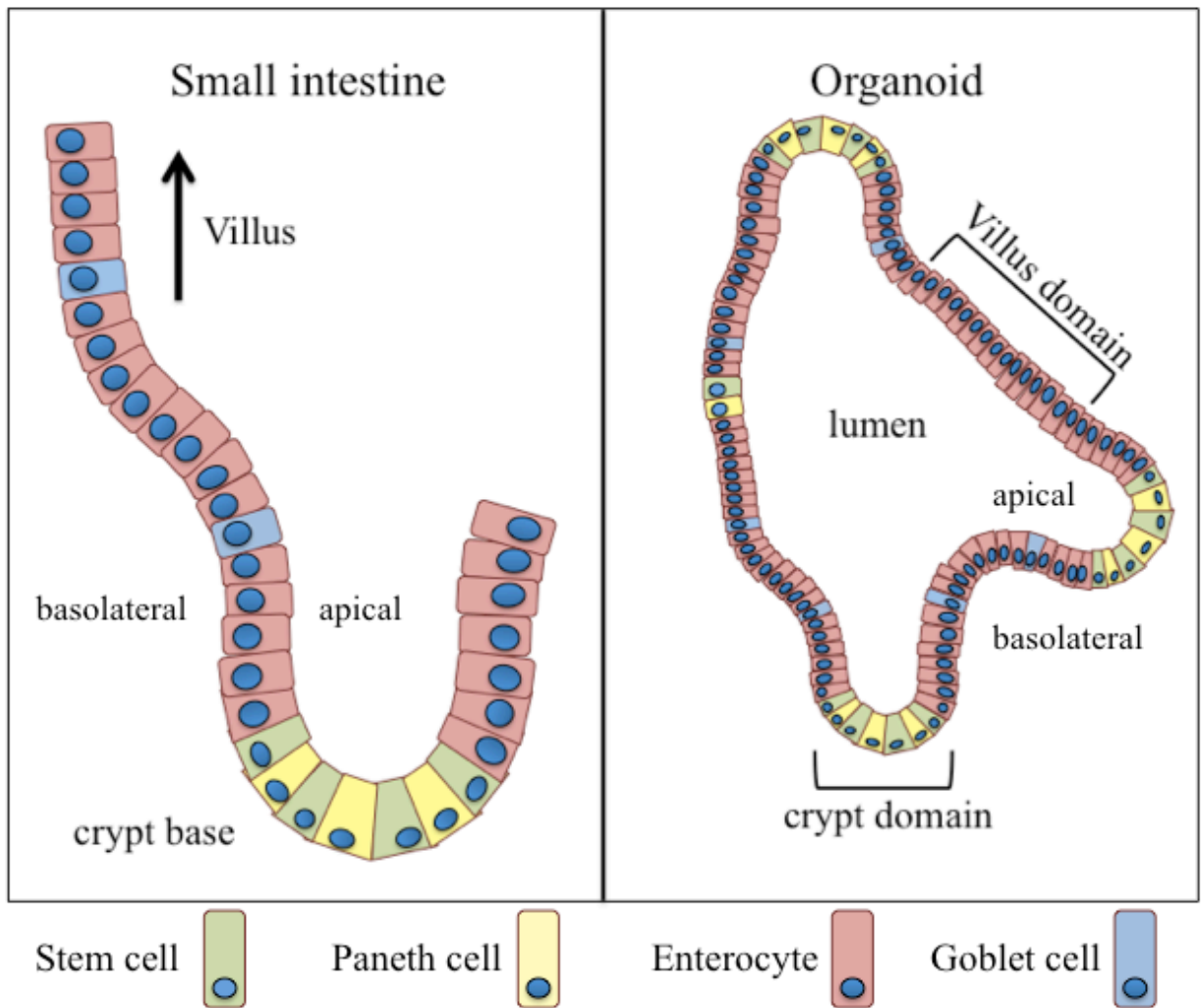


Figure 1.4 Organoids recapitulate small intestinal architecture and epithelial cell diversity.

Stem cells and Paneth cells are interspersed in the crypt base in the small intestine and define the crypt domain of small intestinal organoids. Organoids also contain villus domains composed mainly of enterocytes. The lumen of the organoid is topologically equivalent to the apical side of the small intestine.

structure. Colonic organoids from mice and all human organoids are propagated as cystic structures composed of stem cells and enterocytes and then can be terminally differentiated into other cells types by withdrawal of growth factors¹⁵². Paneth cells have been identified in small intestinal organoids from mice^{40,152}. Therefore, small intestinal organoids represent the first viable culture system for Paneth cells and potentially a new system to study the impact of naturally secreted enteric α -defensins on microbial infection.

1.5 Importance of defensins on microbial pathogenesis *in vivo*

a. Direct impact on bacterial infections

Although purified neutrophil α -defensins are antimicrobial, there is scant evidence to support an *in vivo* role for these peptides. This is partly because experiments are confounded by other antimicrobial neutrophil contents but mostly due to the fact that mice do not express these peptides and, therefore, animal experiments are lacking. To address this, an HNP1 transgenic mouse was created, however it has not been used for any microbiological studies^{153,154}. In contrast, there are numerous studies that support an *in vivo* role for enteric α -defensins upon bacterial infection⁷. One of the earliest studies utilized the *Mmp7*^{-/-} mouse, which lacks mature cryptdins, and showed that these mice succumb more quickly and in greater numbers to oral challenge with STM than wild type mice²². This work was complemented by studies using a knock-in mouse, where the human defensin HD5 was transgenically expressed exclusively in Paneth cells¹⁵⁵. In this model, HD5-expressing mice were protected upon oral challenge with a dose of STM that was lethal for wild type mice. Further supporting a specific role for HD5 in enteric mucosal defense, when the mice were challenged intraperitoneally with STM there was no difference in survival between the genotypes. Unfortunately this important control experiment

of bypassing the intestinal barrier via intraperitoneal injection was never performed in the *Mmp7*^{-/-} mice.

Studies of newborn mice infected with *Shigella* further support a critical role for α -defensins in protection from bacterial infection *in vivo*¹⁵⁶. Mice that were 4 days old were susceptible to *Shigella* infection, while mice that were 7 days old were resistant. Transcriptomic analysis of the mice indicated that AMPs were more highly expressed in the 7-day-old mice as compared to the 4-day-old mice, and histological analysis revealed that 7-day-old mice had Paneth cells while 4-day-old mice did not. Infection of Sox9^{fllox/fllox}-vil-cre mice, which are specifically depleted in Paneth cells, restored the susceptibility of 7-day-old mice to *Shigella*. Therefore, Paneth cell AMPs are responsible for the resistance to *Shigella* infection seen in 7-day-old mice.

The role of HD6 *in vivo* has long been unclear, as this peptide displayed no antibacterial activity *in vitro*. However, mice expressing HD6 in their Paneth cells were protected against intragastric challenge with STM as compared to wild type littermates¹⁵⁷. Intriguingly, the phenotype of infected HD6 transgenic mice was similar to the phenotype of wild type mice infected with a STM mutant that was unable to invade host cells, indicating that HD6 was blocking STM invasion¹⁵⁷. HD6 is uniquely able to self assemble into higher order oligomers¹⁵⁸, and in the HD6 transgenic mouse, extracellular HD6 was assembling around the bacteria and trapping it in net-like structures on the cell surface where it could presumably be targeted for killing by immune cells or other antimicrobial products¹⁵⁷. Although not directly bactericidal, this unique mechanism of bacterial elimination highlights the diversity of ways in which α -defensins can impact bacterial infections.

β -defensins also have a role in bacterial infection *in vivo*. Moser et al. showed that mouse beta-defensin-1 (MBD1) knockout mice had higher bacterial loads of *Haemophilus influenzae* in their lungs 24 hours post infection (hpi) as compared to wild type mice¹⁵⁹. In a separate study, MBD1 knockout mice had increased colonization of *Staphylococcus* species in the bladder compared to wild type littermates, but there was no difference in lung colonization by *S. aureus* between the two genotypes¹⁶⁰. This finding was unexpected, as purified MBD1 was active against *S. aureus in vitro*. Administration of synthetic β -defensins has also been shown to protect from bacterial infection *in vivo*. Piglets administered porcine BD-1 are protected from respiratory challenge with *Bordetella pertussis*, and BD-2 can protect rats against *P. aeruginosa* pneumonia^{161,162}.

b. Direct impact on viral infections[#]

There is less evidence to support a direct role for α -defensins in viral infection as the majority of the work demonstrating the antiviral effect of defensins has been in cell culture. Although one study has shown an increase in lethality from IAV infection in a MBD1 knockout mouse, those results are attributed to an increase in inflammation in the knockout mice rather than direct inhibition of viral replication¹⁶³. Similarly, administration of rhesus θ -defensin protected mice from lethal SARS-coronavirus challenge without affecting lung viral titers, likely due to a reduction in immunopathology in the treated animals¹⁶⁴. Finally, two separate studies have shown that HD5 can protect mice from vaginal challenge with HSV-2, but both studies pre-treated the mice with purified HD5 before virus administration^{90,165}. In fact, there is no example of a direct role for endogenous defensins in blocking virus infection *in vivo*. Indirect evidence for

[#] Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

the importance of defensins *in vivo* comes from association studies looking at defensin levels during various viral disease states. For example, patients with atopic dermatitis have reduced cathelicidin and β -defensins and are at greater risk of developing eczema vaccinatum¹⁶⁶⁻¹⁶⁸. And, HIV-1 positive women had lower HNP1-3 levels and lower anti-HSV-2 activity in their cervicovaginal lavage fluid compared to healthy controls¹⁶⁵. Similarly, cervicovaginal lavage fluid levels of HNP1-3 have been shown to correlate with the anti-HIV-1 activity of the fluid¹⁶⁹. Production of HNP1-3 mRNA and protein in monocyte-derived DCs is higher in HIV-1 infected individuals compared to healthy non-infected controls¹⁷⁰. And, the CD4⁺ T cell counts in those individuals with higher levels of defensin decreased more slowly than the individuals with lower levels of defensin. α -defensins are also detectable in the breast milk of HIV-1 infected mothers, although levels vary from approximately 0.1-7 nM, and are positively correlated with levels of HIV-1 RNA; however, the presence of α -defensins is also positively correlated with higher maternal CD4⁺ T cell counts and a decrease in the risk of HIV transmission from the breastfeeding mother to her infant^{171,172}. The apparent contradiction between higher α -defensin levels correlating to higher HIV viral titers and yet lower transmission might be explained by the fact that the defensin levels were also correlated with a healthier maternal immune system, as indicated by the increased CD4⁺ T cell count. Alternatively, HIV-1 infection may be stimulating α -defensin expression and secretion. Thus, α -defensin levels in HIV-1 infected individuals are correlated with control of viral infection, slowed disease progression, and lower vertical transmission. Additional evidence for a contribution of defensins to HIV-1 transmission and disease progression has been reviewed elsewhere^{135,173-176}.

c. Indirect impact of α -defensins on microbial infections

Impact on intestinal ecology and the immune environment

In addition to direct antibacterial activity, defensins can impact bacterial infections in indirect ways. A clear example of this for enteric infections is the impact of defensin repertoire on the composition of the commensal microbiota. HD5-transgenic, *Mmp7^{-/-}* and wild type mice all have the same bacterial load in the ileum¹⁷⁷. However, there are reciprocal shifts in the dominant bacterial phyla between defensin deficient mice (low Bacteroidetes and high Firmicutes) and mice transgenically expressing human defensins (high Bacteroidetes and low Firmicutes). Therefore, the correlation between defensin expression and increased resistance to STM infection observed in wild type mice as compared to *Mmp7^{-/-}* mice could be due to colonization resistance rather than a direct antibacterial effect. This is especially true in light of evidence suggesting that colonization resistance is impacted by the composition of the microbiota rather than by the overall number of bacteria^{178,179}.

Changes in bacterial phyla in HD5-transgenic mice are associated with the specific loss of segmented filamentous bacteria (SFB) from the commensal microbiota¹⁷⁷. SFB are a member of the Firmicutes phyla and are found in mice and other mammals but are not commonly found in humans¹⁴. Loss of these organisms was associated with a loss of IL-17 producing T helper cells (T_H17 cells) from the population of lymphocytes in the lamina propria¹⁷⁷. As T_H17 cells are an effector cell subset with roles in inflammation and autoimmunity, the modulation of these cells has implications for systemic inflammatory responses and homeostasis^{2,14}. Although defensins impact bacterial infection *in vivo*, it is still not clear if this is due to direct antibacterial activity or due to indirect effects on the microbiota or immune system.

Alteration of the immune response to infection[#]

Defensins are able to augment and direct the immune response to bacteria and viruses in ways that impact the outcome and resolution of infection. Although there are a myriad of mechanisms by which defensins can be immunomodulatory which have been reviewed elsewhere¹⁸⁰⁻¹⁸², some activities which are highly relevant to microbial infections will be discussed. Neutrophil and enteric α -defensins have been found to selectively chemoattract different subsets of T lymphocytes, macrophages, and immature DCs^{15,183-185}. Although the chemokine receptor used by α -defensins has not been identified, the recruitment of these immune cell subsets to the site of viral infection undoubtedly contributes to the outcome of viral infections.

The recruitment of immune cells has been shown to be relevant *in vivo*, as both recombinant and naturally produced defensins augment adaptive immune responses. Intranasal inoculation of mice with ovalbumin (OVA) in combination with HNP1-3 resulted in an increase OVA specific IgG and IgM titers compared to mice that received OVA alone¹⁸⁶. However, despite mucosal inoculation, no OVA specific IgA was observed, indicating that the HNP augmentation may not allow for class switching. A subsequent study co-administered OVA intranasally with HNP1, HNP2, HBD1, or HBD2 individually and found that the predominant IgG isotypes and interleukin profiles were unique to the defensin used, highlighting the complexity of the interaction between defensins and immune cells¹⁸⁷. Finally, the augmented immune response has been shown to be functionally relevant, as intraperitoneal injection of HNPs enhanced the antibody response to a syngeneic tumor challenge and increased the survival time of mice after tumor challenge¹⁸⁸. Thus, although not yet shown in the context of an

[#] Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

infectious challenge, stimulation of the adaptive immune response by defensins likely contributes to antimicrobial immunity.

In addition to inducing antibody secretion, direct contact with defensins can activate immune cells and induce cytokine secretion. HNP1, HNP2, and HD5 have been shown to increase neutrophil uptake of IAV¹¹³. In regards to DCs, HNP1 promotes the activation and maturation of monocyte-derived DCs and production of pro-inflammatory cytokines¹⁸⁹. Defensins can also elicit pro-inflammatory cytokine production from treated cells that could then stimulate DCs^{8,190,191}. Moreover, HNP1 and HNP2 have also been shown to induce CC-chemokine expression and secretion in macrophages, which block HIV infection¹⁹². Therefore, some of the activation of antigen expressing cells could be explained through an indirect mechanism. Overall, α -defensins function to augment and alter the immune responses to microbes, and the response elicited is likely dependent upon the amount of microbial stimuli and the subsequent concentration of defensin that is produced.

Chapter 2: A small intestinal organoid model of enteric pathogen-epithelial cell interactions^{**}

2.1 Background

The complexity of the small intestinal epithelium has been difficult to model in culture, and dissection of epithelium-specific functions *in vivo* is confounded by the influence of signals arising from local and systemic non-epithelial sources. The discovery of a system to culture primary stem cell-derived small intestinal organoids has addressed these issues: First, a defined growth medium allows for the differentiation and maintenance of goblet cells, Paneth cells, enterocytes, enteroendocrine cells, and stem cells that recapitulates the cellular composition of the small intestinal epithelium^{151,193}. Second, organoids have crypt-like domains and villus-like regions and can be stably maintained in sterile culture in the absence of mesenchymal and immune cells. Third, artifacts of transformation inherent in most traditional intestinal epithelial culture models are absent. Organoids have been increasingly used to uncover aspects of cell biology, intestinal development, and wound repair but have not been widely utilized to study pathogen-epithelial cell interactions^{151,194}. To this end, we have developed a new model of enteric infection by accessing the apical aspect of the polarized intestinal epithelium of organoids using microinjection.

As proof of concept, we used our new model to assess the need for α -defensins in epithelial defense against a replicating bacterial pathogen, STM. α -defensins are small, cationic, amphipathic peptides that possess broad antimicrobial activity; however, there have been limited studies of the direct antimicrobial activity of naturally secreted α -defensins in a complex,

^{**} Adapted from Wilson, S.S., Tocchi, A.T., Holly, M.K., Parks, W.C., and Smith, J.G. A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions. *Mucosal Immunology* **8**, 352-361 (2015).

physiologic extracellular milieu⁶. Moreover, their role in modulating bacterial pathogenesis *in vivo* is confounded by indirect effects of α -defensins on the immune system and the composition of the commensal microbiota^{14,177,180}.

To address these shortcomings, our enteric infection model enables the first direct assessment of STM killing during prolonged *ex vivo* co-culture with viable primary epithelial cells capable of naturally producing enteric α -defensins. For this purpose, we established small intestinal organoids from wild type and *Mmp7*^{-/-} mice. As MMP7 is the protease that converts mouse pro- α -defensins into their mature forms, *Mmp7*^{-/-} mice lack functional α -defensins in the small intestine²². Through comparative assays using these two genotypes, we have shown by two approaches that α -defensins substantially contribute to epithelial host defense and restrict growth of STM for at least 20 h in culture. Growth inhibition was seen for multiple strains of STM and at several time-points post-injection. The assay is also responsive to host factors influencing Paneth cell function, as transgenic expression of HD5 in *Mmp7*^{-/-} organoids restored bacterial killing. Nonetheless, although nucleotide-binding oligomerization domain containing 2 (NOD2) deficiency has been linked to reduced α -defensin expression and function in the etiology of Crohn's disease¹⁹⁵, we found that bacterial killing in organoids from *NOD2*^{-/-} mice was not impaired and that α -defensin expression in these organoids was equivalent to wild type. In summary, we have created and validated a novel model to investigate interactions between enteric pathogens and small intestinal epithelial cells that can be extended to additional bacterial and viral pathogens and can be genetically dissected at both the host and pathogen level.

2.2 Results

The organoid lumen is intact and can be accessed by microinjection. For organoids to accurately model the intestine, their lumen and the apical surface of the cells should be

sequestered from the extracellular basolateral environment. An intact organoid lumen would potentially allow for the accumulation of a high local concentration of secretion products including α -defensins. To access this space and mimic apical enteric infection, we utilized microinjection. We established primary small intestinal organoid cultures from wild type C57BL/6 mice^{151,196}. Microinjected PBS caused swelling of organoids, indicating the integrity of the lumen (Figure 2.1A). To demonstrate further that the lumen was functionally intact, we microinjected $\sim 5 \times 10^4$ CFU/organoid of the non-invasive STM strain LT2 $\Delta phoP$ either into the organoid lumen (inside) or in the surrounding Matrigel (outside) proximal to organoids. All samples received exactly 20 injections total. We then incubated the cultures for 2 h, treated with or without 100 $\mu\text{g/ml}$ of the cell membrane-impermeant antibiotic gentamicin for 2 h, and enumerated surviving CFU. We recovered equal numbers of CFU from inside and outside of the organoids in the absence of gentamicin; however, $>10^3$ more CFU were protected from gentamicin when injected inside the organoids (Figure 2.1B).

