The role of ADAMTS enzymes in influenza virus immunity

by

Meagan McMahon
BSc (Biol), BHMSc (Hons)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medicine)

Deakin University
November 2016
I am the author of the thesis entitled 

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submitted for the degree of Doctor of Philosophy 

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Publications

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**Meagan McMahon**, Siying Ye, Daniel Dlugolenski, Ralph A. Tripp, Daniel R. McCulloch and John Stambas. **ADAMTS7 A CRITICAL REGULATOR OF CD8+ T CELL ACTIVATION. In preparation.**

**Meagan McMahon**, Siying Ye, and John Stambas. **ADAMTS ENZYMES IN THE IMMUNE RESPONSE. In preparation.**

Leonard Izzard, Daniel Dlugolenski, Yingju Xia, **Meagan McMahon**, Deborah Middleton, Ralph Tripp and John Stambas. **ENHANCED IMMUNOGENECITY FOLLOWING MIR-155 INCORPORATION INTO THE INFLUENZA A VIRUS GENOME. Submitted.**
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Abstract

Metalloproteinase family members such as MMPs and ADAMs have well-defined roles in host immunity. ADAMTS family members however remain largely uncharacterised in this context. Considering the potential use of ADAMTS enzymes as pharmacological targets, it is critical we understand the functional role of ADAMTS enzymes in cellular processes. This thesis describes the importance of two ADAMTS enzymes, ADAMTS5 and ADAMTS7, in regulating viral immunity.

ADAMTS5 enzymatic activity has a well-characterised role in arthritis pathogenesis with extensive literature supporting these observations. Studies in this thesis have utilised the Adamts5 knockout mouse model to determine the role of ADAMTS5 in influenza virus immunity. In our studies, we found that the absence of ADAMTS5 in mice results in increased influenza virus associated disease characterised by perturbed CD8+ T cell immunity in the spleen and lung. Moreover, CD8+ T cells accumulated in the mediastinal lymph node (MLN), the site of CD8+ T cell activation. We suggest that accumulation of CD8+ T cells is linked to increased versican levels, a key substrate of ADAMTS5. Therefore, we propose that ADAMTS5 plays a crucial role in regulating CD8+ T cell migration from the MLN to periphery following influenza virus infection.

ADAMTS7 has previously been identified in a human protease screen as having a key role in influenza virus pathogenesis. Chapter 5 of this thesis extends these observations in a Adamts7 knockout mouse model to further characterise ADAMTS7’s role in controlling the immune responses in vivo. Our results show that the absence of ADAMTS7 in mice negatively impacts dendritic cell (DC) activation and CD8+ T cell responses-, culminating in delayed viral clearance and increased susceptibility to disease.
Finally, we found that *Adams5*<sup>−/−</sup> and *Adams7*<sup>−/−</sup> mice were potentially more susceptible to Hendra virus infection, suggesting ADAMTS5 and ADAMTS7 are essential mediators for the removal of infectious diseases in mice.
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAHL</td>
<td>Australian Animal Health Laboratory</td>
</tr>
<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloproteinase</td>
</tr>
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<td>ADAMTS</td>
<td>ADAM with ThromboSpondin-1 repeats</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BSL</td>
<td>Bio-Security Level</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organisation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DPI</td>
<td>Days Post Infection</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorter</td>
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<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HDF</td>
<td>Heart Defect</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>Sodium pyruvate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HeV</td>
<td>Hendra Virus</td>
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<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
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<td>LAIV</td>
<td>Live Attenuated Influenza Vaccine</td>
</tr>
<tr>
<td>LB</td>
<td>Laurel Broth</td>
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<tr>
<td>M</td>
<td>Matrix</td>
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<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney</td>
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<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
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<tr>
<td>MHC</td>
<td>Major Histo-compatibility Complex</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
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<td>MLN</td>
<td>Mediastinal Lymph Node</td>
</tr>
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<td>Matrix Metalloproteinase</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
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<td>mRNA</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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PFU  Plaque Forming Unit
PR8  A/PuertoRico/8/34 H1N1
qPCR  Quantitative PCR
RIG  Retinoic-acid Inducible Gene
RISC  RNA-Induced Silencing Complex
RNA  Ribonucleic Acid
ROS  Reactive Oxygen Species
RPM  Revolutions Per Minute
RPMI  Roswell Park Memorial Institute
SDS  Sodium Dodecyl Sulphate
shRNA  Short-hairpin RNA
siRNA  Short-interfering RNA
SEM  Standard Error of the Mean
TCID  Tissue Culture Infectious Dose
TIMP  Tissue Inhibitor of Metalloprotease
TLR  Toll-Like Receptor
TNF  Tumour Necrosis Factor
Vcan^{hdf/+}  Versican^{hdf/+}
VERO  African green monkey kidney epithelial cells
WT  Wildtype
X31  A/HongKong-X31 H3N2
## Nomenclature

### Human

<table>
<thead>
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### Mouse

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<tr>
<td>Vcan</td>
<td>VERSICAN</td>
</tr>
<tr>
<td>Timp</td>
<td>TIMP</td>
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Chapter 1 - Introduction

Influenza is an acute, febrile, and highly contagious respiratory disease which causes significant global morbidity and mortality (1-3). Annually, influenza viruses kill between 250,000 and 500,000 people (2-4), and, although influenza viruses can infect all age groups, it’s the elderly (> 65 years) and the young (< 2 years) who succumb more readily to disease (5, 6). Moreover, influenza virus infection causes a substantial economic burden through increased healthcare costs and decreased workplace productivity, equating to a $USD 87.1 billion global economic burden per year (7).

Though there are therapeutic treatments available for influenza virus infection, current strategies need to be constantly adapted to align with changes occurring in the influenza virus genome that can lead to pandemic potential (see section 1.2). The emergence of pandemic strains constantly reminds us that current therapies (vaccines and antivirals; see section 1.3) can be ineffectual at combating some novel influenza virus strains (8). Consequently, there is a strong need for new and reliable therapeutic targets.

1.1 Influenza viruses

Influenza viruses form part of the Orthomyxoviridea family of viruses, which contain five members: influenza A, B, C and D viruses, thogotovirus and isavirus (9). All three influenza virus types can infect humans, yet, it is influenza A and B viruses that contribute significantly to global mortality (10). Influenza A viruses contain 8 negative sense single stranded ribonucleic acid (RNA) segments that encode 12 main proteins (11, 12). These proteins can be separated into structural and non-structural (NS) proteins (Figure 1.1). The structural proteins include the matrix (M) 1 protein and the M2 ion transporter encoded by the M gene, the neuraminidase (NA) and haemagglutinin (HA) glycoproteins and the components
of the RNA-polymerase complex which include the polymerase basic (PB) 1, PB2 and Acid polymerase (PA), and the RNA nucleoprotein (NP) (11, 12). NS proteins include NS-1, the nuclear export protein (NEP), the PB1-frame 2 and the recently defined PA-X protein (13). Other proteins encoded by some influenza virus strains include the N-terminally truncated version of the polypeptide (14), the PB2-S1 (15) and the M42 (16).

Of importance to virus classification and replication are the surface glycoproteins, the HA and NA, which assist with virus infection. Currently there have been 18 HA and 11 NA variants identified (17, 18). HA is a homo-trimeric protein that allows influenza viruses to attach to sialic acid receptors on lung epithelial cells and enter the host cell in order to replicate (17). Human influenza viruses bind to sialic acids in an α2-6 conformation, whereas avian influenza viruses bind α2-3 sialic acid receptors (19). The NA is a tetrameric protein which cleaves sialic acids attached to HA glycoproteins facilitating its release from the infected cell (11). Due to their location on the surface of influenza virus, HA and NA are more susceptible to selection pressures imposed by the host immune response. As such, the HA and NA undergo constant mutation.