Enteric α -defensins are found in secretory granules of Paneth cells in the small intestine^{4,197}. Unlike humans, mice express an expanded repertoire of enteric α -defensins, termed cryptdins¹¹. Mouse α -defensins are produced as pro-peptides and cleaved and activated to their mature form by MMP7 in Paneth cells²². Accordingly, *Mmp7*^{-/-} mice have normal expression of pro-defensins in their Paneth cells granules but are functional knockouts of mature α -defensins in the small intestine^{22,198}. We established organoids from *Mmp7*^{-/-} mice (C57BL/6) and found that absence of MMP7 did not alter the integrity of the organoid lumen (Figure 2.1B). Overall, these data show that the organoid lumen is intact and can be accessed via microinjection.

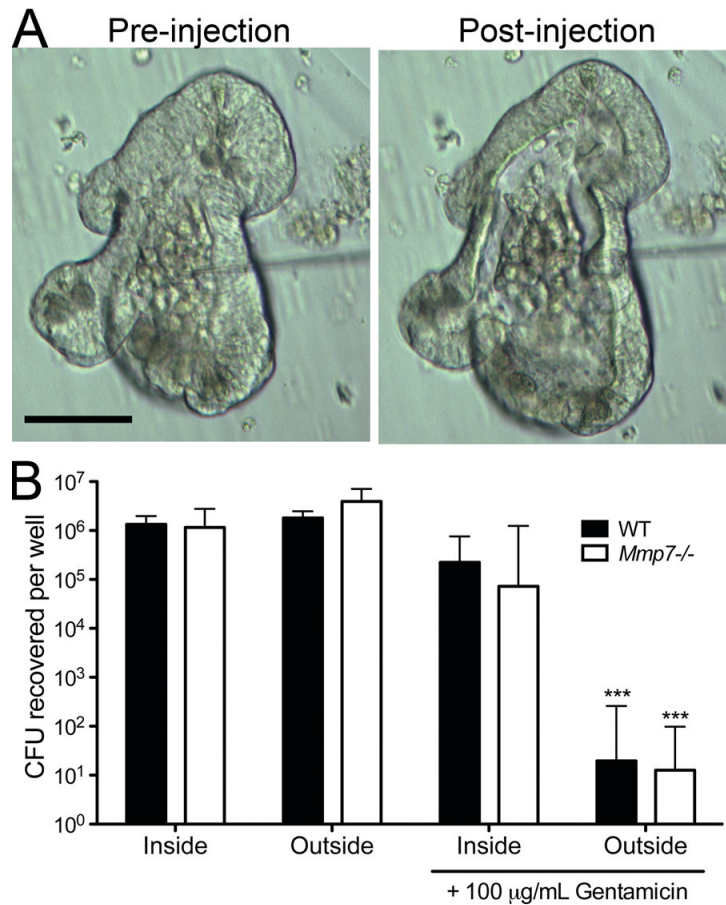


Figure 2.1. The organoid lumen is intact and can be accessed by microinjection. (A) Before and after images of microinjection of PBS into the organoid lumen. Scale bar is 50 µm (B) Survival of STM after injection into the organoid lumen (inside) or into the surrounding Matrigel (outside) in the presence and absence of 100 µg/mL gentamicin. Data for wild type (WT, black bars) and *Mmp7*^{-/-} (white bars) organoids are the antilog of the average of log-transformed CFU from three independent experiments ± SD. For statistical significance, one-way analysis of variance (ANOVA) with Tukey post-tests was used to compare all pairs of columns. Only the indicated bars (***) were significant, and they were significant in comparison with every other condition except each other.

Bacterial growth is inhibited in wild type but not *Mmp7*^{-/-} organoids. We next asked if α -defensins present in the organoid lumen could inhibit STM growth. Wild type and *Mmp7*^{-/-} organoids were injected with STM expressing GFP (LT2 Δ *phoP* GFP, 5x10³ CFU/organoid) and imaged at 0 h and 5 h post-injection. In three separate experiments, a reduction in fluorescence occurred by 5 h post-injection in wild type organoids but not in *Mmp7*^{-/-} organoids (Figure 2.2A-C). Rather, diffusion of the bacteria within the organoid lumen from the site of injection was seen in *Mmp7*^{-/-} organoids. The surviving CFU were quantified at 20 h post-injection, and a 3.9-log reduction in CFU was seen in wild type organoids compared to *Mmp7*^{-/-} organoids (Figure 2.2D). To correlate quantification of bacterial killing by fluorescence and CFU, we imaged and then immediately isolated bacteria from parallel cultures of wild type and *Mmp7*^{-/-} organoids at 0, 5, and 9 h post-infection. Under these conditions we found a close correlation between the two assays with a 1.3-log reduction in survival and a 1-log reduction in average fluorescence intensity in wild type compared to *Mmp7*^{-/-} at 5 h post-injection that increased to a 1.9-log reduction in survival and a 1.3-log reduction in average fluorescence intensity in wild type compared to *Mmp7*^{-/-} at 9 h (Figure 2.3). These results indicate that changes in CFU over this time period reflect bacterial dynamics in the lumen.

We then assessed the magnitude of α -defensin-mediated killing in organoids from multiple independent preparations. Wild type and *Mmp7*^{-/-} organoids were injected with LT2 Δ *phoP* (5x10³ CFU/organoid), and surviving CFU from 20 injected organoids were pooled and assayed at 9 h post-injection. We observed a 1.1-log reduction in wild type organoids compared to *Mmp7*^{-/-} organoids (Figure 2.2E). In addition, CFU in the *Mmp7*^{-/-} organoids increased in relation to the inoculum, indicating that the bacteria were able to grow in the organoid lumen (Figure 2.2E). We then examined the effect of carbamylcholine chloride (CCh), a known

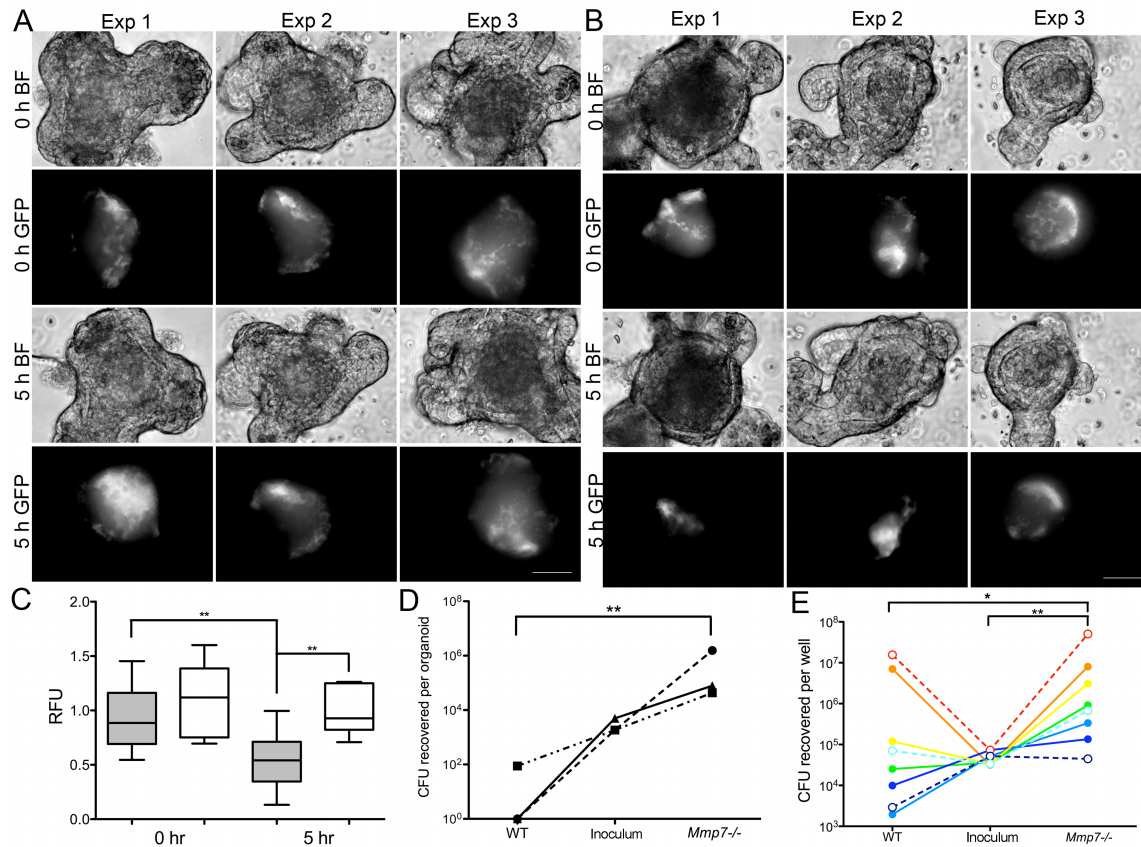


Figure 2.2. Bacterial growth is inhibited in wild type but not $Mmp7^{-/-}$ organoids.

Representative images from 3 independent experiments of STM LT2 $\Delta phoP$ GFP microinjected into (A) Wild type organoids and (B) $Mmp7^{-/-}$ organoids. Images were taken at 0 and 5 h post-injection, and the bright field (BF) image corresponds to the 0 h GFP image. Signal above threshold is shown for each image. (C) Data is the relative fluorescence intensity (RFU) of 3 injected organoids per experiment (9 total) for wild type (grey) and $Mmp7^{-/-}$ (white). Whiskers are the minimum and maximum of the data, and the horizontal line is the mean. (D) CFU recovered 20 h post-injection. Each line corresponds to one experiment in A and B, and each data point is the average CFU from 2-3 organoids. (E) CFU recovered 9 h post-injection. Each line represents one independent experiment, and each data point is the total CFU from 20 organoids injected in a single well. Dashed lines with open symbols represent experiments using organoids pre-treated with 10 μ M CCh. Scale bars are 50 μ m (A and B). * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Bonferroni's Multiple Comparison Test was used for 2.2C and one-way ANOVA with Tukey post-test for 2.2D and 2.2E.

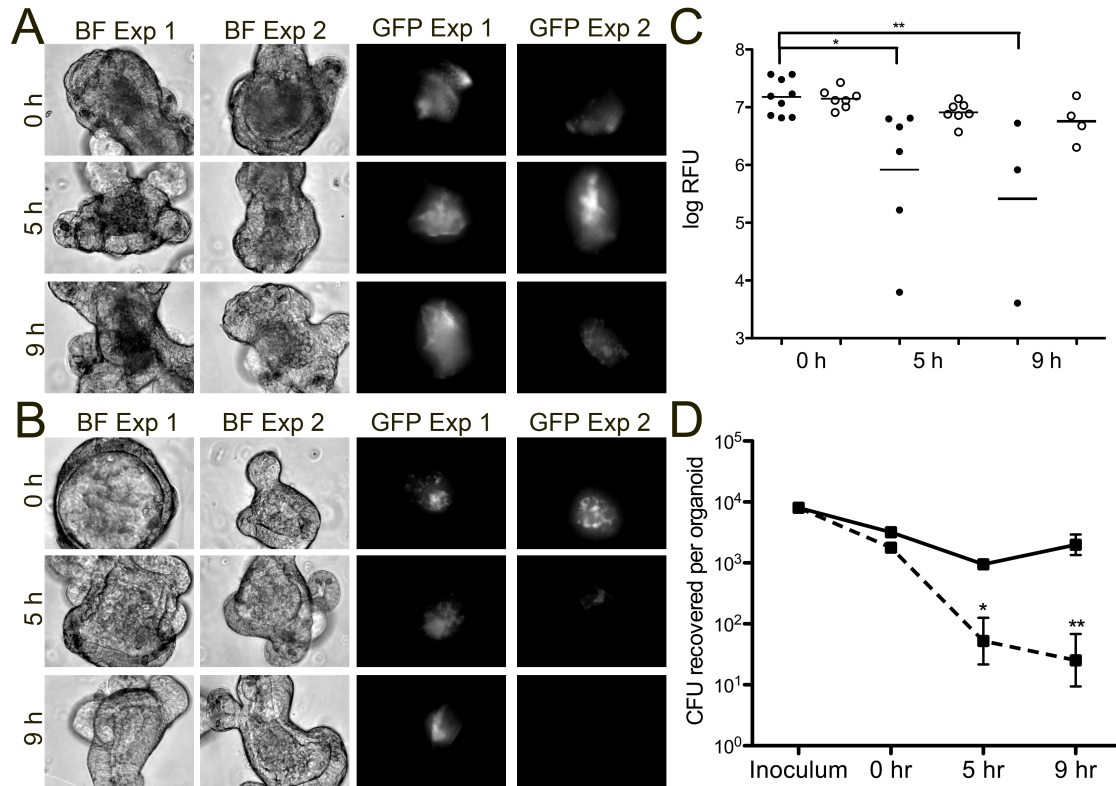


Figure 2.3. Fluorescence reduction in wild type organoids correlates with a reduction in CFU. Images from 2 independent experiments of STM LT2 *AphoP* GFP microinjected into (A) *Mmp7*^{-/-} and (B) wild type organoids. Images are representative of the range of data, were taken at 0, 5 and 9 h post-injection, and both the bright field (BF) image and the GFP image are shown. Signal above threshold is shown for each GFP image. (C) Data are the log transformed relative fluorescence units (RFU) of injected wild type (closed) and *Mmp7*^{-/-} (open) organoids at 0, 5 and 9 h post-infection. Each symbol represents the data from one injected organoid, and the cumulative data from two independent experiments is shown. (D) CFU recovered 0, 5 and 9 h post-injection from wild type (dotted line) and *Mmp7*^{-/-} (solid line) organoids. Data is the average CFU recovered per organoid from at least 3 independent experiments. **p*<0.05, ***p*<0.01, by one-way ANOVA with Tukey post-tests.

stimulator of Paneth cell secretion⁶, and found that although CCh induced organoid swelling, indicative of an effect on secretion, it did not alter the magnitude of bacterial killing (dashed lines in Figure 2.2E). Taken together, these results by two distinct measures support the conclusion that STM were viable in the organoid lumen only in the absence of functional α -defensins.

Organoids from *Mmp7*^{-/-} mice lack mature α -defensins. To support our hypothesis that impaired STM killing in *Mmp7*^{-/-} organoids was due to a specific absence of functional α -defensins, we assessed the relative levels of pro- and mature α -defensins in wild type and *Mmp7*^{-/-} organoids. Organoids from both genotypes grew at similar rates and contained equivalent proportions of goblet and Paneth cells, which were readily apparent (Figures 2.4A-B and Figure 2.5A-C). To confirm the production of pro- α -defensins, we stained organoids from both mouse strains for mouse α -defensin-5 (Crp5). We observed specific staining for Crp5 in the Paneth cells of both wild type and *Mmp7*^{-/-} organoids. No staining was observed with the control antibody (Figure 2.4A). Thus, Paneth cells in organoids from both wild type and *Mmp7*^{-/-} mice produce granules containing pro- α -defensins.

Next, the presence and activation state of α -defensins in wild type and *Mmp7*^{-/-} organoids were directly assessed by acid-urea PAGE (AU-PAGE). Equivalent amounts of total protein extracted from wild type and *Mmp7*^{-/-} organoids were compared to extracts of crypt-enriched fractions from wild type and *Mmp7*^{-/-} mice. Purified Crp23, an abundant α -defensin expressed in C57BL/6 mice, was included as a control¹¹. Extracts from freshly isolated crypts and organoids derived from wild type mice contained species with mobilities consistent with mature α -defensins. In contrast, extracts from *Mmp7*^{-/-} crypts and organoids did not (Figure 2.4D).

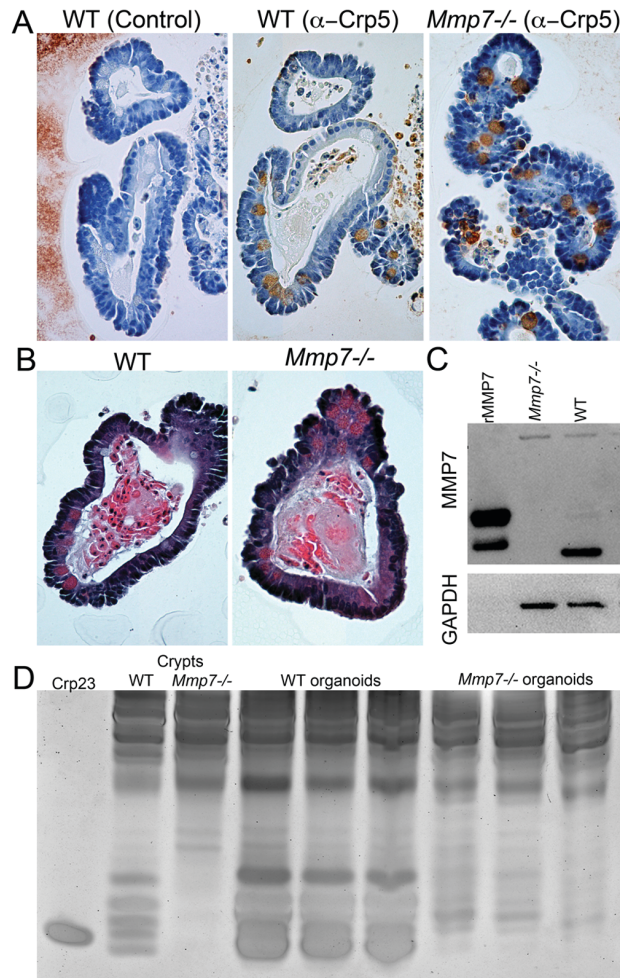


Figure 2.4. Organoids from $Mmp7^{-/-}$ mice lack mature α -defensins. (A)

Immunohistochemistry of wild type and $MMP7^{-/-}$ organoids stained with goat anti-Crp5 antibody or control goat IgG. (B) Wild type (WT) and $Mmp7^{-/-}$ organoids stained with hematoxylin and eosin. (C) Immunoblots of lysates from wild type and $Mmp7^{-/-}$ organoids probed for MMP7. Recombinant MMP7 (rMMP7) was used as a positive control, and an antibody to GAPDH was used as a control for loading. The upper band in the rMMP7 lane corresponds to pro-MMP7 (30 kDa) and the lower band is the active form (20 kDa). The mobilities of active rMMP7 and active MMP7 in the lysates differ due to the presence of additional mass from the purification tag. (D) Equal amounts (100 μ g) of lyophilized extract from freshly isolated crypts or organoids from wild type and $Mmp7^{-/-}$ mice were analyzed by AU-PAGE. Each lane of organoid extract is from a separate preparation of organoids from different mice. Purified mouse α -defensin-23 (Crp23, 1 μ g) indicates the mobility of mature α -defensins.