1.2 Antigenic drift and shift of influenza viruses

A single exposure to influenza virus is usually sufficient to provide immunity against future infection by a homologous strain. However, mutations within the HA and the NA result in structural changes within the genome rendering influenza virus unrecognizable to the host immune system, thereby allowing re-infection (20). Mutations accumulating within the HA and NA sequence result in antigenic drift, eventually resulting in a new strain of influenza virus. Antigenic drift generally occurs every 2-3 years and causes seasonal epidemics (21).
Figure 1.1 Influenza A Viruses.
Influenza A viruses contain 8 negative sense RNA gene segments bound to a RNA polymerase. These segments are enclosed by a lipid bilayer and M proteins. The HA and NA glycoproteins are found on the surface of the virus and facilitate virus attachment and release from a host cell.
Major changes in the HA or NA occurs through a process known as antigenic shift. Antigenic shift results in novel surface glycoproteins being expressed following virus reassortment (5). Some animals, such as pigs, contain both α2,3 and α2,6 sialic receptors (receptors for HA host cell attachment) which are accessible to both avian and human influenza viruses (22). Due to this, pigs can be co-infected with multiple viruses. Co-infection leads to genetic reassortment of influenza virus genes, resulting in the formation of novel influenza viruses (23, 24). These viruses sometimes express new proteins for which the human host population is often naïve. These new viruses have, on occasion, had the ability to become zoonotic and initiate new pandemics. Pandemics such as the 1918 “Spanish flu”, the 1957 “Asian Flu”, and the 1968 “Hong Kong Flu” as well as the first pandemic of this century, the 2009 H1N1 “Swine Flu”, have all resulted from antigenic shift mutations (23). Although seasonal epidemics can kill up to 500,000 people each year, the pandemics mentioned above are estimated to have caused 40 million (1918), 4 million (1957), 1 million (1968) and 300,000 (2009) deaths, respectively (25, 26).

More recently, global health organizations, such as the World Health Organization, have been concerned about the potential for high pathogenic H5N1 and low pathogenic H7N9 avian influenza viruses to become zoonotic. H5N1 avian influenza viruses currently have a mortality rate of ~60 % in humans (3, 27), however little human to human transmission has been definitively determined (28). Additionally, H7N9, originally detected in chickens, has emerged in China and has an observed 27 % mortality rate in humans (29). Although, like H5N1 influenza viruses, little human-to-human transmission has been observed. Due to these potential pandemic threats, it is imperative that therapeutic agents are designed to be effective against a broad range of influenza virus strains as current strategies against these zoonotic agents have failed to ameliorate disease.
1.3 Intervention strategies targeting influenza viruses

1.3.1 Antivirals

Antivirals are therapeutic agents that limit replication of influenza viruses. M2 and NA inhibitors are two classes of antivirals used to reduce the impact of influenza virus infection. M2 inhibitors block the ion channel, stopping protons from entering the virion and preventing acidification of the viral interior (30). Amantadine and rimantadine were the two most commonly used forms of M2 inhibitors. However, resistance to these drugs has been observed in all influenza virus subtypes indicating that they are not be effective for controlling virus replication (31, 32).

NA inhibitors block the enzymatic activity of the NA enzyme, preventing cleavage of the surface-attached HA receptors which become reattached during viral exit (33). NA inhibitors are analogues for sialic acids, and can block the NA active site (33). Here, they inhibit the NA-mediated cleavage of host cell sialic acids which have become bound to HA’s upon cell exiting, preventing budding from the infected cell (33). This results in viral accumulation at the cell surface and a reduction in the number of infectious virus particles released (34). Tamiflu (oseltamivir) and Relenza (zanamivir) are the two commonly prescribed NA inhibitors. Scientists initially predicted that little or no resistance would be observed towards these drugs, as the NA active site is essential for the virus life cycle. However, resistance towards NA inhibitors has been observed in some seasonal influenza A virus subtypes as well as in the recent 2009 H1N1 “swine flu” pandemic (35-37).

1.3.2 Vaccinations

Influenza vaccination was introduced in the 1950s, with both inactivated and live formulations now commercially available and used globally. The inactivated formulation is
prepared by taking the allantoic cavity of embryonated chicken eggs and inactivating the infectious supernatant using beta-proproriolactone or formaldehyde (20, 38). Additionally, virus can also propagated in cell culture [e.g. Madin Darby Canine Kidney (MDCK) and African green monkey kidney epithelial (VERO) cell lines (39, 40)], although this is not as common. Current inactivated vaccines afford protection against 3-4 circulating influenza virus strains, usually comprising 2 influenza A and 1-2 influenza B subtypes (41). As these formulations contain only inactivated virus it is suitable for those over 4 years of age, incorporating susceptible populations such as the elderly and pregnant women (20).