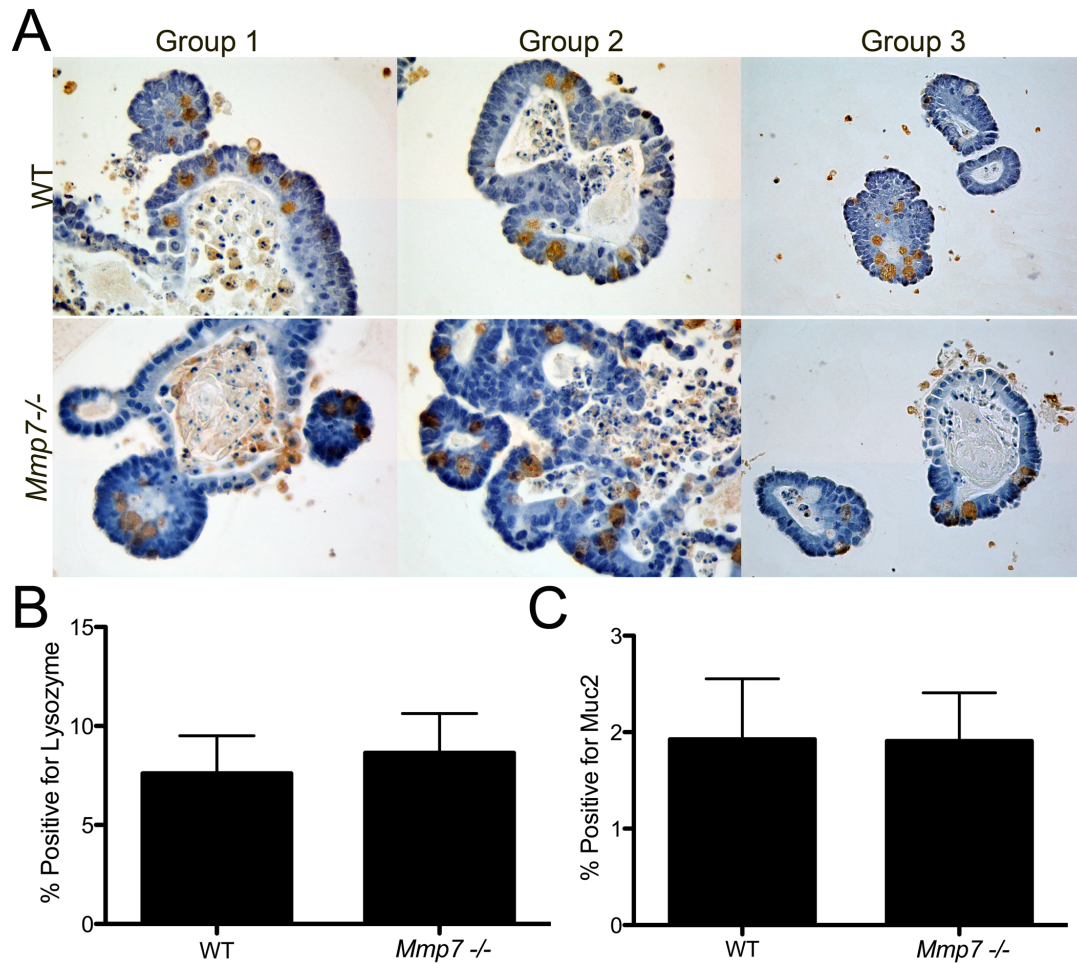


Figure 2.5. Wild type and *Mmp7*^{-/-} organoids have similar morphology and cellular composition. (A) Immunohistochemistry of wild type and *Mmp7*^{-/-} organoids stained with goat anti-Crp5 antibody. Representative images from 3 independent organoid preparations are shown. Percentage of Paneth cells (B) and Goblet cells (C) in wild type and *Mmp7*^{-/-} organoids was determined by flow cytometry. No significant difference in percentage of either Paneth cells or Goblet cells between genotypes was observed. Results are expressed as the mean \pm SEM of 3 separate experiments. Data in B and C were log transformed and analyzed by repeated measures one-way ANOVA with Tukey post-tests.

Furthermore, abundant active MMP7, which is present in Paneth cell granules *in vivo* and responsible for activation of pro- α -defensins²², was produced in wild type but absent from *Mmp7*^{-/-} organoids (Figure 2.4C). These findings provide further evidence that organoids recapitulate the state of α -defensin maturation reported for wild type and *Mmp7*^{-/-} mice¹⁹⁸.

Increased sensitivity of Δ *phoP* STM mutants confirms a role for α -defensins in luminal killing. To further support the critical and specific role of α -defensin-mediated bacterial killing in this model, we compared the survival of wild type and LT2 Δ *phoP* in organoids from both genotypes. As discussed in Chapter 1, the STM PhoP-PhoQ two-component regulatory system is critical for sensing and responding to antimicrobial peptides, and STM *phoP* mutants are more sensitive to purified α -defensins¹⁹⁹. For these experiments, we chose a later time point (20 h post-injection) to amplify any survival difference. A dose-response study identified 50 CFU/organoid as a minimal dose that reliably yielded quantifiable surviving CFU. Significant bacterial killing was observed for both strains in wild type organoids compared to *Mmp7*^{-/-} organoids (Figure 2.6A). Consistent with the role of PhoP in sensitizing STM to α -defensins, the difference was greater in LT2 Δ *phoP* (4.6-log reduction) than in wild type LT2 (1.7-log reduction).

Next, we assessed the ability of organoids to defend against STM 14028s, a strain commonly used in mouse models of *Salmonella* pathogenesis²². We introduced a chromosomal *sipB* mutation that renders the bacteria unable to attach to and therefore invade epithelial cells and assessed the impact of PhoP²⁰⁰. After 20 h, we observed a 6-log reduction in CFU in the 14028s Δ *phoP* background but not in the wild type strain (Figure 2.6B). Thus, the increased killing of the Δ *phoP* mutants of both STM strains confirms the importance of α -defensins in

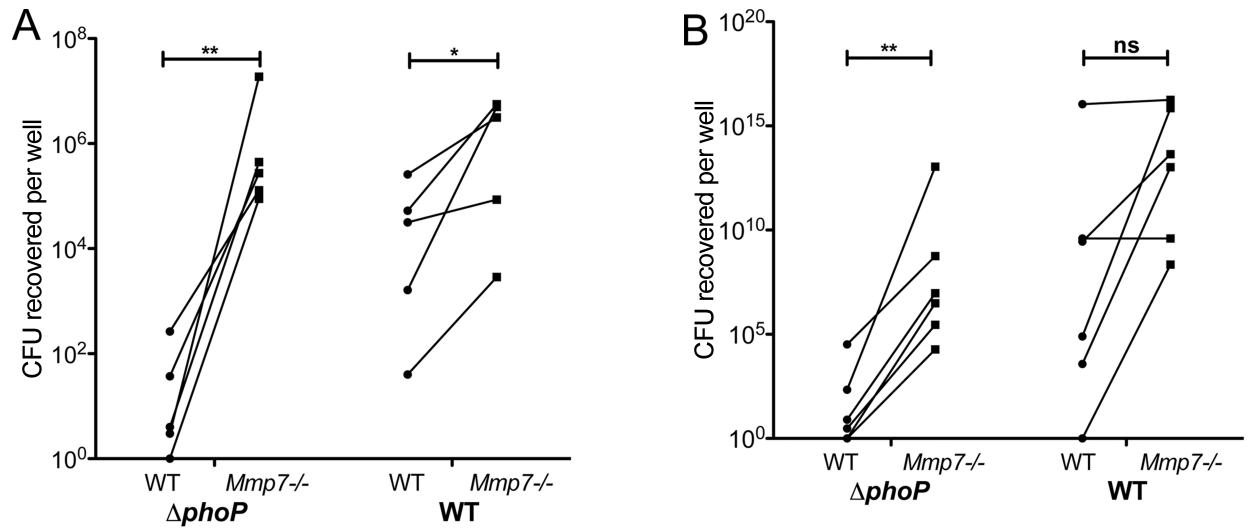


Figure 2.6. Long-term killing of multiple STM strains. STM LT2 and STM LT2 $\Delta phoP$ (A) or 14028s $\Delta sipB$ (WT) and 14028s $\Delta sipB \Delta phoP$ (B) were injected into wild type and $Mmp7^{-/-}$ organoids, and surviving CFU were enumerated 20 h post-injection. Each line represents an independent paired experiment, and each point is the pooled data from a well of 20 injected organoids. * $p < 0.05$, ** $p < 0.01$. ns, not significant, data were log transformed and analyzed by paired t test.

mediating killing in our model.

Transgenic expression of HD5 rescues bacterial killing. Having established the importance of bacterial genotype in α -defensin mediated killing in our system, we sought to measure the effect of additional host genotypes relevant to Paneth cell function. We established organoids from *Mmp7*^{-/-} mice that express transgenic HD5 (*Mmp7*^{-/-} *DEFA5*^{+/-}). Mice expressing transgenic HD5 on a wild-type background (*Mmp7*^{+/+} *DEFA5*^{+/+}) are resistant to oral challenge with STM; however, there has been no investigation into the antibacterial activity of *Mmp7*^{-/-} *DEFA5*^{+/-} Paneth cells¹⁵⁵.

We first examined the processing of HD5 in organoids. In humans, proHD5 is cleaved by endogenous Paneth cell trypsin; however, trypsin is not present in mouse Paneth cells³³. Although HD5 can also be processed by MMP7 *in vitro*³³, mature HD5 has also been shown *in vivo* in the *Mmp7*^{-/-} *DEFA5*^{+/-} background and has been attributed to alternative host and microbial proteinases¹⁹⁸. We observed processed HD5 in organoids and isolated crypts from both *Mmp7*^{-/-} *DEFA5*^{+/-} and *Mmp7*^{+/+} *DEFA5*^{+/+} mice via western blot (Figure 2.7A).

We then asked if transgenic expression of HD5 augmented Paneth cell antibacterial activity. As in our previous assays, 5x10³ CFU per organoid of STM LT2 *ΔphoP* was injected into wild type, *Mmp7*^{-/-}, and *Mmp7*^{-/-} *DEFA5*^{+/-} organoids. Surviving CFU were enumerated 7 h (Figure 2.7B) and 16 h post-injection (Figure 2.7C) to capture the dynamics of STM killing by HD5. After 7 h, survival of the inoculum was reduced 50-fold upon injection into wild type organoids. Although differences among genotypes were not significant, we observed the greatest survival in *Mmp7*^{-/-} organoids and intermediate survival in *Mmp7*^{-/-} *DEFA5*^{+/-} organoids. By 16 h post-injection, STM grew in organoids from all three genotypes; however, survival between

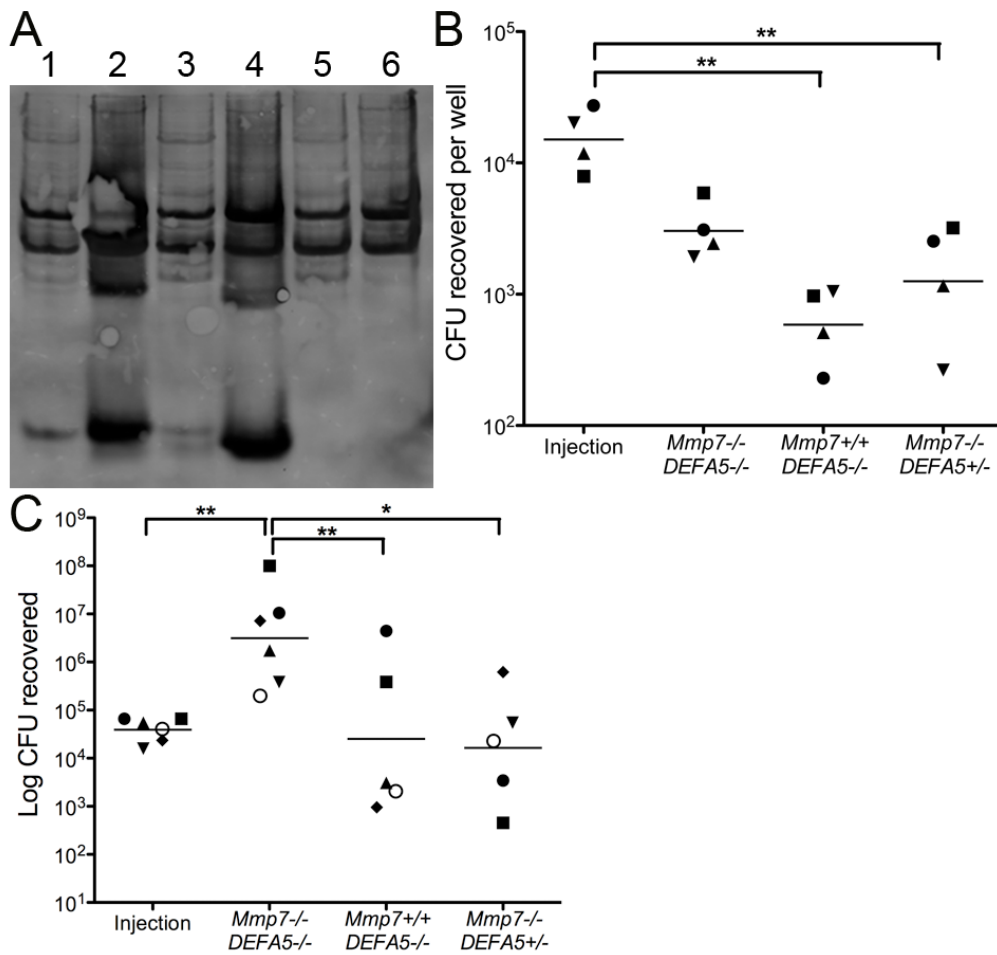


Figure 2.7. HD5 maturation and STM killing in *Mmp7*^{-/-} *DEFA5*^{+/-} organoids. (A) AU-PAGE immunoblot of extracts from *Mmp7*^{-/-} *DEFA5*^{+/-} organoids (lane 1, 500 μg protein) and freshly isolated crypts (lane 2, 250 μg protein), *Mmp7*^{+/+} *DEFA5*^{+/+} organoids (lane 3, 500 μg protein) and freshly isolated crypts (lane 4, 250 μg protein), and wild type (WT) organoids (lane 5, 500 μg protein) and freshly isolated crypts (lane 6, 250 μg protein) were probed for HD5. (B and C) STM LT2 *ΔphoP* was injected into WT, *Mmp7*^{-/-} and *Mmp7*^{-/-} *DEFA5*^{+/-} organoids, and surviving CFU were enumerated 7 h (B) and 16 h (C) post-injection. Each symbol represents an independent paired experiment and is the pooled data from a well of 20 injected organoids. *p<0.05, **p<0.01. Data was log transformed and analyzed by repeated measures one-way ANOVA with Tukey post-tests.

wild type and *Mmp7*^{-/-} (2.1-log difference) was significant. Moreover, the transgenic expression of HD5 restored wild type killing in the *Mmp7*^{-/-} *DEFA5*^{+/-} organoids, with a 2.3-log difference in survival between *Mmp7*^{-/-} and *Mmp7*^{-/-} *DEFA5*^{+/-}. Consistent with Figure 2.3, there is an initial decrease in CFU in all of the organoid genotypes at 7 h, with a larger reduction in organoids that contain mature α -defensins. By 16 h there is growth across the organoid genotypes from the 7 h time point, which is much larger in the *Mmp7*^{-/-} background (3-log increase) than in the wild type (1.1-log increase) or *Mmp7*^{-/-} *DEFA5*^{+/-} (1.6-log increase) organoids.

NOD2 deletion has no effect on bacterial killing. We next addressed the importance of NOD2 expression on the capacity of organoids to impact bacterial survival. NOD2 is an intracellular receptor for peptidoglycan, and loss of function mutations in *NOD2* are associated with ileal Crohn's disease and correlated with reduced expression of enteric α -defensins^{195,201}. In mouse models, NOD2 deficiency is linked to reduced α -defensin expression and impaired antibacterial activity of crypt secretions, indicating that decreased α -defensin expression might be an inherent phenotype of *NOD2*^{-/-} mice²⁰²⁻²⁰⁴. However, a more recent study demonstrated the levels and activities of α -defensins in *NOD2*^{-/-} mice are similar to those in wild type mice²⁰⁵.

To address these questions, we compared the levels of activated α -defensins between organoids from *NOD2*^{-/-} and wild type mice and found that they were equivalent (Figure 2.8A). To assess the impact of NOD2 expression on bacterial killing, we injected 5x10³ CFU of STM LT2 *ΔphoP* into *NOD2*^{-/-} and wild type organoids and enumerated surviving CFU at 16 h post-injection. We saw no significant difference in recovered CFU between the organoid genotypes

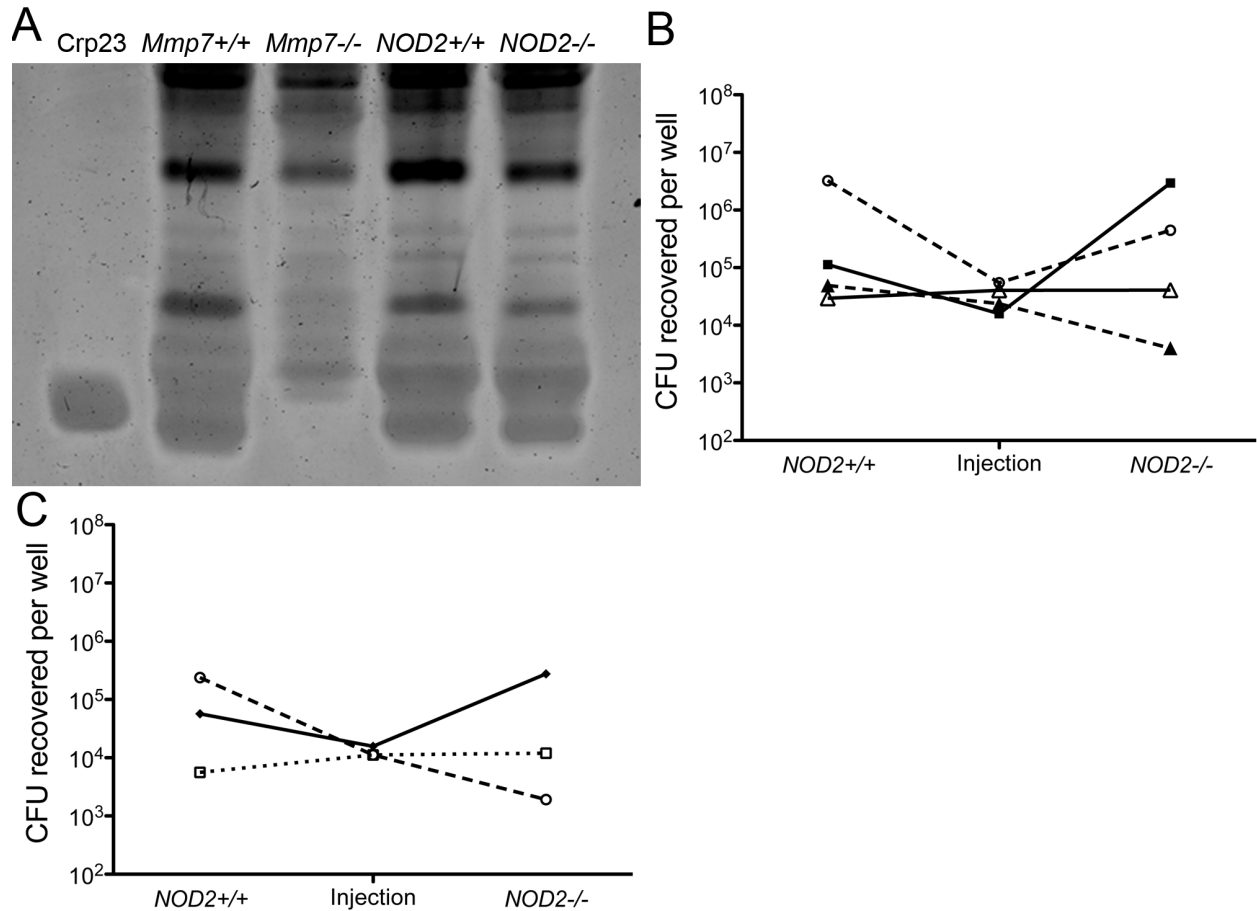


Figure 2.8. α -defensin expression and antibacterial activity of *NOD2*^{-/-} organoids. (A) Equal amounts (100 μ g) of lyophilized extracts from organoids from wild type, *Mmp7*^{-/-}, *NOD2*^{+/+}, and *NOD2*^{-/-} mice were analyzed by AU-PAGE. Purified mouse α -defensin-23 (Crp23, 1 μ g) indicates the mobility of mature α -defensins. CFU recovered 16 h post-injection of STM LT2 *ΔphoP* mixed without (B) or with (C) 100 μ g MDP. Each line represents one independent experiment, and each data point is the total CFU from 20 organoids injected in a single well. For comparison, data were log transformed and analyzed by repeated measures one-way ANOVA with Tukey post-tests.