The second type of commercially available formulation is the live attenuated influenza vaccine (LAIV) used throughout Europe and North America. This vaccination is a nasal spray which introduces a live attenuated virus to the nasal cavity. Unlike the inactivated vaccine, which affords only humoral immune response (IgG and IgA antibodies), the LAIV activates both the humoral and cellular immune responses (influenza-specific CD4+ and CD8+ T cells) (38, 42). The importance of these immune responses in influenza virus infection will be explained in greater detail in Section 1.7. This type of vaccination is currently only available in America and Europe and is recommended to individuals aged between 5 and 60. However, this year the Centre for Disease Control (America) recommended that the inactivated vaccine be prescribed over the LAIV, due to reduced effectiveness observed of this vaccine between 2013 and 2016.

Additionally, the constant evolution of influenza viruses necessitates an annual review of vaccine components (20). However, the circulating strain is not always identified leading to a mismatch, additionally the time taken to select the circulating strain can further result in mismatches. Due to this, as well as the specific characteristics of the persons vaccinated (e.g. health and age), influenza vaccines have a range of efficacy between 60-90% in people aged between 20 and 65 (43, 44). This efficacy drops to 40-60% in the elderly (>65 years) (44-47).
1.3.3 Novel therapeutic targets

As described above, current therapeutic treatments mostly target the globular HA head. Because genetic drift and shift cause changes in the influenza virus genome (mostly at the HA globular head), current therapeutics may not always be effective against novel circulating influenza subtypes. Hence, discovering new therapeutic targets (including those that target the virus and the host) is important in this ongoing struggle to limit the burden of influenza virus infection.

1.3.3.1 Immune evasion pathways

Influenza viruses can use immune evasion mechanisms to facilitate infection and replication. These pathways include the Raf/MEK/ERK and NF-κB pathways (48). The Raf/MEK/ERK pathway is necessary for normal cell growth, differentiation and survival (49, 50). Additionally, several studies have detected that upregulation of the Raf/MEK/ERK pathway is necessary for influenza virus replication (51). Inhibition of the pathway at the MAPK/ERK level leads to lower viral loads in infected MDCK cells (51) through impaired NEP function resulting in nuclear retention of viral RNA. Furthermore, *in vivo* inhibition of MEK1 and MEK2 (proteins activated in the Raf/MEK/ERK pathway) using the U0126 inhibitor resulted in lower virus titres in influenza-infected mice (52).

The NF-κB pathway is involved in the production of cytokines associated with host defense against infection. *In vitro* studies have indicated that inhibiting the NF-κB pathway following influenza virus infection reduces viral load (53, 54). This is caused by viral RNA retention in the nucleus. Activation of this pathway is required for normal cell lysis, but it also supports viral release (53). In the context of influenza virus infection, *in vivo* inhibition of the NF-κB pathway, using the BAY 11-7082 NF-κB inhibitor, resulted in decreased virus titres in influenza virus infected mice (52). These studies identify the potential for modulating host cell
factors such as Raf/MEK/ERK and NF-κB to prevent influenza virus replication. Importantly, the USA Federal Drug Administrator has already approved cancer treatments for human use known to inhibit these pathways simplifying their potential use as therapeutic agents against influenza virus infection (54-56).

Influenza virus infection is a lytic disease and clearance requires both arms of the immune system (see section 1.6 and 1.7). Immune cells assist with the clearance of influenza virus infected cells through activation of the NADPH pathway resulting in the release of reactive oxygen species (ROS), including Nox2 a subunit of the NADPH complex involved in hydrogen peroxide and a hydroxyl radical production (57, 58). The release of ROS promotes antiviral activity and the timely removal of infection. Although ROS production is beneficial in normal immune responses, over-reactive release can result in excessive inflammation leading to lung damage, contributing to mortality. The NADPH inhibitors, gp91dstat and apocynin, are attractive therapeutics for the reduction of influenza-associated inflammation. Inhibition of ROS production in vitro reduced viral titres following H5N1 and H7N9 avian influenza virus infection of A549 epithelial cells (59). Use of apocynin in vivo during A/HongKong-X31 (X31) (H3N2) infection correlated with in vitro findings (58). Furthermore use of ROS scavengers upon 1918 influenza virus infection in mice further supports the use of ROS intervention strategies to ameliorate disease burden (60). These studies were supported in vivo where apocynin treatment resulted in increased recovery time and decreased in neutrophil infiltration in influenza virus infected mice (58, 59, 61).

These studies highlight the potential importance of modulating the host response to control influenza virus infection. The development of novel therapeutic agents that modulate host responses could be used in combination with existing antiviral treatments that target the virus to a) prevent virus replication, b) aid the immune system in clearing infection and c) prevent further transmission of influenza viruses.
1.4 Mouse models of influenza virus infection

Mouse models are commonly used to assess influenza virus replication, virulence and the host immune responses. Mice on the DBA/2J, BALB/c and C57BL/6 backgrounds have been used in influenza virus related experiments, however it is of importance to note that mice are not natural hosts of influenza viruses (62). Nonetheless, mouse adapted influenza virus strains have been developed and can be used to assess the impact of infection on the host (63, 64).

Like humans, mice can be intranasally infected with influenza virus (63, 64). Intranasal infection of mice mimics human infection and allows the analysis of primary and secondary immunity. Upon intranasal infection, mice show typical signs of disease, including weight loss, hunching and ruffled fur (62, 63). The effects of influenza virus infection in mice can be measured through recording weight loss, assessing virus titers from respiratory organs and characterizing the innate and adaptive immune responses in various tissues (63). Whilst the ferret has been extensively used to assess transmission of human influenza viruses our ability to characterize innate and adaptive immunity is constrained by a lack of reagents for this species. Conversely, reagents to analyze influenza-specific innate and adaptive immunity in mice are readily available. One such example is tetramer staining which is used to enumerate influenza-specific CD8+ T cells in C57BL/6 and BALB/c mice (65-69). Moreover, the availability of knockout mice has contributed to our detailed understanding of influenza-specific immunity.

1.5 Influenzas virus pathogenesis in mice – an overview

When mice are infected with a sub-lethal dose of influenza virus [e.g. X31 (H3N2)], mice start losing weight at 2-3 days post infection (dpi) (representing the peak of virus titres)
and continue this weight loss until 6-8 dpi (63). This time point normally represents the induction of the adaptive immune response (see section 1.7) from which point mice begin to regain weight, coinciding with a reduction of viral load and clearance of virus by day 10 (Figure 1.2) (70). This is typically depicted as a U-shaped weight loss curve.

Innate immune cells play a pivotal role in the clearance of influenza virus infection. These cells increase rapidly within the first 2 days of infection and peak at 4 dpi (71). DCs, a key cell type involved in antigen presentation, are numerically at their highest levels in the lung, 18 hours’ post infection (72-75). These cells then migrate to draining lymph nodes to activate naïve T cells (see section 1.6.5).

Finally, T and B cells are required for the clearance of influenza virus infection (see section 1.7). Removal of influenza virus infected cells by the adaptive immune response shows a biphasic response (71). An initial clearance is observed at early time points, which is mediated by innate immune cells. The latter stages of clearance are dominated by T and B cells. Influenza-specific T cell responses peak between 7 and 10 dpi, whereas B cell numbers and Influenza-specific antibodies peak 14 dpi. Following the removal of infection, influenza-specific T and B cells will undergo apoptosis, leaving behind small stable memory populations (68).
Figure 1.2. Immunity to Influenza Viruses. Influenza virus titres peak at 3 dpi and viral replication is restrained by the innate and adaptive immune system. This figure is not to scale.
1.6 The innate immune response to influenza virus infection

The protection afforded by the innate immune system against influenza virus infection involves numerous defense mechanisms, such as mucins and surfactants that are secreted into the respiratory airways (76, 77). This is in addition to the accumulation of cells associated with the innate immune system [neutrophils, macrophages and natural killer (NK) cells] that induce apoptosis, removal of influenza virus infected respiratory epithelial cells and the eventual activation of the adaptive immune response (77).