(Figure 2.8B). To ensure that NOD2 was fully activated, we repeated these experiments with the addition of muramyl di-peptide (MDP) to the inoculum. We again saw no significant difference in recovered CFU between the genotypes (Figure 2.8C). Thus, NOD2 expression had no effect on intrinsic α -defensin levels or Paneth cell antibacterial function in this model, although the magnitude of killing in these C57BL/6J organoids was weaker than that of C57BL/NHsd organoids in our other experiments.

2.3 Discussion

We have established a model to mimic apical enteric infection that utilizes microinjection of small intestinal organoids. Prominent features of this model include an intact lumen that prevents diffusion of apically secreted products into the bulk volume of the culture medium and the ability to selectively infect either the apical or basolateral aspect of the polarized epithelium. Thus, this model allows for a broad range of studies of the interactions between microbes, epithelial cells, and their secreted products. As proof of concept, we determined the impact of naturally secreted α -defensins on bacterial survival in the organoid lumen. Growth of non-invasive STM was significantly reduced in wild type organoids compared to *Mmp7*^{-/-} organoids. Killing was observed at multiple time points after injection, by two independent measures, and for two STM strains. In accordance with the survival data, organoids from wild type mice produced mature α -defensins, whereas those from *Mmp7*^{-/-} mice did not. In addition, the decreased relative survival of strains lacking the PhoP regulon and the ability of transgenic expression of HD5 to restore killing are consistent with an α -defensin-dependent mechanism. These results indicate that naturally secreted α -defensins in the complex extracellular milieu of the organoid lumen are

capable of killing bacteria and that α -defensin activity is sustained, protecting the epithelium of the organoid over time.

This model represents a significant improvement over standard methods to determine antibacterial activities of α -defensins and can be easily applied to studies of other epithelial products. Standard assays for determining the antibacterial activities of enteric α -defensins were discussed in the introduction, and predominately use purified peptides and are performed under low-salt conditions, as bacterial killing is generally inhibited in buffers with physiologic levels of salt^{8,10}. A limited number of *ex vivo* studies have examined the ability of naturally produced α -defensins or defensin-containing extracellular fluids to inhibit bacterial growth⁸. For enteric α -defensins, Ayabe et al. performed experiments which first supported the notion that secreted α -defensins are capable of directly killing bacteria⁶. However, this study utilized freshly isolated small intestinal crypts, which are unstable and only amenable to short-term experiments. Additionally, bacterial killing was demonstrated under low salt conditions and overall was modest (<10-fold) compared to the level of killing we saw with wild type organoids (up to a 6-log reduction).

Thus, our approach supports the antibacterial findings of previous studies with substantial improvements. First, our model enables the study of defensins in a complex milieu without purification and without altering buffer constituents. Second, multiple experiments can be performed with equivalent, stably cultured organoids, which are stable for months to years. In contrast, while *ex-vivo* human and mouse biopsy specimens can be apically infected with bacterial, they are only viable for 12-48 hours²⁰⁶. Third, the fate of bacteria injected into a single organoid can be tracked over time. Fourth, the assay is very sensitive, as it can be amplified through prolonged co-culture of the microbes and organoids and is effective over a wide range of

input CFU. Finally, organoids can be genetically manipulated or established from genetically engineered mice, allowing the role of specific proteins and pathways in enteric defense to be assessed^{194,207}.

Given these advantages, we applied our enteric infection model to investigate the ability of organoids from *NOD2*^{-/-} mice to inhibit bacterial growth compared to wild type organoids. In genome wide association studies, NOD2 mutations contribute the greatest genetic risk for Crohn's disease, although the exact role that NOD2 mutations play in the etiology of Crohn's disease is unclear²⁰⁸. Initial studies reported a reduction in α -defensin transcript levels in *NOD2*^{-/-} mice, which was mirrored in human Crohn's disease patients with NOD2 mutations^{195,204}. This defect was functionally confirmed through a reduction in antibacterial activity in *NOD2*^{-/-} crypt secretions^{202,203}. However, recent studies have reported wild type levels of α -defensins in both mice and humans with NOD2 deficiency and support a role for NOD2 in stem cell survival^{205,209,210}. We found that *NOD2*^{-/-} organoids contain wild type levels of α -defensins. Moreover, we observed no defect in their ability to restrict growth of luminal bacteria. These results are most consistent with the absence of an effect of NOD2 on either α -defensin expression or Paneth cell function.

Despite the strength of our approach, there are some limitations to the design and interpretation of these and future applications. First, although the epithelial cell diversity of the small intestine is recapitulated in organoids, the spatial relationships of the crypt-villus axis are not uniformly preserved. In addition, organisms are introduced in close proximity to crypt cells in the organoid, whereas *in vivo* they would need to traverse a greater distance from the bowel lumen to reach an anatomically equivalent location. The compact spatial organization also presents a technical challenge, in that there is a finite volume that can be injected into the

organoid lumen, which could potentially be limiting for organisms that cannot be concentrated sufficiently. Second, we have shown that the organoid lumen is a discreet environment. We were unable to directly quantify the concentration of α -defensins at basal levels or in response to stimuli in the organoid lumen. This issue is specific to defensins, as it is difficult to biochemically or histologically distinguish intracellular defensins stored in granules and those released into the lumen by degranulation, a mechanism well supported by the literature^{4,211}. A recent imaging study demonstrated secretion of lysozyme into the lumen of organoids over a time frame equivalent to that of our experiments²¹². This supports our interpretation that α -defensins are secreted even by unstimulated Paneth cells, since α -defensins are stored in Paneth cell granules with lysozyme^{4,6,34}. Third, the degree to which the organoid lumen recapitulates the small intestinal lumen, particularly in regards to pH and osmolality, is unclear. The organoid lumen likely more accurately models the confines of intestinal crypts rather than the much larger bowel lumen. Fourth, although our study demonstrates that naturally secreted α -defensins substantially contribute to inhibiting bacterial growth, it does not preclude a role for the microbiota and the immune system in impacting the phenotype of *Mmp7*^{-/-} mice after enteric infection^{22,177}. Additionally, as *Mmp7*^{-/-} mice lack both mature α -defensins and CRS, both of which are antibacterial, we cannot formally exclude a role for CRS in the observed killing seen in wild type organoids^{213,214}. Finally, we encountered a quantitative issue in some experiments due to turbidity of the organoid medium 20 h post-infection in the *Mmp7*^{-/-} cultures, most often with the 14028s strain. We hypothesize that the absence of α -defensins permitted bacterial overgrowth that compromised the integrity of the organoid lumen. This complication impacts the absolute quantification of experimental CFU but does not alter the qualitative interpretation of the experiments.

Overall, we believe that our organoid model has significant potential for future applications. It is readily applicable to studying the direct impact of antimicrobial peptides and proteins on pathogen growth, which can likely be extended to viruses and parasites. Although we limited our assay to non-invasive strains of STM that are more sensitive to defensin killing, organoids could be used to interrogate invasive bacteria and their effects on host cells, and additional bacterial mutants could be studied alone or in combination. The veracity of the system can be further increased by inducing the differentiation of M cells and by co-culturing with immune cells^{207,215}. Finally, human organoids can be cultured under similar conditions, permitting translational studies of acute infection and chronic diseases such as inflammatory bowel disease¹⁵². Thus, the establishment of this model of enteric infection enables a broad range of studies of host-pathogen interactions.

Chapter 3: Capsular polysaccharides protect *Klebsiella pneumoniae* from α -defensin killing[‡]

3.1 Background

The opportunistic pathogen *Klebsiella pneumoniae* is a member of the gut microbiota of healthy individuals. Although *K. pneumoniae*'s mucoid capsular polysaccharide (CPS) coat is considered a key virulence factor, the precise role of exopolysaccharides during colonization of the gastrointestinal tract is unclear²¹⁶. To elucidate the role of CPS in colonization of the gastrointestinal tract, the Garrett lab at Harvard School of Public Health assessed the biogeography of *K. pneumoniae* in gnotobiotic mice (data not shown). They found that mucoid *K. pneumoniae* were outcompeted by naturally arising variants with reduced CPS (non-mucoid variants, NMVs) throughout the majority of the gastrointestinal tract of gnotobiotic mice. The only exception was the distal small intestine, where high mucoid *K. pneumoniae* levels were observed. Therefore, the distribution of mucoid *K. pneumoniae* positively correlated with regions of high Paneth cell AMP expression. To directly test the contribution of Paneth cell α -defensins to selective pressure to maintain CPS, we determined if there were survival differences between mucoid and NMVs of *K. pneumoniae* after exposure to purified enteric α -defensins or upon infection of small intestinal organoids.

3.2 Results

CPS protects *K. pneumoniae* from killing by Paneth cell α -defensins. Based on the correlation between mucoid *K. pneumoniae* and high host cryptdin (Crp, mouse enteric α -

[‡] Adapted from Wardwell-Scott, L., Wilson, S.S., Smith, J.G., and Garrett, W.S. The *Klebsiella pneumoniae* Capsular Polysaccharide Coat Affords Survival in a Diverse Microbial Community and Protection from Host Antimicrobial Peptides in the Gastrointestinal Tract.

defensin) expression, we hypothesized that CPS provides *K. pneumoniae* with an advantage in the presence of Paneth cell α -defensins. We tested the sensitivity of mucoid and NMV *K. pneumoniae* to α -defensins. Although we observed killing of both mucoid and NMV strains with all α -defensins tested, there was increased killing activity against NMVs compared to mucoid *K. pneumoniae*. Crp23 did not significantly inhibit the growth of NMV 2, however all other NMV strain were inhibited 3-6-fold as compared to mucoid *K. pneumoniae* (Fig. 3.1A). HNP1 inhibited growth of NMV *K. pneumoniae* 2-3 fold as compared to mucoid strains (Fig. 3.1B) while HD5 inhibited growth of NMV *K. pneumoniae* 2-8 fold as compared to mucoid strains (Figure 3.1C).

NMV *K. pneumoniae* growth is inhibited by naturally secreted α -defensins. To address the effects of CPS on survival of *K. pneumoniae* upon exposure to naturally secreted Paneth cell α -defensins, we compared the growth of mucoid and NMV variants after microinjection into wild type and *Mmp7*^{-/-} small intestinal organoids, as in Chapter 2. A 2-4 fold growth inhibition was seen with all NMVs in the wild type organoids compared with growth in *Mmp7*^{-/-} organoids (Fig. 3.1D). In contrast, mucoid *K. pneumoniae* growth actually increased in WT organoids (Fig. 3.1D). These data support a model in which the increased sensitivity of NMVs to AMPs, specifically α -defensins, imposes selective pressure in the mouse ileum during colonization by *K. pneumoniae*.

3.3 Discussion

Understanding the biological implications of α -defensin resistance for individual gut bacterial species has proven challenging due to the complex microbial environment of the

gastrointestinal tract. Prior work suggested that resistance to AMPs, such as polymixin B and HNP1, by *K. pneumoniae* and other encapsulated bacteria may be driven by entrapment of cationic peptides in the bacterial capsule, but visualization of how this shapes *K. pneumoniae* populations *in vivo* has remained elusive^{217,218}. By employing a gnotobiotic mouse model and a small intestinal organoid system, we show that Paneth cell α -defensins shape *K. pneumoniae* populations, specifically their CPS production, and that mucoid *K. pneumoniae* is maintained in α -defensin rich regions of the gastrointestinal tract.

Not only did mucoid *K. pneumoniae* tolerate direct exposure to physiologic levels of purified α -defensins better than NMVs, but mucoid *K. pneumoniae* also displayed considerably enhanced growth in the presence of naturally secreted Paneth cell α -defensins in a small intestinal organoid system. Therefore, this study provides direct evidence for α -defensins in the maintenance CPS *K. pneumoniae* during colonization of the gastrointestinal tract. This may inform future studies of transmission and pathogenicity.

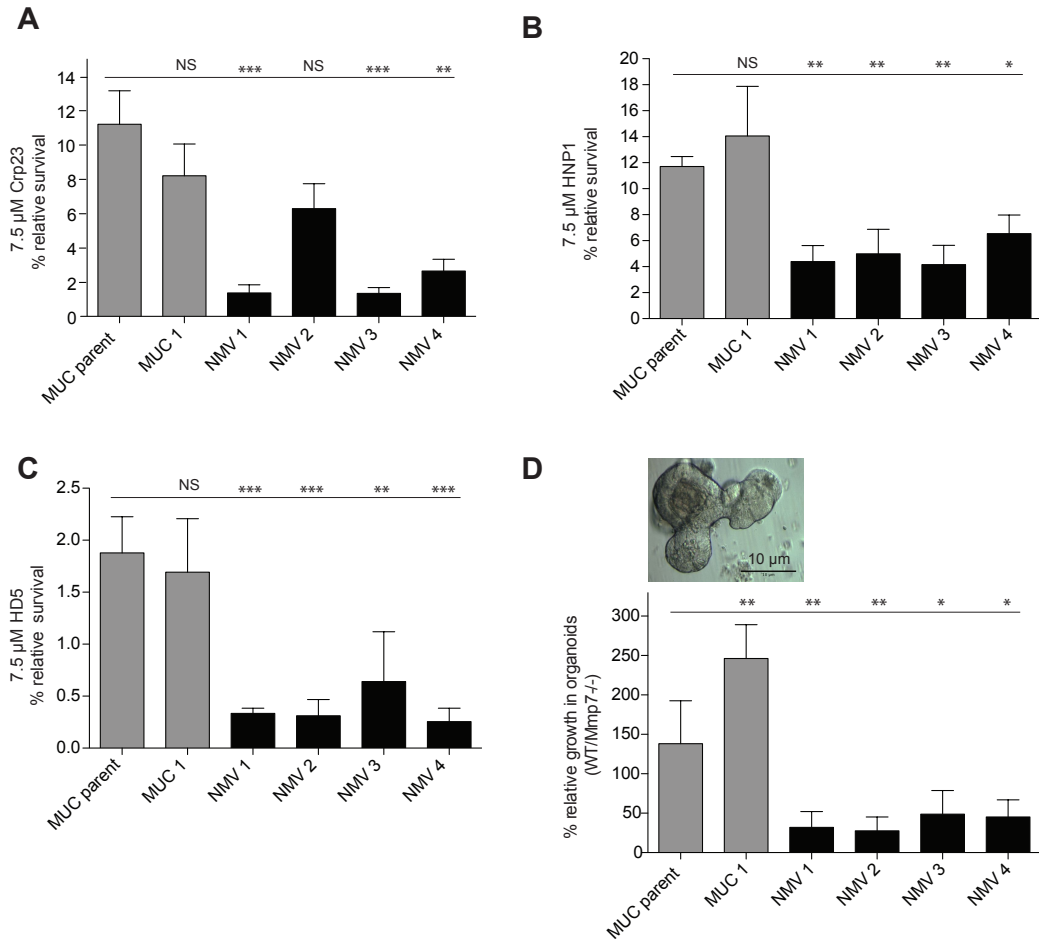


Figure 3.1. CPS protects *K. pneumoniae* against killing by α -defensins. Relative survival of mucoid (MUC) and NMV *K. pneumoniae* in the presence of murine Crp23 (A), human HNP1 (B), and human HD5 (C). Relative survival compared to untreated samples. (D) Image of a small intestinal organoid in culture (top). Relative survival of CPS variants in organoids with functional Paneth cell cryptdin production (WT) compared with organoids defective in cryptdin production (*Mmp7*^{-/-}). ***p < 0.001, **p < 0.01, *p < 0.05, one-way ANOVA with Dunnett's posttest.

Chapter 4: Enteric virus infection is enhanced in a small intestinal organoid system and *in vivo*

4.1 Background

In previous studies of defensin-virus interactions, we have shown that while some species of HAdVs are inhibited by α -defensins, there are other species where infection is enhanced by α -defensins⁸¹. We hypothesized that resistance to α -defensin neutralization may be a feature of viruses that frequently encounter α -defensins in the course of natural infection and therefore have evolved to resist neutralization by these ancient peptides. However, as humans produce both enteric and neutrophil α -defensins, determining the levels of α -defensins that viruses are exposed to during infection is complicated. As the mouse does not have neutrophil α -defensins and only expresses enteric α -defensins, identifying the natural pathogens of mice that would normally encounter α -defensins is more straightforward. Additionally, HAdVs do not replicate in mouse cells, which impacts our ability to test whether enhancement occurs *in vivo*. Therefore, we utilized two natural pathogens of mice, mouse adenovirus-1 (MAdV-1), which is tropic for endothelial cells and causes neurological symptoms in infected mice, and MAdV-2, which causes no overt disease and is naturally tropic for the small intestine²¹⁹.

Using purified mouse and human enteric α -defensins, we show that mouse adenoviruses recapitulate the dichotomy seen with human adenoviruses, with MAdV-1 being sensitive to defensin-neutralization and MAdV-2 being resistant or enhanced by these same peptides. This enhanced entry occurred in a receptor-independent manner. Using our previously established small intestinal organoid model⁴⁰, more virus was produced when MAdV-2 was microinjected into wild type versus *Mmp7*^{-/-} organoids, demonstrating that naturally secreted α -defensins are capable of enhancing viral infection. Increased shedding was observed in wild type mice

compared to *Mmp7*^{-/-} mice after oral infection with MAdV-2 in two separate experiments, indicating that small intestinal organoids are predictive of *in vivo* effects. Therefore, α -defensins enhance enteric viral infection in cell culture, in small intestinal organoids, and *in vivo*.

4.2 Results

Mouse adenoviruses recapitulate the dichotomy seen with human adenoviruses. We tested the activity of the mouse and human enteric α -defensins against MAdV-1 and MAdV-2. HD5 inhibited MAdV-1 infection by 100-fold, while the mouse enteric α -defensins Crp3 and Crp2 were less potent, inhibiting infection approximately 6-fold at the highest concentration tested (Figure 4.1A). Crp23, which differs from Crp2 by only one amino acid, only reduced MAdV-1 infection 2-fold. In contrast, infection by MAdV-2 was enhanced or unaffected by these same α -defensins (Figure 4.1B). Incubation with Crp2, Crp3, or Crp23 led to a maximal 1.7-fold, 1.8-fold, and 2-fold increase in infection, respectively. MAdV-2 infection was also moderately enhanced by HD5 at lower doses, while a modest 2-fold reduction in infection was observed at higher doses of HD5. Interestingly, Crp4, which is potently antibacterial, had no effect on either MAdV-1 or MAdV-2, highlighting the specificity of defensin-pathogen interactions.