1.6.1 Detection of influenza viruses

Upon infection, pattern recognition receptors recognize various aspects of pathogens including, carbohydrates, proteins and nucleic acids (78, 79). The main pattern recognition receptors that detect influenza virus are the retinoic acid-inducible gene 1 (RIG-I), toll-like receptors (TLRs) (TLR7 and 8) and Nucleotide-binding oligomerization domain-containing (NOD) receptors (78, 80).

RIG-I receptors detect the single-stranded RNA 5’triphosphate of influenza virus in the cytosol of infected cells (81-83). Like RIG-I receptors, NOD-1 receptors recognise viral RNA in cytosolic compartments. TLR7 and 8 recognise single-stranded RNA in endosomes of influenza-infected cells (84-86). The recognition of viral components results in the release of pro-inflammatory cytokines (tumour necrosis factor (TNF) α, interleukin (IL) 1α and β, 6, 8, 18, interferon (IFN) α, β and γ) that promote the activation of innate immune cells (87). Interestingly, the RIG-I receptor can be blocked by the NS-1 protein expressed in the influenza virus infected cell (88), preventing the RIG-I signaling cascade and the eventual release of pro-inflammatory cytokines, such as IFNα and IFNβ (81, 89).
1.6.2 Neutrophils

Neutrophils are typically the first immune cell type to arrive in influenza virus infected lungs, arriving as early as 6 hours post infection (90, 91). Neutrophils secrete a range of pro-inflammatory molecules and immune mediators, such as ROS, defensins and TNFα. These mediators have potent antiviral activity towards influenza virus infected cells (92-94). Neutrophils also generate extracellular traps following virus infection. These traps contain an array of excreted enzymes (such as elastase, cathepsin G and histones) to trap pathogens before they enter cells (93).

As neutrophil knockout mice are embryonic lethal, understanding the importance of this cell type during influenza virus infection has been experimentally achieved through the use of neutrophil targeting antibodies (95, 96). Neutrophil depleted mice infected with X31 (H3N2) or A/PuertoRico/8/34 H1N1 (PR8) (H1N1) influenza viruses show increased weight loss, pneumonia and death, indicating that these cells are essential for clearance of influenza virus (91, 95). Although these studies confirm that neutrophils play a critical role in limiting disease severity in mice, excessive neutrophil infiltration results in an overproduction of inflammatory mediators (e.g. ROS, TNFα) resulting in increased lung damage and pathology (91, 97). However, the prolonged presence of neutrophils and extracellular traps exacerbate damage to infected tissue (92, 98) resulting in acute lung injury and acute respiratory distress syndrome. This syndrome is a complication of severe influenza virus infection and is characterised by alveolar damage and respiratory failure (99), which can lead to death.

1.6.3 Macrophages

Resident lung macrophages detect infection, become activated and remove influenza virus infected cells. Removal of influenza virus infected cells occurs through phagocytosis (100, 101) and the production of innate cytokines, such as IFNα and β and IL12 (102). As
macrophage, knockout mice are embryonic lethal, antibody depletion has been used to assess the contribution of macrophages to influenza virus immunity. Macrophage depletion results in uncontrolled virus replication, severe pneumonia and increased mortality in C57BL/6 mice infected with BJx109 (H3N2) influenza virus (100, 103, 104). In support of these findings in mice, macrophage depletion in pigs using dichloromethylene diphosphonate also causes increased weight loss, lower antibody titres and decreased CD8+ T cell numbers following A/New Caledonia/20/99 (H1N1) influenza virus infection (103). Macrophage depletion in mice also drives compensatory neutrophil infiltration into the lungs (105), leading to the over-production of pro-inflammatory molecules that contribute to the destruction of lung tissue through the release of superoxide radicals (106).

1.6.4 NK cells

NK cells are recruited to the lungs between 2-5 dpi and can recognise and lyse infected cells (71, 107). NK cells can detect influenza viruses via two mechanisms. Firstly, they bind neutralizing antibodies that have recognised influenza virus (108). NK cells then lyse infected cells through a mechanism known as antibody dependent cell-mediated cytotoxicity, resulting in the perforation and death of influenza virus infected cells (108, 109). Alternatively, NK cells recognize influenza virus HA glycoprotein through NKp44 and NKp46 cell surface receptors. Recognition of virus infected cells by NK cells also results in the release of cytokines, such as IFNγ, TNFα and IL15, which initiate the migration of other inflammatory cells to influenza virus infected lungs (110, 111). The secretion of IL15 also positively stimulates NK cell activity and the expansion and maintenance of NK cells in mice infected with influenza virus (112). The role of NK cells in influenza virus immunity has been highlighted using the Ncr1−/− mouse model. In these mice the NCR-1 gene (NKp46 in humans), the NK cell receptor gene, was replaced with a green fluorescent reporter cassette (113). This mutation prevented the
recognition of PR8 (H1N1) influenza virus infected cells, culminating in to a lethal infection (113).

1.6.5 DCs

DCs play an important role in innate immunity and are crucial for activating the cell mediated immune response (see section 1.7.2) (74, 114). DC populations involved in clearance of influenza virus infection include conventional DCs, interstitial DCs, and plasmacytoid DCs. These DCs produce an array of cytokines (IL1, 6 and 12, TNFα and IFNs) that promote antiviral responses (115-117). However, it is the conventional DC subsets (CD11b\textsuperscript{hi}CD103\textsuperscript{lo} or CD11b\textsuperscript{lo}CD103\textsuperscript{hi}) that present antigen to naïve T cells. Upon influenza virus infection, these DCs acquire influenza virus antigen from infected cells or they themselves become infected. These DCs then traffic to the lymph node (18 hour – 5-dpi). Here, the resident lymph node CD8\textsuperscript{+} DCs receive this antigen from the migratory population. Lymph node CD8\textsuperscript{+} DCs then present antigen to naïve CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells via Major Histo-compatibility complex (MHC) Class I and Class II molecules (118), leading to their activation and expansion (119). Once T cells reach effector sites in the lung, airway CD103\textsuperscript{hi} DCs continue antigen presentation to promote activation (120).

Antibody depletion of CD11b\textsuperscript{lo} and CD11b\textsuperscript{hi} DC subsets in mice further supports the importance of DCs in influenza pathogenesis (73). Depletion of CD11b\textsuperscript{lo} and CD11b\textsuperscript{hi} DCs with clodronate-liposome results in delayed influenza virus clearance [A/Japan/305/57 (H2N2)] and significant weight loss in mice (121), leading to ineffective T cell priming.
1.7 The adaptive immune response to influenza virus infection

The adaptive immune response is critical for efficient elimination of influenza virus and involves humoral and cellular immunity. Two cell types critical to this arm of the adaptive immune response are B cells and T cells. Lack of B and T cells in Scid and Rag2−/− mice leads to mortality when infected with a sub-lethal dose of PR8 (H1N1) influenza virus (122, 123), identifying the importance of these cells in the clearance of influenza virus infection.

1.7.1 Humoral immunity

Natural responses towards influenza virus can also produce non-neutralising antibodies towards the M1, M2 and NP proteins (124, 125). These antibodies, rely on the Fc portion of the variable region in the antibody to recognise infected cells (126). This recognition, results in complement-mediated destruction or antibody-dependent cell-mediated cytotoxicity and ultimately death of the infected cell. However, these are not influenza-specific antibodies.

At the resolution of influenza virus infection (> 10 days), B cells become the most predominant, responding cell and limit infection through the production influenza-specific antibodies (127). These antibodies mostly target the easily-accessible cell surface glycoproteins of the influenza virus, the HA and NA and to a lesser extent the M1 protein (128-130). HA-specific antibodies bind the trimeric head of the HA and neutralise virus by preventing attachment to cells (128, 130). NA antibodies do not neutralise virus, but prevent further spread of infectious virus particles, in a similar fashion to NA inhibitors by blocking the virus release from cells (see section 1.3.2) (129). Influenza-specific antibodies can also activate the NK mediated antibody-dependent cell-mediated cytotoxic pathway to lyse and remove influenza-infected cells (129, 130). The importance of B cells in influenza virus clearance is evidenced in experiments using μMT−/− mice (131). μMT−/− mice lack mature B cells preventing an effective antibody response (131). Infection of μMT−/− knockout mice with X31
(H3N2) influenza virus results in delayed clearance, and infection with a sub lethal dose of PR8 (H1N1) influenza virus results in decreased survival compared to WT controls (129, 131).