To visualize and quantify infection without staining, we created a viral construct that would express a fluorescent protein during infection using a BAC recombineering method to fuse green fluorescent protein (GFP) to the minor capsid protein IX²²⁰. A similar construct on the MAdV-1 background had already been created by the Spindler Lab. Comparing the growth of MAdV-2 and MAdV-2.IXeGFP in a multi-cycle infection assay, we observed that MAdV-2 and MAdV-2.IXeGFP grow with similar kinetics, but that MAdV-2.IXeGFP does not replicate

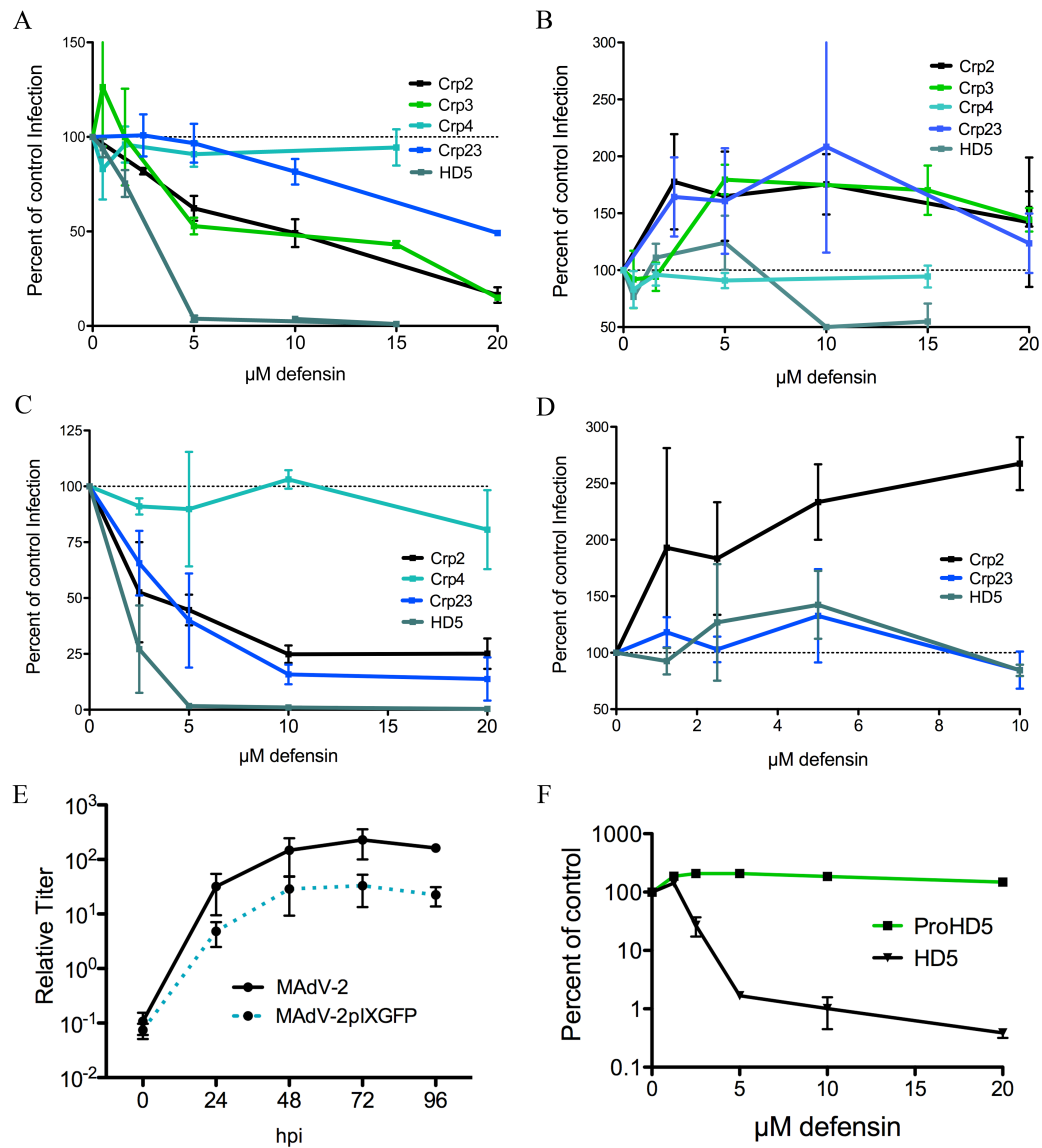


Figure 4.1. Defensins neutralize infection by MAdV-1 and enhance infection by MAdV-2. CMT93 cells were with infected with (A) MAdV-1, (B) MAdV-2, (C and F) MAdV-1.IXeGFP or (D) MAdV-2.IXeGFP that was pre-incubated with the indicated concentrations of α -defensins. Data is expressed relative to control cells infected in the absence of α -defensin (100%). Results are the means of at least three independent experiments \pm SD. (E) CMT93 cells were infected in parallel with MAdV-2 or MAdV-2.IXeGFP. Beginning at 0hrs, cells and supernatant were collected from one well daily for 5 days and viral progeny tittered on a fresh monolayer of CMT93 cells. Infection levels were quantified by staining for the major capsid protein hexon, and are presented relative to the input virus (0 hpi). Results are the means of two independent experiments \pm SD.

to the same levels as MAdV-2 (Figure 4.1E). It is possible that less infectious MAdV-2.IXeGFP virions are produced because the GFP fusion may affect capsid formation or render the capsid less stable. Nonetheless, we tested the activity of both mouse and human enteric α -defensins against the previously created MAdV-1.IXeGFP and the newly engineered MAdV-2.IXeGFP. Consistent with the results using wild type MAdV-1, we found that MAdV-1.IXeGFP was strongly neutralized by α -defensins, with HD5 inhibiting infection over 100-fold, Crp2 inhibiting infection 4-fold, and Crp23 inhibiting infection 7-fold (Figure 4.1C). Conversely; Crp4 had no effect on MAdV-1.IXeGFP, consistent with the phenotype seen with MAdV-1. MAdV-2.IXeGFP infection was enhanced by all α -defensins tested, up to 1.3-fold by Crp23, 1.4-fold by HD5 and 2.6-fold by Cpr2 (Figure 4.1D). Perhaps reflecting differences in detection sensitivity, the observed neutralization and enhancement of the GFP expressing viruses were in both cases more profound than that seen with their wild type counterparts. Therefore, as the addition of the GFP fusion did not interfere with the defensin phenotype of either virus, MAdV-1.IXeGFP and MAdV-2.IXeGFP were used in subsequent assays.

Enhancement is observed with naturally secreted α -defensins. Inhibition of viral infection by α -defensins is well studied. We have mechanistic information for the inhibition of both enveloped and non-enveloped viral species by α -defensins, including for HAdVs²²¹. Enhancement of viral infection has been less reported and is less studied^{81,98}. Therefore, it was possible that enhancement was an artifact of using purified proteins and would not be observed with naturally secreted α -defensins. To address this issue, we took advantage of the small intestinal organoid infection model presented in Chapter 2⁴⁰. In small intestinal organoids, Paneth cell contents, including α -defensins and lysozyme, are constantly released and

accumulate in the organoid lumen²¹². Data from Chapter 2 shows that α -defensins produced by small intestinal organoids are active and potently antibacterial, and that this activity is lost in organoids from *Mmp7*^{-/-} mice that lack mature α -defensins.

To test whether naturally secreted α -defensins impact MAdV-1 or MAdV-2 infection in the organoid model, we first needed to confirm that the pro-defensins expressed in *Mmp7*^{-/-} organoids had no antiviral activity. Using MAdV-1.IXeGFP, we show that pro-HD5 has no effect on MAdV-1 infection (Figure 4.1F). The results indicate that the antiviral activity against MAdV-1 is only seen with mature α -defensins, and therefore, our organoid model could be applied to studies of mouse adenovirus.

We next asked if the viruses of interest could infect and replicate in the organoids. Whole wild type organoids were dissociated with a needle and syringe and infected with MAdV-1.IXeGFP or MAdV-2.IXeGFP. The mixture was then re-embedded in Matrigel and cultured over 8 days. Virus released into the supernatant was titered on CMT93 cells as a means to quantify viral replication. MAdV-1.IXeGFP appeared to persist in culture over 8 days (Figure 4.2). Conversely, we detected a 3-log increase in levels of MAdV-2.IXeGFP, and replication appeared to plateau around day 3 post-infection. This outcome was somewhat unanticipated; although MAdV-1 is mainly known to infect macrophages and endothelial cells, it does replicate in CMT93 cells, which are a murine colorectal cancer cell line. As only MAdV-2 was able to replicate in organoids, the impact of naturally secreted α -defensins on viral enhancement was exclusively studied from this point.

To determine if naturally secreted α -defensins impact infection, wild type or *Mmp7*^{-/-} organoids were infected with MAdV-2.IXeGFP via three distinct routes and infection levels

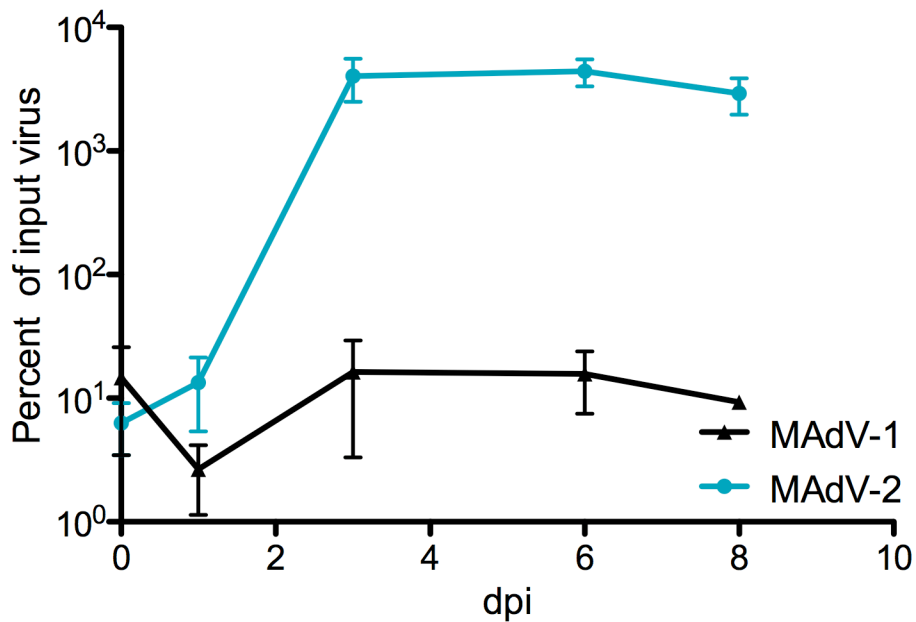


Figure 4.2 MAdV-2 replicates in small intestinal organoids. Wild type small intestinal organoids were dissociated with a needle and syringe and incubated at 4°C with $1-2 \times 10^5$ infectious units of MAdV-1.IXeGFP or MAdV-2.IXeGFP. Infected organoids were resuspended in Matrigel and plated in parallel wells of a 24-well plate. Beginning at 0 hpi, cells and supernatant were collected from one well at the indicated times post-infection and viral progeny titered on a fresh monolayer of CMT93 cells. Data is expressed relative to input virus (100%) and results are the means of at least three independent experiments \pm SD.

were measured daily for 5 days by titering output virus on CMT93 cells (Figure 4.3A). To model natural apical infection and to expose the virus to the highest concentrations of α -defensin possible, we utilized microinjection to directly access the organoid lumen (Figure 4.3A). To further maximize defensin concentration, organoids were treated with the cholinergic agent CCh, which induces Paneth cell degranulation, for 30 min prior to injection. After microinjection, 3 times more virus was produced in wild type organoids than in *Mmp7^{-/-}* organoids on day 2, which increased to 5 times more virus on day 3 (Figure 4.3A). When organoids were infected basolaterally (avoiding the accumulated α -defensins) or infected after organoid disruption (which disperses the α -defensins) there was no difference in infection levels between the genotypes. We also analyzed the total number of infected cells after microinjection in the two genotypes of organoids by FACS and found that more cells were infected in wild type organoids than in *Mmp7^{-/-}* organoids (Figure 4.3B). This difference reached statistical significance at 72 hpi, when we also saw the biggest difference in output virus. Taken together, these results support the conclusion that naturally secreted α -defensins increase MAdV-2 infection.

α -defensins increase both the number of cells initially infected and the spread of virus throughout the well. The increased virus production seen after infection of wild type organoids could be explained by an increase in initial infection or could be due to α -defensins impacting subsequent rounds of infection. We first used the MAdV-2.IXeGFP construct to look at the total number of infected cells in individual wild type and *Mmp7^{-/-}* organoids after microinjection. Ten organoids per well were microinjected with MAdV-2.IXeGFP mixed with FITC-dextran in order to mark microinjected organoids. At 24 hpi infected cells per organoid were enumerated from live cultures using a fluorescence microscope outfitted with filters for the visualization of both

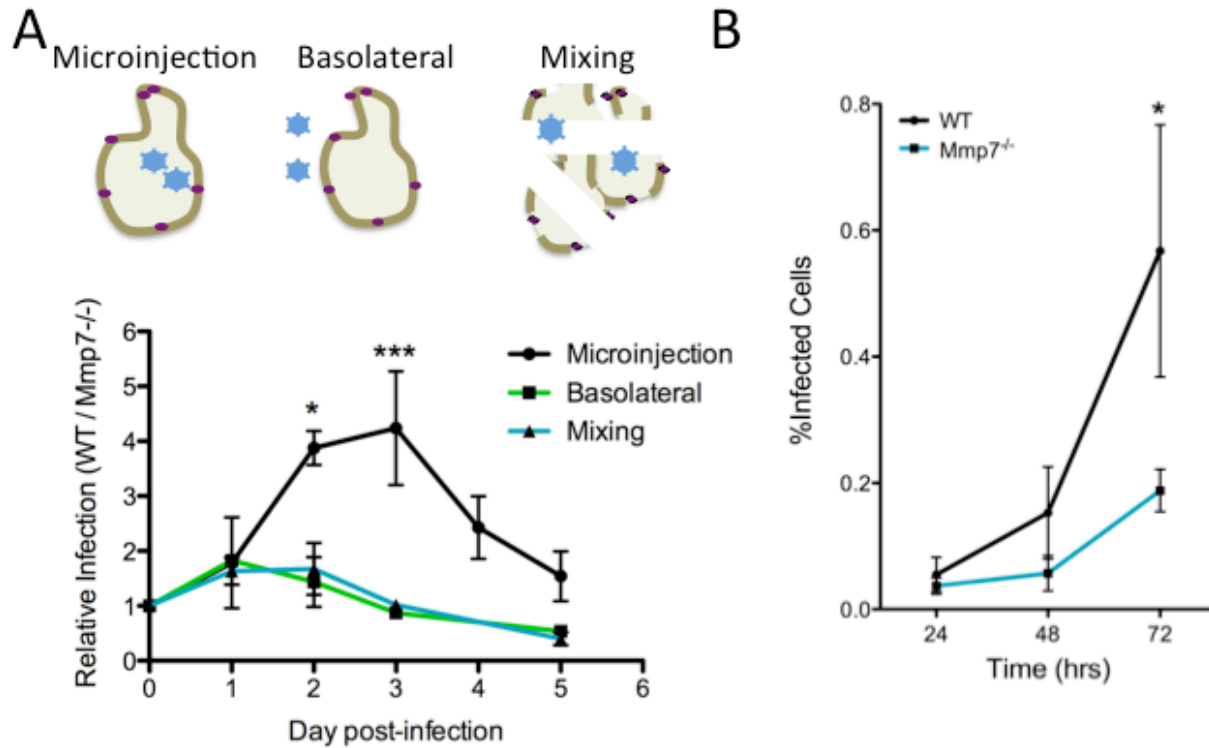


Figure 4.3. MAdV-2 infection is enhanced in microinjected, wild type small intestinal organoids. (A) Wild type and *Mmp7*^{-/-} organoids were infected with MAdV-2 via microinjection, basolateral infection, or mixing. Infected cells and supernatant were collected daily and viral progeny titered on CMT93 cells. Data is the relative infection of wild type organoids compared to *Mmp7*^{-/-} organoids from 3 independent experiments. (B) Ten wild type and ten *Mmp7*^{-/-} organoids were infected with MAdV-2.IXeGFP via microinjection. At the designated time points, organoids were dissociated into single cells, and GFP positive cells were quantified by FACS. Results are the values from three independent experiments \pm SD. * $p < 0.05$, *** $p < 0.01$, analyzed by one-way ANOVA with Tukey post-tests.

GFP and FITC-dextran. Analysis was restricted to organoids that were still stained with FITC-dextran to ensure that included organoids were infected by microinjection. Overall, approximately twice as many infected cells were observed in wild type organoids compared to the *Mmp7*^{-/-} organoids (Figure 4.4A). Since 24 hours is essentially a single round of MAdV-2 infection, this data suggests that the mechanism of enhancement by α -defensins in the wild type organoids is due to an initial increase in infected cells.

In these cultures, deletion of MMP7 is not limited to Paneth cells. To formally rule out a role for MMP7 other than activation of α -defensins in the observed enhancement, we enumerated infected cells in colonic organoids. As previously discussed, Paneth cell expression is limited to the small intestine. Therefore, colonic organoids allow for the impact of MMP7 expression on enhancement to be addressed in the absence of α -defensins. The lack of α -defensin expression in colonic organoids was confirmed by qPCR, which showed abundant α -defensin transcripts in small intestinal but not colonic organoids (Figure 4.4B). After microinjection, no difference in initially infected cells was seen between wild type and *Mmp7*^{-/-} colonic organoids (Figure 4.4A). Moreover, the number of infected cells in colonic organoids and *Mmp7*^{-/-} small intestinal organoids was similar; indicating that the increase in infected cells observed in wild type small intestinal organoids was due exclusively to α -defensins.

We next looked at whether the observed initial increase in infected cells lead to increased spread of MAdV-2.IXGFP from organoid to organoid within a well. Ten small intestinal or colonic organoids of each genotype were microinjected with MAdV-2.IXeGFP and visualized live using a fluorescence microscope. The number of GFP- positive organoids was quantified via fluorescence microscopy every 24 hours for 4 days. Total GFP-positive wild type small intestinal organoids increased on average 3.4-fold over 96 hours (Figure 4.4C). In agreement with previous

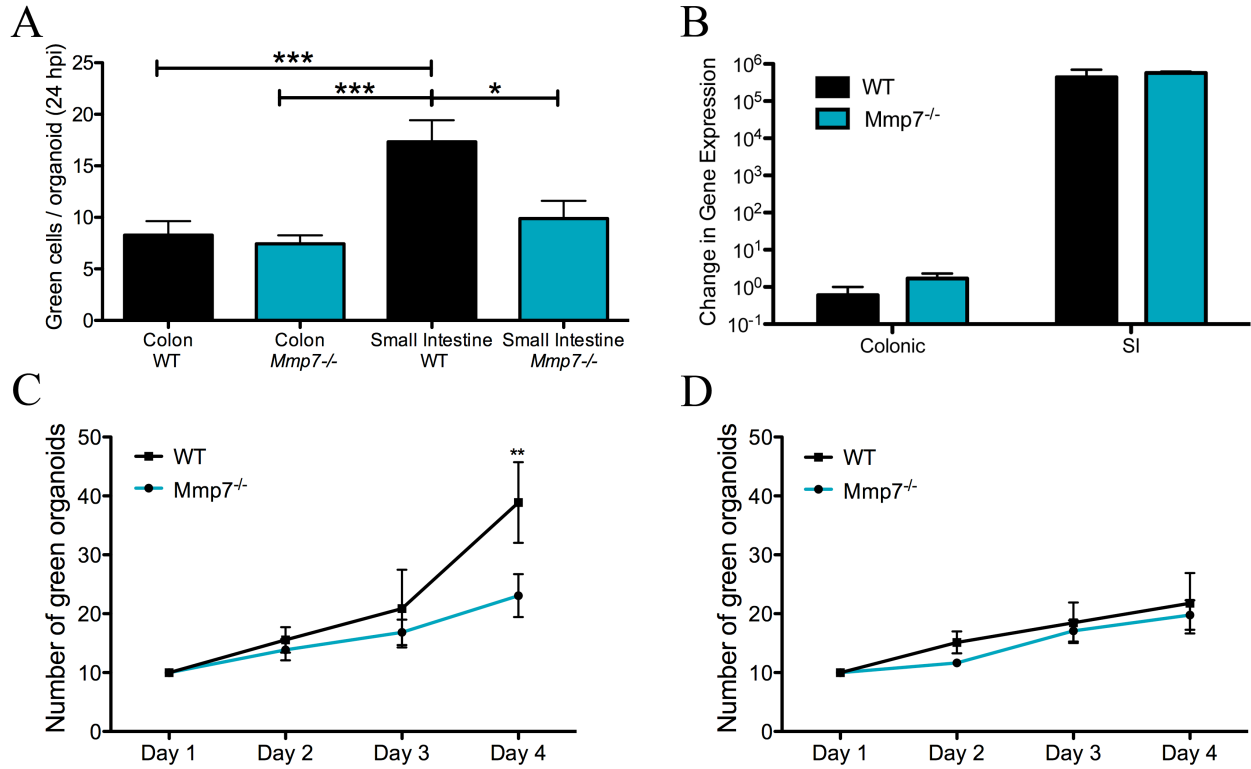


Figure 4.4. An increase in initially infected cells and an increase in viral spread are both observed in wild type organoids. (A) Total GFP-positive cells in WT and *Mmp7*^{-/-} small intestinal and colonic organoids at 24 hpi. Data is the average number of GFP positive cells from ten microinjected organoids from at least 9 independent experiments \pm SEM. (B) qPCR for MMP7 and Crp4 in organoids 4 days after passage. Data are the average of two replicate wells of organoids and is relative to expression immediately after passaging. Total GFP-positive WT and *Mmp7*^{-/-} (C) small intestinal and (D) colonic organoids per well quantified daily for 4 days. Shown is the average number of GFP positive organoids from three individual wells with ten microinjected organoids each. Data is the average from 3 independent experiments \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, from repeated measures one-way ANOVA with Tukey post-tests.

data, the total number of GFP-positive *Mmp7*^{-/-} small intestinal organoids, or colonic organoids of either genotype only increased on average 1.3-fold in the same timeframe (Figure 4.4D). Therefore, α -defensins increase the number of initially infected cells, and this leads to an increase in organoid to organoid spread.

Enteric α -defensins allow viruses to enter cells in a receptor independent manner. Previous studies have demonstrated that HAdV entry is blocked when whole virus competes for binding to cell surface receptors with a molar excess of recombinant fiber knob, which is a domain of the viral attachment protein⁸¹. We took advantage of this fact in order to address the mechanism of enhancement that we observed in our assays. CMT93 cell monolayers were incubated with a molar excess of recombinant MAdV-2 fiber knob. The presence of MAdV-2 fiber knob specifically blocked infection by MAdV-2 up to a 8.3-fold in a dose dependent manner while having no effect on infection by MAdV-1 (Figure 4.5A). A MAdV-2 fiber knob concentration of 1.5 μ M was chosen for subsequent assays, as it was the minimum concentration with maximal inhibitory activity.

To determine if α -defensins allow for receptor-independent MAdV-2 entry, MAdV-2 was pre-incubated with an enhancing α -defensin before being added to cells pretreated with MAdV-2 fiber knob. When MAdV-2 was pre-incubated with 5 μ M Crp2, a 2-fold increase in infection was observed, independent of the presence of MAdV-2 fiber knob (Figure 4.5B). In contrast, incubation of MAdV-2 with MAdV-2 fiber knob alone resulted in a 6.6-fold reduction in infection. This effect was specific to the MAdV-2 fiber knob, as fiber knob from HAdV-16 had no effect on infection by MAdV-2. No treatment tested had any impact on MAdV-1 infection, indicating that MAdV-2 fiber knob was specifically blocking MAdV-2 receptor-dependent entry.

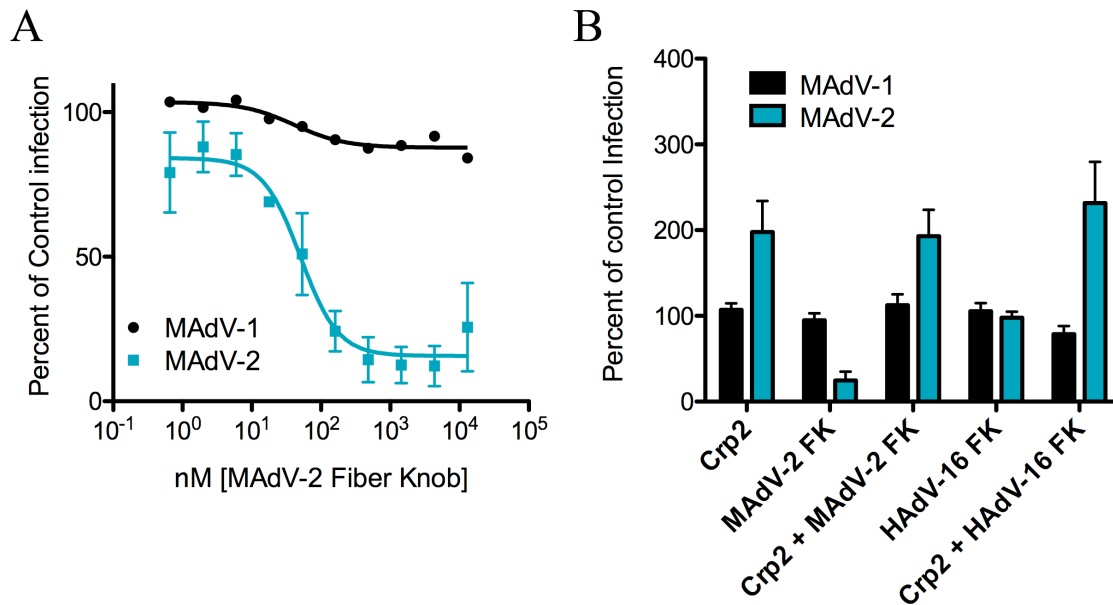


Figure 4.5. α -defensins allow MAdV-2 entry in a receptor-independent manner. (A) MAdV-1.IXeGFP or MAdV-2.IXeGFP was added CMT93 cells pretreated with MAdV-2 fiber knob, and infection was quantified 48 hpi. (B) MAdV-1 and MAdV-2 were pre-incubated with 5 μ M Crp2 or left untreated, and then added to CMT93 cells that had been pre-treated with 1.5 μ M MAdV-2 fiber knob (FK), 16 nM HAAdV-16 FK, or left untreated. Infection was quantified 48 hpi. Data is expressed relative to control cells infected in the absence of α -defensin or FK (100%). Results are the means of at least three independent experiments \pm SD except for MAdV-1 in panel A, which is one replicate.

Crp2 did not inhibit MAdV-1 infection due to the low concentration used for these experiments. Taken together, these results indicate that the presence of α -defensins allows MAdV-2 to enter cells in a receptor independent fashion.

α -defensin activation *in vivo* increases MAdV-2 shedding. To investigate viral enhancement *in vivo*, wild type and *Mmp7*^{-/-} mice were infected orally with 1x10⁷ iu/mouse of MAdV-2. Infection was monitored by quantifying viral genomes shed in fecal pellets at 6, 20, and 28 hpi. Wild type mice shed significantly more virus than *Mmp7*^{-/-} mice over the course of the experiment (Figure 4.6A and 4.6C). To see whether this increase in shedding was sustained over a longer time course, wild type and *Mmp7*^{-/-} mice were infected orally with 1x10⁶ iu/mouse of MAdV-2. Fecal pellets were collected at 2, 4, and 7 dpi, and viral genomes were quantified. Over one week, wild type mice shed more virus than *Mmp7*^{-/-} mice (Figure 4.6B and 4.6D). Therefore, α -defensin-mediated enhancement of MAdV-2 infection results in increased viral shedding *in vivo*.

4.3 Discussion

Through our studies we have shown that MAdV-2 infection is enhanced by purified α -defensins *in vitro*, by naturally secreted α -defensins in small intestinal organoids and *in vivo*. Numerous recent papers have implicated the microbiota in promoting viral infection²²²⁻²²⁵. Kuss et al demonstrated that antibiotic pre-treatment reduced viral replication and mortality in poliovirus infected mice²²². Reovirus pathogenesis was also reduced in antibiotic treated mice from this study, indicating that the effect applied to multiple viral families. A subsequent paper showed that enhanced poliovirus infection was not dependent on live bacteria, but rather on binding of the virus to bacterial surface polysaccharides, which leads to capsid stabilization and an increase in infection²²³. A separate group demonstrated that

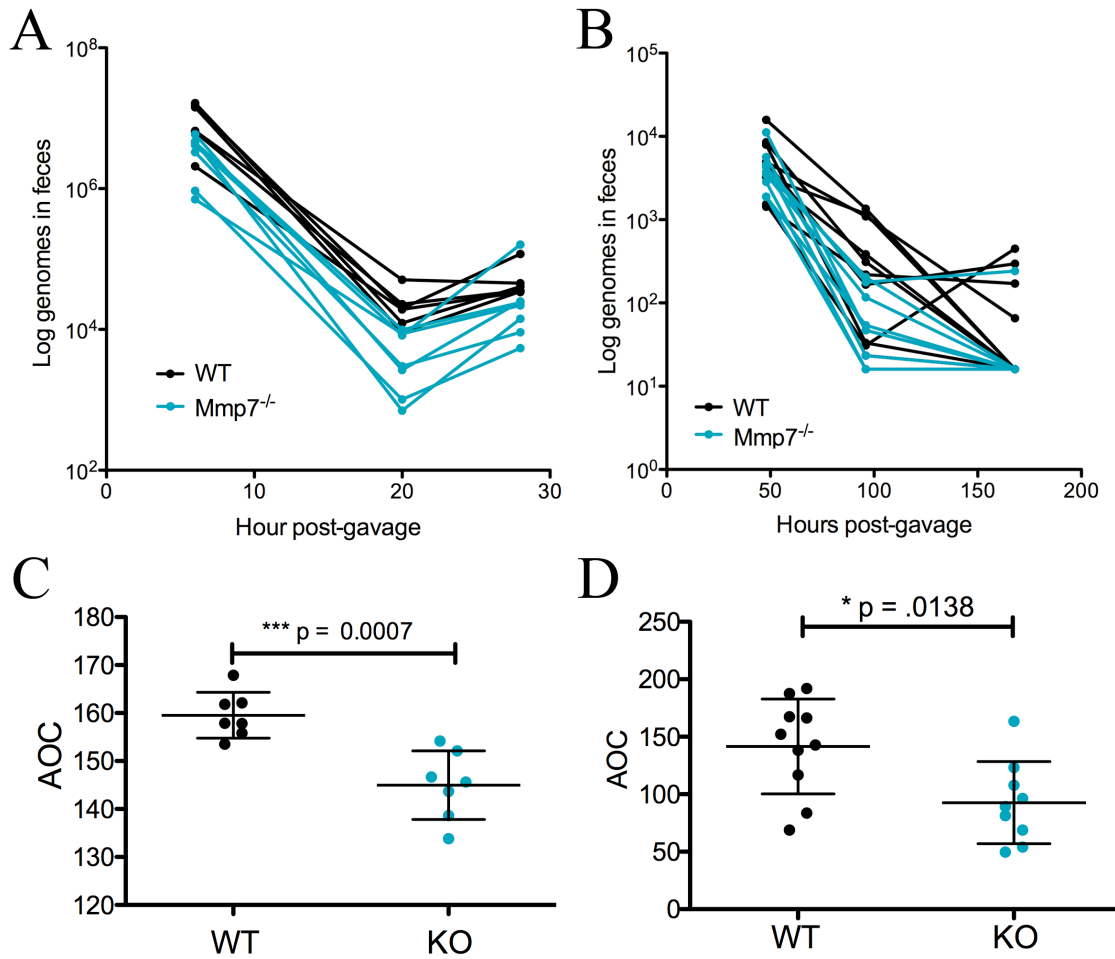


Figure 4.6. Fecal shedding of MAdV-2 is increased by enteric α -defensin activation. Data are viral genomes/fecal pellet at the indicated time points post infection for each wild type or *Mmp7*^{-/-} mouse after oral infection with (A and C) 1×10^7 iu/mouse or (B and D) 1×10^6 iu/mouse of MAdV-2. Total virus shed per mouse was calculated by determining the area under the curve from each mouse after oral infection with MAdV-2. N=7-8 mice per group. P values are noted from an unpaired t-test.

infection by the vertically transmitted Mouse Mammary Tumor Virus (MMTV) is impacted by the presence of LPS²²⁵. During natural infection, LPS bound to MMTV particles activates TLR4 and stimulates production of the anti-inflammatory cytokine IL-10. This signaling cascade is necessary for the induction of host tolerance and potentially allows MMTV to avoid triggering an antiviral response during infection. Transmission of MMTV was not seen in germ-free or antibiotic treated mice. More recently, antibiotic treatment has been shown to prevent acute mouse norovirus infection and clear persistent mouse norovirus infection *in vivo*²²⁴. This is consistent with previous results showing that murine norovirus can directly bind to bacterial glycans²²⁶. Taken together, these studies support a critical role for the gut microbiota in facilitating enteric viral infection.

Our results support a new and distinct host-dependent mechanism that augments enteric viral infection. We observed that infection by an enteric mouse pathogen was enhanced by α -defensin peptides that are typically potent antivirals in cell culture. The fact that these α -defensins are highly expressed where natural viral infection occurs implies that MAdV-2 may have evolved to hijack a host-derived peptide normally associated with inhibiting viral infection and utilize it to increase its own infection. There are a few other examples of α -defensins enhancing viral infection: the promotion of HIV attachment by HD5 and HD6 and the enhancement of some HAdVs by HNP1 and HD5, as mentioned in Chapter 1^{81,82}. Defensin-mediated enhancement of HIV is particularly intriguing, as unlike the viruses mentioned previously, LPS binding to HIV gp120 decreases virus binding to target cells²²⁷. Therefore, it is possible that the role of α -defensins in augmenting viral infection may complement or counteract the impact of bound bacterially derived products.

Our results using organoids indicate that α -defensins are capable of enhancing viral

infection. Although our data indicates that α -defensins allow receptor-independent entry of MAdV-2, the precise mechanism of this enhancement was not determined. Potential alternative routes of entry mediated by α -defensins are summarized in Figure 5.1. As α -defensins are highly cationic, enhancement may simply be due to charge neutralization allowing more virus to bind to the cell in the presence of α -defensins, similar to the effects of polybrene or other cationic compounds²²⁸. Indeed, we and others have documented increased cell binding due to defensins even for viruses that are subsequently neutralized^{81,96,123}. However, increased cell binding is unlikely to be the sole mechanism of enhancement, as we have observed enhancement of HAdVs and MAdV-2 even when the virus is pre-bound to cells in the cold, unbound virus is washed away, and then defensin is added and infection allowed to proceed. This protocol eliminates any impact of defensins on the initial amount of virus bound to cells, and strongly indicates that observed enhancement of infection is not simply due to increased cell binding.

There is evidence that some α -defensins can permeabilize eukaryotic membranes, leading to increased infection⁹¹. To enhance MAdV-2 infection, defensins could be forming pores at the cell surface and allowing more virus to enter the cell. More intriguingly, enhancement could be due to α -defensins that enter the cell bound to MAdV-2 and subsequently permeabilize the endosomal membrane, resulting in more virus reaching the nucleus. Binding studies using HAdV have shown that there is a 3-30-fold reduction in defensin binding to enhanced HAdV serotypes compared to binding to the neutralized serotype HAdV-5⁸¹. However, these results from a biochemical assay could translate into defensins being able to enter the endosomal pathway bound to both neutralized and non-neutralized AdVs. At this point, α -defensins would remain bound to and prevent uncoating of neutralized AdVs, while α -defensins that enter the cell bound to enhanced AdVs would now interact with the endosomal membrane and potentially disrupt it.

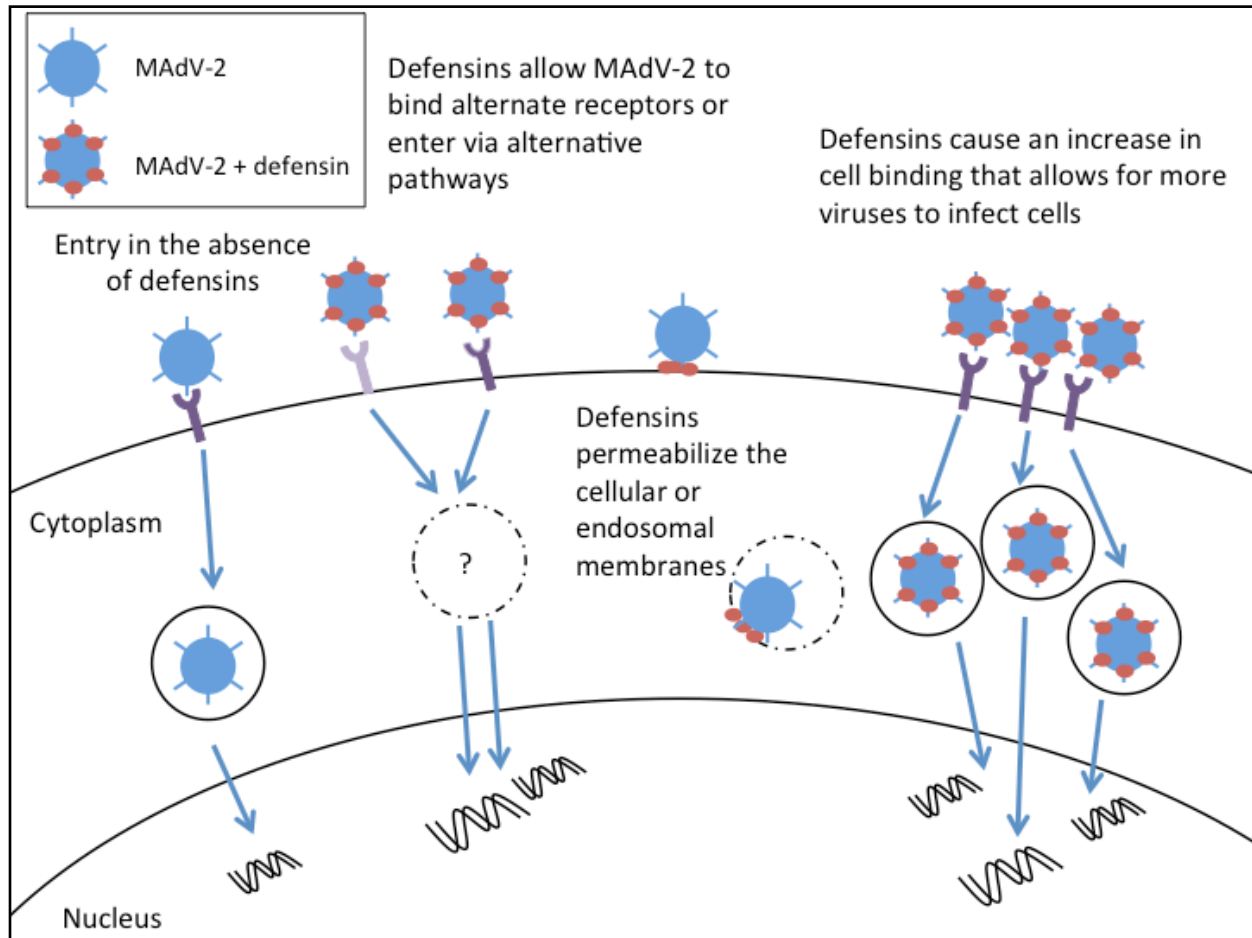


Figure 4.7 Potential mechanisms for the α -defensin-mediated enhancement of MAdV-2.

Based on previous experiments and our own data, there are three major ways that α -defensins could increase infection by MAdV-2: (1) Defensins could alter MAdV-2 binding to the cell (dark purple receptor is the putative receptor, light purple receptor is alternate receptor interaction mediated by α -defensins), (2) increase MAdV-2 binding to the cell or (3) permeabilize cellular membranes. These mechanisms are not mutually exclusive.

This would functionally increase the amount of virus reaching the nucleus and lead to enhanced infection. Although α -defensins could interact with endosomal constituents while still bound to MAdV-2, it is also possible that enhancement results from α -defensins bound to MAdV-2 in a low affinity interaction subsequently entering higher affinity interactions with endosomal membrane components. Binding affinity studies using recombinant penton from HAdV-5 and HD5 have been performed²²⁹. Similar studies using the penton from an enhanced serotype need to be performed to address this possibility.

It is possible that α -defensins bound to MAdV-2 facilitate an interaction with an alternate cell surface receptor. Testing for the utilization of an alternate cell surface receptor with MAdV-2 is currently challenging, as the receptor utilized by MAdV-2 is unknown. This question can not easily be addressed by instead performing experiments with enteric HAdVs, as the receptors utilized by these viruses are also not completely known^{230,231}. Therefore, the unknown and likely novel receptors utilized by enteric AdVs would have to be determined before these experiments could be performed. An alternative would be to investigate binding to alternative receptors using the enhanced HAdV-19, as this serotype is well characterized. However, this is not an enteric virus, therefore the relevance of finding to the mechanism of enteric virus enhancement would be unclear. However, pursuing this line of investigation should remain a priority, as if defensin-bound viruses are able to utilize alternate receptors to enter cells this would have implications for our understanding of enteric virus tropism. It may even suggest that it is the distribution of α -defensins in the gastrointestinal tract itself that dictates the location of enteric virus infections. This idea is further bolstered by the fact that neutrophil and enteric α -defensins are hypothesized to interact with chemokine receptors that have not yet been identified^{15,183-185}. Identification of a

cell surface receptor for enteric α -defensins would also inform this question of alternate receptor usage.

Rather than alter receptor usage α -defensin binding may instead alter intracellular trafficking of enhanced viruses. To test this, one could infect cells simultaneously with a neutralized and an enhanced virus, for example MAdV-1 and MAdV-2. If the viruses utilize the same entry pathway, they should colocalize with both each other and with shared intracellular markers. However, if the viruses fail to colocalize both with each other and with the same intracellular markers, then this implies enhancement could be due to the viruses entering a different and more productive entry pathway.

Chapter 5. Significance and future directions

5.1 Enhancement of enteric viruses by α -defensins

Preliminary data with other viruses

The data in Chapter 4 only demonstrates the enhancement of one virus by α -defensins. Therefore, a key unanswered question is whether enhancement by α -defensins extends to other viruses. In preliminary studies we have shown that purified α -defensins enhance the infection of multiple other enteric viruses. HD5, Crp2 and Crp23 enhance infection by two strains of mouse rotavirus, EMcN and EDIM (data not shown). As was observed for AdV infection, enhancement of mouse rotavirus occurred both after pre-incubated of the virus with α -defensins and when the virus was pre-bound to the cell and α -defensin subsequently added. Infection of the mouse norovirus strain MNV-1 is also enhanced by HD5, although 2-fold inhibition by Crp2 was seen at the highest doses (data not shown). In contrast, when we tested the activity of HD5 against a second non-enteric mouse virus, mouse papillomavirus, infection was inhibited over 100-fold (data not shown). Therefore, of the six mouse viruses tested to date, the enteric viruses MAdV-2, EDIM, EMcN, and MNV-1, representing three distinct viral families are enhanced by enteric α -defensins. In contrast, non-enteric viruses MAdV-1 and mouse papillomavirus are neutralized. Given the very restricted distribution of α -defensin expression in the mouse, the fact that all enteric viruses follow this paradigm is intriguing. Although it remains to be seen if mouse rotavirus and mouse norovirus infection is enhanced in organoids or *in vivo*, preliminary data suggests that enhancement may be a common feature of enteric mouse viruses.

The experiments in chapter 4 and mentioned above were all performed with mouse viruses. Therefore whether or not enteric α -defensins enhance human enteric viruses infection is unclear. Preliminary data has demonstrated enhancement of the human rotaviruses WA, HAL

and DS-1 by HD5. We have also previously published that one of the two naturally enteric HAdVs is enhanced in cell culture by HD5⁸¹. As tools to genetically manipulate human organoids have been described, the creation of *HD5*^{-/-} human organoids would present a unique opportunity to address whether human enteric viruses are enhanced by naturally secreted α -defensins, analogous to the observed enhancement of MAdV-2 in mouse small intestinal organoids^{232,233}. Enhancement of enteric viruses by enteric α -defensins potentially represents a novel paradigm in host-pathogen interactions. Further investigation is warranted to determine if enhancement of diverse viruses species by purified α -defensins is recapitulated in small intestinal organoids or *in vivo*.

5.2 The future of intestinal organoids in microbiological research

Using intestinal organoids to reproduce previous results

Intestinal organoids are becoming a widely utilized tool in both microbiological and biomedical research. However, a number of questions about the relevance of research conducted in intestinal organoids remain unknown or incompletely explored. Multiple groups have tried to address the extent to which organoids recapitulate observations made using both traditional cell culture and *in vivo* models. Using mouse organoids derived from the jejunum and ileum, Zhang and colleagues show that STM infection of organoids results in bacterial invasion, disruption of tight junctions²³⁴, induction of NF- κ B mediated inflammatory responses, and a reduction in stem cell numbers, all of which have been previously demonstrated²³⁴⁻²³⁷. Using human intestinal organoids it was confirmed that microinjection of a wild type *Clostridium difficile*, but not a nontoxigenic strain, results in organoid disruption, consistent with results previously obtained from cell lines and hamster infection models^{238,239}. *Helicobacter pylori* infection of human gastric organoids has similarly confirmed that specific cell types are more susceptible to

colonization and that colonization results in NK-kB and IL-8 production²⁴⁰⁻²⁴². Finally, mouse intestinal organoids have been used to show that changes in gene expression induced upon exposure to the microbiota constituents *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, are in line with those from other mouse studies, which underscores the usefulness of the organoid model²⁴³. While showing that organoids recapitulate some aspects of previous cell culture and *in vivo* results is no doubt important, if this is the extent of the inquiry, then experiments in organoids represent no real improvement over traditional cell culture techniques. In addition, these confirmatory studies might actually mask the true utility of organoids by ignoring results that are different between organoids and cell lines. Therefore, future studies seeking to confirm phenotypes should be cognizant of these concerns, and highlight the discrepancies they uncover as well as the similarities.

Growth of previously unculturable organisms

Intestinal organoids represent the first opportunity for long-term culture of many intestinal epithelial cell types, there is the potential that organoids will similarly allow for the growth of previously unculturable organisms. This is especially true for many human enteric viruses, which grow either very poorly in culture (HAdVs, rotavirus) or not at all (norovirus)²⁴⁴. Culturing of human clinical rotavirus samples can be achieved through multiple rounds of tissue culture adaptation in non-human primate primary cells, followed by growth in cell lines²⁴⁵. Finkbeiner et al have shown that human organoids support the replication of primary rotavirus isolates directly from infected feces, negating the need for any tissue culture adaptation in non-human cells²⁴⁶. Preliminary results from our lab show that enteric HAdVs grow well in human

organoids, but not in mouse organoids, indicating that this system accurately reflects *in vivo* tropism.

An important consideration for the cultivation of organisms is the kind of intestinal organoid used. Although mouse intestinal organoids are exclusively derived from intestinal tissue, there are currently two distinct ways to make human intestinal organoids^{151,152,247,248}. One method utilizes inducible pluripotent stem cells (iPSC) and the other relies on intestinal crypts isolated from biopsies or surgical specimens, but the specific growth factors needed to drive intestinal organoid formation are consistent between the protocols. Although they are not dependent on tissue availability, iPSCs are embryonic in phenotype and can only be passaged for a finite period of time²⁴⁷. These organoids also take weeks longer to generate cultures and display less tissue organization than crypt-derived organoids. Finally, iPSC organoids include mesenchymal cells, which are completely absent in crypt-derived organoids²⁴⁴. This mesenchymal cell contamination is especially relevant for work that seeks to establish culture conditions for unculturable viruses, as it may confuse results. Indeed, rotavirus replication in the iPSC organoids was detected in both epithelial and mesenchymal cells²⁴⁶. Future studies seeking to determine whether enteric pathogens can grow in organoids would be advised to utilize the exclusively epithelial crypt-based organoids before claiming epithelial cell specific growth.

The benefits of a reductionist system

Interpretation of enteric experiments *in vivo* is complicated, as input from epithelial cells, the microbiota, and the immune system must all be considered. However, organoids represent a reductionist system, composed exclusively of epithelial cells, making them an ideal platform to address questions regarding the response of epithelial cells upon encountering microbes. As

organoids can easily be made from wild type and knockout mice, they represent a powerful tool to study epithelial intrinsic signaling pathways relevant for infection. To this end, mouse colonic *IL22ra1* knockout organoids have been utilized to look at the IL22 receptor dependent signaling pathway, activation of which is critical for resistance to *Citrobacter rodentium* infection²⁴⁹. Infection of mouse gastric organoids with *H. pylori* has been shown to induce NF-κB mediated Sonic hedgehog expression, which initiates gastritis associated with infection²⁵⁰. This process was long hypothesized to happen but had proven difficult to show *in vivo*. The same group was then able to show a previously unappreciated role for CD44 in *H. pylori* mediated epithelial cell proliferation, again using mouse gastric organoids²⁵¹. These studies have all benefitted from the newfound availability of primary epithelial cells that intestinal organoids provide, and future work will only contribute to our understanding of the role of epithelial specific responses to microbes.

Organoids can also be used to address questions about the sufficiency of microbes or host-genotypes for the production of observed phenotypes. Our published work in mouse small intestinal organoids, described in Chapter 2 and Chapter 3, demonstrates that secreted α-defensins in a natural milieu are capable of restricting bacterial growth. However, this work does not exclude a role for the microbiota or the immune system in defensin-mediated phenotypes *in vivo*. Mouse small intestinal organoids have also been used to explain the observed upregulation of *fut2* in the ileum of Na⁺/H⁺-exchange isoform 3 null (*NHE3*^{-/-}) mice²⁵². Increased *fut2* transcription observed *in vivo* is not due to host genotype directly, but rather due to a specific expansion of *Bacteroides thetaiotaomicron* in the terminal ileum. After microinjection of *B. thetaiotaomicron* into wild type and *NHE3*^{-/-} ileal organoids, *fut2* was upregulated independent of genotype, showing that *B. thetaiotaomicron* is sufficient for *fut2* induction. A separate study

looked at the ability of stem cells from wild type and $NOD2^{-/-}$ mice to form organoids. Stimulation of stem cells with the NOD2 agonist MDP was sufficient to induce a protective response in these cells, which increased the number organoids that were eventually formed ²¹⁰. No increase in organoid number was observed after stimulation of $NOD2^{-/-}$ stem cells. Human iPSCs have also been used to demonstrate that *C. difficile* infection is sufficient to reduce the production of mucins and NHE3 expression, both of which have been observed in humans infected with *C. difficile* ^{251,253}. Performing similar experiments *in vivo* is not possible due to the presence of commensal microbes. Even in germ-free mice, the presence of immune cells makes interpretation of experiments complicated. Therefore, these studies highlight a major benefit of intestinal organoids, which can be used to conclusively link a phenotype to a specific microbe or host genotype.

With a reductionist system like intestinal organoids, one potential experimental direction is to add back individual components in an attempt to address specific questions. In this vein, the conditions to derive Peyer's patch microfold cells (M cells) in mouse organoids have been described ²⁵⁴. As M cells are known to be important for the infection of numerous pathogens such as STM, retroviruses, norovirus, and poliovirus, studies in organoids with and without M cells may help to fully illuminate the role of these cells in enteric infections ²⁵⁵. Our preliminary data indicates that murine bone marrow derived macrophages can be successfully co-cultured in Matrigel with small intestinal organoids (data not shown). Therefore, future studies examining the specific interactions between immune cells and organoids should be performed. Experiments looking at immune cell chemotaxis would be especially suited to the organoid system, as organoids could be microinjected with microbes or left untreated, and the number of immune cells in association with the respective organoids tracked over time. In general, the potential

experiments that can be performed in organoids using this kind of an add back approach are endless and will greatly inform our current understanding the importance of epithelial cells in the intestine.

Organoids as predictors of in vivo effects

The relevance and future of organoid infection models will be impacted by their ability to predict *in vivo* effects. As discussed in Chapter 4, this is one of the most important conclusions that can be drawn from observed enhancement of MAdV-2 infection. We clearly demonstrate that there is an increase in viral replication specific to wild type small intestinal organoids, which is not observed in colonic organoids or *Mmp7*^{-/-} organoids. Based on these results, one could predict that orally infected wild type mice would produce and shed more virus than *Mmp7*^{-/-} mice, which is precisely what we observed in multiple experiments. One other published paper also demonstrates that organoids are predictive of an *in vivo* phenotype²¹⁰. After establishing the protective effect of NOD2 stimulation on intestinal stem cells in small intestinal organoids, Nigro et al were able to demonstrate that MDP administration can also protect stem cells *in vivo* in a NOD2 dependent manner. Therefore, the data so far support the idea that mouse organoids are highly predictive of *in vivo* effects.

These studies also imply that results from human intestinal organoids would be directly applicable to the human intestine. It is well established that drugs do not impact all cells uniformly and instead that cells from different tissues can have very different responses to the same compound²⁵⁶. In addition, drug efficacy and toxicity testing in animal models is confounded by an inability to differentiate between direct and indirect effects. The use of cell lines can resolve these issues; however, by definition immortalized cells are distinct from the

primary cells from which they were derived. It has been shown that TNF α and chemotherapeutic drugs are toxic to human organoids and mouse intestines *in vivo*, but that toxicity in the colorectal cell line, Caco-2, was observed at doses 10-30 times higher²⁵⁷. This study implies that human organoids are more predictive of *in vivo* effects than cell lines. As human organoids are a renewable source of primary human cells, they represent an unprecedented opportunity to test candidate pharmaceuticals in a system that more accurately reflects humans than cells lines or animal testing. This is especially relevant as 80-90% of drugs currently fail when tested in humans due to toxicity or lack of efficacy²⁵⁸.

Future Perspectives

One of the early limitations to studies in organoids was cost, as a completely defined cell culture media using purified proteins is extremely expensive in comparison to traditional cell culture media. However, many of the necessary growth factors can now be produced as conditioned medias, driving down costs^{196,259}. In addition, for mouse small intestinal organoids, there is now a commercially available complete culture media. The benefits to performing studies in non-transformed epithelial cells are immense, and it is likely that enteric research will move to preferentially utilizing organoids over cell lines.

Chapter 6. Materials and Methods

Purified defensin and fiber knob production. Crp23 was generated from a synthesized 80% pure linear peptide (CPC Scientific) as previously described⁹⁶. HD5 was refolded as above or purchased from Hycult Biotech (HC2121). HNP1 was purchased from Abcam (ab9934). Crp2 was a gift from Wuyuan Lu (University of Maryland) and Crp 3 and Crp4 were a gift from Andre Ouellete (University of Southern California). All α -defensin concentrations were quantified by UV absorbance at 280 nm using calculated molar extinction coefficients.

MAdV-2 fiber knob was generated from a bacterial expression plasmid (pET28(+)) encoding MAdV-2 residues 517-586 with an N-terminal 6 \times His tag (a gift from Mark J van Raaij, Spanish National Center for Biotechnology). MAdV-2 fiber knob was expressed in BL21-CodonPlus (DE3)-RIPL cells (Stratagene) upon induction at an OD₆₀₀ of 0.6–0.8 using 0.4 mM IPTG for 4 hr at 37°C. Cell pellets were frozen at –80°C in lysis buffer, lysed by sonication, and purified by a TALON column (Clontech) as previously described^{260,261}. Fractions containing the highest concentrations of eluted protein were pooled and concentrated/desalted into desalting buffer (50 mM Na₂HPO₄/NaH₂PO₄, 130 mM NaCl, 10% glycerol, pH 8.0). Fiber knob oligomerization was confirmed by non-reducing gel analysis.

Bacterial strains, culture conditions and antibacterial assays. STM strains used are listed in Table 1. Mutants were generated in STM 14028s by P22 phage transduction. *K. pneumoniae* WGLW5 (mucoïd parent; BEI HM-749) was previously cultured from the stool of BALB/c *Tbet*^{-/-} *x Rag2*^{-/-} (TRUC) mice²⁶². MUC 1, NMV 1, NMV 2, NMV 3, and NMV 4 are derivatives of WGLW5 isolated from stool after a 4 wk passage of WGLW5 through a germ-free mouse. Strains were grown in LB-Miller (STM) or LB (*K. pneumoniae*) at 37 °C with aeration.

Antibiotics were added at 50 µg/mL.

For *in vitro* killing assays, overnight *K. pneumoniae* cultures were diluted 20x into fresh LB and incubated at 37°C for 1 h with shaking. Cultures were diluted to OD600 0.5 with 10 mM PIPES and 1% tryptic soy broth (PIPES-TSB). Bacteria were washed 2x and resuspended in 1 mL PIPES-TSB. After a 1:10 dilution in PIPES-TSB, 25 µl bacteria were mixed with 7.5 µM HNP1, HD5, or Crp23. Samples were incubated at 37°C for 2 h with shaking before serial dilution onto MacConkey agar. Percentage of relative survival was determined by comparison to untreated bacterial samples.

Table 1. Strains and plasmids used

Strain or plasmid name	Genotype or Plasmid Backbone vector	Source
BC155	<i>S. typhimurium</i> LT2	Brad Cookson (University of Washington, Seattle)
BC132	<i>S. typhimurium</i> LT2 <i>phoP</i> ::Tn10Cam	Brad Cookson (University of Washington, Seattle)
BC156	<i>S. typhimurium</i> 14028s	Brad Cookson (University of Washington, Seattle)
BC1563	<i>S. typhimurium</i> 14028s <i>fljBA</i> ::FRT <i>sipB</i> ::FKF KanR	²⁶³
SSW1	BC156 <i>sipB</i> ::FKF KanR	(Wilson citation)
BC162	BC156 <i>phoP</i> ::Tn10Cam	Brad Cookson (University of Washington, Seattle)
SSW3	BC162 <i>sipB</i> ::FKF KanR	⁴⁰
pDW5	<i>pPtetA</i> ::GFP	²⁶⁴
SSW4	BC132 pDW5	⁴⁰

Viruses and cell culture conditions. Wild type MAdV-1 was originally obtained from Steven Larsen (Indiana University Medical Center) and MAdV-1.IXeGFP was a kind gift from Katherine Spindler (University of Michigan). Wild type MAdV-2 was a gift from Susan Compton (Yale University School of Medicine). The replication-competent MAdV-2 construct with a GFP reporter fused to the minor capsid protein IX (MAdV-2.IXeGFP) was produced

using recombineering with a bacterial artificial chromosome containing the genome of MAdV-2 (pKSB2 MAdV-2, a kind gift from Silvio Hemme)²²⁰.

All mouse adenoviruses were propagated in CMT93 cells (a kind gift from Susan Compton, Yale University School of Medicine). CMT93 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich), 4 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM nonessential amino acids (complete DMEM). Virus for all studies were concentrated from supernatant by polyethylene glycol precipitation and purified by CsCl gradient ultracentrifugation as described²¹⁹. The particle concentration of purified virus was determined using a Bio-Rad Protein Assay (Bio-Rad) with a bovine serum albumin standard.

The infectious titers of wild type MAdV-1 and MAdV-2 stocks were determined by infecting CMT93 cells with serial dilutions of virus. The cells were then fixed in 2% paraformaldehyde, permeabilized with 20 mM glycine and 0.5% Triton X-100 in phosphate buffered saline (PBS), and stained with anti-hexon antibody 8C4 (Fitzgerald Industries International) and an Alexa Fluor 488-conjugated secondary antibody (Life Technologies). Hexon positive cells were enumerated by flow cytometry. For GFP expressing MAdVs, cells were infected and fixed as above, and GFP positive cells were enumerated by flow cytometry. The infectious titer of the viral stock was calculated using the Poisson distribution.

Virus growth curves. To compare growth kinetics of MAdV-2 and MAdV-2.IXeGFP, parallel wells of CMT93 cells seeded in 24-well plates were infected at an MOI of 3. Infected cells were collected at 0, 24, 48, 72 and 96 hpi and freeze-thawed three times to make lysates. Lysates were serially diluted on fresh monolayers of CMT93 cells seeded in black wall, clear bottom 96-well

plates (Perkin-Elmer, San Jose, CA) and infection quantified via hexon staining 48 hpi as previously described. The daily viral titer for each virus was calculated using the Poisson distribution.

Defensin and fiber knob assays inhibition assays. CMT93 cells were seeded into black wall, clear bottom 96-well plates and infected with serial dilutions of MAdV-1, MAdV-2, MAdV-1.IXeGFP or MAdV-2.IXeGFP. At 48 hpi plates of GFP expressing MAdVs were fixed and scanned as described above. Cells infected with wild type viruses were fixed and stained with the anti-hexon primary antibody and an Alexa Fluor 488-conjugated secondary antibody as above. Infection was quantified by measuring total monolayer fluorescence using a Typhoon 9400 variable mode imager (GE Healthcare, Piscataway, NJ). A virus concentration producing 30% maximal signal was chosen for further studies where enhanced infection was anticipated, and a virus concentration producing 80% maximal signal was chosen for further studies where inhibition of infection was anticipated.

For defensin studies, serum-free DMEM (SFM) alone or containing increasing concentrations of mouse or human defensins was incubated with purified virus for 45 min on ice. The mixture (35 μ l/well) was added to a confluent monolayer of CMT93 cells and incubated at 37°C for 2 h with rocking, washed, and cultured with complete DMEM for 48 h. For initial fiber knob inhibition studies (Figure 4.5A) increasing concentrations of MAdV-2 fiber knob were mixed with virus in SFM and a total volume of 50ul, added to cells, and incubated at 37°C for 2 h. Cells were then washed and cultured with complete DMEM for 48hr. For experiments in Figure 4.5B, virus was pre-incubated with a final concentration of 5 μ M Crp2 in a total volume of 37.5 ul for 45 mins on ice. In parallel, a confluent monolayer of CMT93 cells was pre-treated

for 45mins at 37°C with SFM containing 1.5 μM MAdV-2 fiber knob, 16 nM HAdV-16 fiber knob, or BSA as a protein control in a final volume of 50ul SFM. After 45 minutes on ice, 12.5 ul of SFM containing 1.5 μM MAdV-2 fiber knob, 16 nM HAdV-16 fiber knob or BSA was added to the virus and defensin mixture. The pre-treated CMT93 monolayer was then washed twice with SFM, and 45 ul of the virus, defensin, and fiber knob or BSA mixture added to cells and incubated for 2 h at 37°C. Cells were then washed twice with complete media and 48 hpi plates were fixed and scanned for eGFP signal as above, and background-subtracted total well fluorescence was quantified using ImageJ software.

Mice. All mouse experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and following the International Guiding Principles for Biomedical Research Involving Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Wild type and *Mmp7*^{-/-} mice were on a C57BL/6NHsd background²⁶⁵. *Mmp7*^{+/+} *DEFA5*^{+/-} mice on a C57BL/6 background²⁰³ were obtained by cryorecovery from Jackson Labs and were interbred to yield *Mmp7*^{+/+} *DEFA5*^{+/+} mice. *Mmp7*^{+/+} *DEFA5*^{+/-} mice were then backcrossed onto the *Mmp7*^{-/-} background to yield *Mmp7*^{-/-} *DEFA5*^{+/-} mice. *NOD2*^{-/-} (stock number 5763) and control wild type C57BL/6J mice were obtained from Jackson labs.

Organoid production and maintenance. Small intestinal organoids were cultured from crypt enriched ileal fractions from 6-10 wk old wild type and *Mmp7*^{-/-} mice as previously described¹⁵¹. Briefly, the distal 10 cm of the small intestine was removed and flushed with 0.04% sodium hypochlorite in PBS. After removal of mucus and villi, the epithelium was dissociated for 90 min

at RT in a solution of 3 mM EDTA and 0.5 mM DTT in Hank's Buffered Salt solution (HBSS). Crypt enriched fractions were identified following vigorous shaking into sequential changes of fresh, ice cold, sterile $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. Cells were then concentrated by centrifugation at $300 \times g$ for 5 min at 4 °C, and the pellet was resuspended in 300 μl of HBSS containing 0.5 mM Rock inhibitor (Fisher) and 10 μM Jagged-1 (Anaspec). After a second round of centrifugation, the cell pellet was resuspended in growth factor reduced Matrigel (BD Biosciences). 50 μl aliquots were plated in the center of 24 well plates and overlaid with 500 μl of Complete Crypt Culture Medium (CCCM)¹⁹⁶. Once established, culture media was supplemented with 200 μl CCCM every 2-3 days. Organoids were subcultured every 6-7 days¹⁹⁶.

Colonic organoids were cultured from crypt enriched colonic fractions from 6-10 wk old wild type and *Mmp7*^{-/-} mice as above with the following modifications. Crypts were dissociated via incubation for 90 min at RT in a solution of 3 mM EDTA, 0.5 mM DTT, and 1x Antibiotic Antimycotic Solution (Sigma-Aldrich) in HBSS. As colonic crypts have a tendency to adhere to plastic surfaces, all plastic wear was pretreated either with 10% FBS for 30 minutes on ice, or 10% FBS was pre-pipetted into pipette tips and stripettes. Pelleted crypts were resuspended in 5mLs TrypLE (Life Technologies) and incubated at 37 °C for 5 min, followed by addition of an equal volume of DMEM/F12 (Gibco) and pelleting before resuspension in HBSS containing Rock Inhibitor and Jagged-1. Finally, colonic crypt culture media is identical to small intestinal organoids media, with the following additions: murine Wnt-3A (0.1 $\mu\text{g}/\text{mL}$, Sigma-Aldrich), CHIR99021 (2.5 μM final, Stemcell Technologies), and valproic acid (1 mM, Sigma-Aldrich)

Organoid bacterial infection assays. Organoids were subcultured as above 3-4 days prior to microinjection, deposited in 30 μl Matrigel on glass coverslips in 12-well tissue culture dishes,

and overlaid with 1 mL CCCM. Before injection, organoids on coverslips were washed twice with Advanced DMEM/F12 and overlaid with 1 mL CCCM without antibiotics. A Nikon Ti microscope with Nomarski optics fitted with a rotating glide stage and a FemtoJet Microinjector (Eppendorf) was used for microinjection. Injection needles were pulled from glass capillaries (1B100-4, World Precision Instruments) on a horizontal bed puller (Sutter Instruments). The tip ends were broken using tweezers.

For injection, overnight cultures of bacteria grown in LB-Miller broth were subcultured for an additional 2 h at 37 °C with aeration under selection. 1 mL of subculture was washed twice and serially diluted in PBS to the desired concentration. The CFU of each inoculum was quantified from 20 injections into PBS prior to organoid injection. To enumerate surviving CFU, media was removed from wells and centrifuged to recover bacteria, while organoids were removed from Matrigel using Cell Dissociation Solution (BD Biosciences) at 4 °C for 30 min. Organoids in solution were added to the bacterial pellet, centrifuged at $300 \times g$ for 5 min, and resuspended in 100 μ l sterile water for 5 min to lyse eukaryotic cells. The sample was then vortexed for 15 sec before serial dilution in PBS and plating on LB agar plates. Plates were incubated at 37 °C, and colonies were counted after 18-20 h to determine CFU.

20 organoids per sample were injected one time each with STM. Each injection contained 5×10^4 CFU for Figure 2.1B; 5×10^3 CFU for Figure 2.2A-E, Figure 2.7A-C, Figure 2.8B-C, Figure 6B-C, and Supplementary Figure 1; and 50 CFU for Figure 2.6A-B. Note that we have graphed the total inoculum delivered to the entire well in all figures except Figures 2.2D and Figure 2.3D in which the inoculum delivered to a single organoid is graphed. Samples were then incubated in CCCM without antibiotics (1 h for Figure 2.3A-D, Figure 2.7B-C, and Figure 2.8B-C or 2 h for Figure 2.1B, Figure 2.2A-E, and Figure 2.6A-B) and then for an equal amount

of time with 100 µg/mL gentamicin. Where indicated, 10µM CCh (Sigma-Aldrich) was added to the culture well 30 min before injection. For Figure 2.1B, CFU were enumerated at this point. For subsequent experiments, injected organoids were washed twice with Advanced DMEM/F12 and grown in CCCM without antibiotics. Surviving CFU was enumerated at 0, 5, 7, 9, 16, or 20 h post-injection, as indicated in each figure legend.

Organoid viral infection assays. For infection by mixing, organoids were subcultured prior to infection and overlaid with 500 uL CCCM in a 24-well tissue culture dish. After 3-4 days, organoids in Matrigel plugs from 6 wells from a 24-well dish were disrupted with a pipette tip, and the solution was then dissociated with a 25-gauge needle and 1 mL syringe. Cells were then pelleted by pulse centrifugation in a mini-centrifuge, and resuspended in 250ul of CCCM. MAdV-2.IXeGFP ($1-2 \times 10^5$ infectious units) in 250ul of CCCM was added to the cells, and incubated at 4°C for 30 minutes rocking to allow for virus binding. Cells were then washed twice in CCCM to remove unbound virus, and resuspended in 30ul CCCM and 120ul Matrigel. 30ul was plated into 5 wells of a 24 well plate, allowed to dry and then overlaid with 500ul CCCM. For basolateral infections, media was removed from 2-3 day old organoids seeded in 24-well plates and replaced with 500ul of fresh CCCM containing MAdV-2.IXeGFP ($1-2 \times 10^5$ infectious units).

For viral infection by microinjection, organoids were split and re-embedded in Matrigel, deposited on glass coverslips and overlaid with 1 mL CCCM in a 12-well tissue culture dish 3-4 days before infection. Prior to injection, wells were treated with 10 uM CCh (Sigma-Aldrich) for 30 min. The microscope used for injection and needles were the same as for bacterial microinjection. Each needle was loaded with MAdV-2.IXeGFP (Figure 4.3) or with virus mixed

1:5 with 80,000 MWCO FITC-dextran (Figure 4.4, Life Technologies). After needle tips were broken with tweezers in halocarbon oil (Sigma-Aldrich), 10 organoids per coverslip were injected. The same needle was used for injection of all wild type and *Mmp7*^{-/-} organoids from a single experiment. After injection, wells were washed twice with 1X DMEM/F12 (Gibco) and overlaid with 1 mL CCCM. Samples were collected daily for 5 days, and day 0 samples were collected immediately after media addition.

For Figure 4.4A and Figures 4.4C-D, infected (GFP positive) cells and organoids were counted using a fluorescence microscope with a GFP filter. For Figure 4.2 and Figure 4.3, samples were collected by disrupting the Matrigel plug with a P1000 pipette tip. Cells and supernatant were transferred to an eppendorf tube and frozen at -80°C. After three freeze thaw cycles, viral lysate was serially diluted on CMT93 cells previously seeded in black-well, clear bottomed 96 well plates. At 48 hpi plates were fixed and scanned for eGFP signal as above, and background-subtracted total well fluorescence was quantified using ImageJ software.

Immunohistochemistry. Small intestinal organoids were removed from Matrigel using Cell Dissociation Solution as above, concentrated by centrifugation, and fixed in 10% neutral buffered formalin (NBF). Fixed organoids were then resuspended in Histogel (Thermo-scientific), stored overnight in 10% NBF, and embedded in paraffin. Deparaffinized samples were incubated with goat anti-Crp5 (1:8000, a kind gift from Dr. Andre Ouellette²¹⁴) or goat IgG (0.6 µg/ml, Life Technologies) followed by diaminobenzidine precipitation and hematoxylin counterstaining. Non-sequential sections from the same blocks were stained with hematoxylin and eosin.

Western blot. Small intestinal organoids were cultured for 6 days and removed from Matrigel using Cell Dissociation Solution as above. Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors. After separation by SDS-PAGE, immunoblots were probed for MMP7 (ab5706, Abcam) or GAPDH (sc-32233, Santa Cruz) and visualized with chemiluminescence. To generate rMMP7, the *Mmp7* gene was cloned into a pET27 vector with a C-terminal Strep tag. Upon expression in *E. coli* strain BL21, rMMP7 was purified from solubilized inclusion bodies by affinity chromatography using StrepTactin Sepharose (IBA GmbH).

FACS analysis. Wild type and *Mmp7*^{-/-} organoids were dissociated by incubation in Accutase Cell Detachment Solution (BD) for 20 minutes, followed by physical disruption via pipetting with a 25 G needle on a 1 mL syringe. This solution was then filtered to obtain a single cell suspension and fixed in 2% paraformaldehyde. For cells infected with MAdV-2.IXeGFP, cells were analyzed at this point for infection via quantification of GFP expression. For detection of specific cell varieties, cells were next quenched and permeabilized in PBS containing 20 mM glycine and 0.5% Triton-X 100. For Paneth cell detection, cells were incubated with a 1:200 dilution of rabbit-anti-lysozyme antibody (A 0099, Dako) at 4 °C for 45 min, followed by incubation with a 1:500 dilution of goat-anti-rabbit alexa fluor 647. For goblet cell detection, cells were incubated with a 1:50 dilution of rabbit-anti-Mucin 2 antibody (sc-15334, Santa Cruz) at 4 °C for 45 min, followed by incubation with a 1:200 dilution of goat-anti-rabbit alexa fluor 647. All primary and secondary antibodies were diluted in FACS buffer (PBS with 1% FBS and

0.05% sodium azide) and all washes were performed with FACS buffer. Data was acquired using a BD FACS Canto II and analyzed using FACS Diva (BD) and FlowJo (Tree Star).

AU-PAGE and AU-PAGE western blot. Organoids or crypt-enriched fractions from mouse ileum were concentrated by centrifugation, resuspended in 30% acetic acid, and sonicated. After incubation overnight at 4 °C with agitation, samples were diluted 3-fold with water. Insoluble material was removed by centrifugation at $100,000 \times g$ for 2 h at 4 °C, protein concentrations of the supernatants were determined by Bio-Rad Protein Assay (Bio-Rad), and equivalent amounts of each sample were lyophilized. Lyophilized samples were dissolved in 5% acetic acid and separated by 17% AU-PAGE¹⁹⁸. Folded Crp23 was created from a synthesized 80% pure linear peptide (CPC Scientific) by the same procedure as previously reported for the α -defensin HD5⁹⁶. Proteins were visualized with SYPRO Ruby (Life Technologies). Gels were imaged using a Typhoon 9400 variable mode imager (GE Healthcare). For western blot, samples were separated by 12.5% AU-PAGE and semi-dry transferred to nitrocellulose membranes. Membranes were immediately fixed in glutaraldehyde and blocked in 5% milk, before overnight RT incubation in rabbit anti-HD5 antibody (kind gift from Edith Porter¹⁹⁷) at a 1:1000 dilution. Membranes were incubated in goat-anti-rabbit Alexa Fluor 488 (Life Technologies) and imaged using a Typhoon 9400 variable mode imager (GE Healthcare).

Quantitative PCR. Total RNA was extracted from small intestinal and colonic organoids after 0 days and 4 days in culture. Media was removed from the well and the Matrigel plug containing organoids was immediately resuspended in 1mL of RNAbec (Tel-test) and frozen at -80C. Tubes were thawed at room temperature and RNA extracted according to the manufacturer's

instructions. RNA pellets were resuspended in 10ul of DEPC water. RNA was primed with Oligo DT (Integrated DNA Technologies) and reverse transcribed using Superscript III (Life Technologies). qPCR was performed using cDNA, Ssofast EvaGreen Supermix (Life Technologies), and the primers listed in Table 2. Reaction conditions consisted of 45 cycles of PCR with 55°C annealing temperatures using an iCycler (Bio-Rad). Relative change in gene expression is expressed as compared to organoids immediately after split (Day 0) and is normalized to the housekeeping gene *RPL5*.

Table 2. Primers used.

Name	Sequence
RPL5 F	5'- GGAAGCACATCATGGGTCAGA -3'
RPL5 R	5'- TACGCATCTTCATCTTCCTCCATT -3'
Defcr4 F	5'- CCAGGGGAAGATGACCAGGCTG -3'
Defcr4 R	5'- TGCAGCGACGATTTCTACAAAGGC -3'
M2FF2	5'- GTCCGATTCGGTACTACGGT -3'
M2FR2	5'- GTCAGACAACCTCCAGGGT -3'

Mouse infections and fecal genome quantifications.

Mice were housed under specific pathogen-free conditions and were infected under ABSL2 conditions between 5 and 7 weeks of age. Mice were infected via oral gavage with purified virus diluted in sterile PBS. For experiments in Figure 4.6A and 4.6C mice were housed 5 per cage and segregated by genotype. Fecal pellets were collected beginning immediately before infection and every 6 h for the duration of the experiment. For the experiments in Figure

4.6B and 4.6D mice were individually housed beginning 3 or 4 d prior to infection and for the duration of the experiment. Fecal samples consisted of ten fecal pellets that accumulated in the cages of single-housed mice since the previous collection. Accordingly, on the day of infection and after every fecal collection, mice were transferred to new cages with clean bedding.

Viral DNA was extracted from fecal pellets using the QIAamp DNA Stool Mini Kit (Qiagen) into a total volume of 200 μ l. MAdV-2 genomes were quantified by qPCR using the SsoFast EvaGreen Supermix (Bio-Rad) against a standard curve of pKSB2-MAdV2 using primers M2FF2 and M2FR2 (sequences in Table 2). Reaction conditions consisted of 40 cycles of PCR with 55°C annealing temperatures using an iCycler (Bio-Rad). The limit of detection was defined by the number of viral copies detected in feces from uninfected mice.

Statistics. Experiments were analyzed using Prism (v. 5.0d, GraphPad). Specific statistical tests used are indicated in the relevant figure legend. In all analyses, $p < 0.05$ was considered significant. In the figures, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.005$.

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Education

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Publications

1. Wardell-Scott, L., **Wilson, S.S.**, Smith, J.G. and Garrett, W. The gut microbiota and host anti-microbial peptides influence *Klebsiella pneumoniae* population dynamics. Under review.
2. **Wilson, S.S.**, Tocchi, A., Holly, M.K., Parks, W.C. & Smith, J.G. A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions. *Mucosal Immunology* 8(2):352-61 (2015).
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Conference presentations

1. **Wilson S.S.**, Bromme, B.A., Holly, M.K., and Smith, J.G. (2014) α -defensin enhancement of mouse adenovirus infection. Poster presentation delivered at the International Adenovirus Meeting, San Diego, CA., July, 2014.
2. **Wilson, S.S.**, Tocchi, A., Parks, W.C., and Smith, J.G. (2014) A Small Intestinal Organoid Model of Enteric Pathogen-Epithelial Cell Interactions. Oral presentation delivered at the annual meeting of the American society for Microbiology, Boston, MA, May 2014. Abstract selected for Young Investigator Oral Presentation.
3. **Wilson, S.S.** and Smith, J.G. Evaluating interactions between epithelial cells and enteric pathogens in a small intestinal organoid model. Oral presentation delivered at the annual meeting of the Northwest Chapter of the American society for Microbiology. Seattle, WA, November 2012.4)
4. **Wilson, S.S.** and Smith, J.G. Cell Type Specific Neutralization of Adenovirus Infection by α -defensins. Poster presentation delivered at the annual meeting of the American society for Virology. Madison, WI, July 2012.