1.7.1.1 Influenza-specific antibody subtypes

IgM antibodies that recognize the HA and NA glycoproteins are the earliest influenza-specific antibody subtype produced by B cells (132) and facilitate complement mediated destruction of influenza virus infected cells (133). C5a, a molecule involved in the complement cascade, can be targeted using a peptide antagonist for its receptor, delaying influenza virus clearance (129, 134, 135). Secretory IgA antibodies are found in respiratory mucous and can facilitate binding of influenza viral proteins in order to prevent the formation and release of viral progeny (136, 137). Additionally, influenza-specific IgG antibodies move from the serum to the respiratory tract to neutralise influenza viruses and provide long-lasting protection against infection (138).

1.7.1.2 Heterosubtypic immunity

Homologous immunity describes protection afforded by antibodies raised against one strain of virus. Importantly, strong antibody-mediated protection can also be afforded against heterologous strains of influenza virus (termed heterosubtypic immunity) (139-141), although most antibodies produced towards influenza virus are not heterosubtypic. Recent studies have identified antibodies in influenza virus infected that target the HA glycoprotein stalk which have the ability to protect against multiple strains of influenza virus (142, 143). These antibodies are now the subject of intense research in the hope of generating a universal antibody-based vaccine.
1.7.2 Cell mediated immunity

1.7.2.1 CD4+ T cells

CD4+ T cells are known as “T helper cells” as they are involved in stimulating B cell proliferation and production of influenza-specific antibodies (144). B cells proliferate through the "help" CD4+ T cells provide by expressing cytokines such as IL4 and 13 (145, 146). The importance of CD4+ T cells in the immune response to influenza virus infection has been highlighted using MhcII− and CD4− mouse models. MhcII− mice infected with influenza virus are unable to efficiently activate CD4+ T cells (147) and show a diminished capacity to produce influenza-specific antibodies (148). Depletion of CD4+ T cells during the primary response also results in delayed CD8+ T and B cell responses upon secondary challenge (148). Additionally, CD4+ T cells are also vital for the establishment of heterosubtypic immunity. In a study assessing the importance of B cells and CD8+ T cells in heterosubtypic immunity, it was identified that CD4− mice lost more weight than CD8− mice upon heterologous and homologous challenge, although they did not succumb to disease (140).

1.7.2.2 CD8+ T cells

In the influenza virus mouse model, defined CD8+T cell precursor frequencies lead to the formation of a well-characterised immunological hierarchy for recognition of antigenic peptides. The CD8+ T cell response following influenza virus infection is focused on internal influenza proteins, with the NP366-374 and PA224-233 peptides the immunodominant antigenic determinants presented on MHC Class I molecules in the C57Bl/6 mouse model (66). Subdominant CD8+ T cells responses involve the PB1703-711, PB1-F262-70 and NS2114-121 antigenic peptides (66). Primary infection with influenza virus results in co-dominant NP366-374 and PA224-233+ CD8+ T cells responses, whereas NP366-374+ CD8+ T cell numbers are 6-10 fold higher than PA224-233 CD8+ T cell numbers following secondary infection (68, 149).
Throughout both primary and secondary infection, the subdominant antigenic responses (PB1\textsubscript{703-711}, PB1-F2\textsubscript{62-70} and NS2\textsubscript{114-121}) are lower than those observed for NP\textsubscript{366-374}+ and PA\textsubscript{224-233}+ -specific CD8+ T cells responses. Cytotoxic CD8+ T cells directly kill influenza virus infected cells. Activated CD8+ T cells secrete cytokines, including IL2, 4, 5, 9, 10, 17, 21, 22, IFNγ, and TNFα, and chemokines, such as CCL3 and 4 and CXCL9 and 10, to promote macrophage and neutrophil infiltration and function (87). CD8+ T cells kill influenza virus infected cells by releasing perforin and granzyme (150) or through TRAIL or Fas ligand interactions to induce apoptosis in influenza virus infected cells (151, 152). When B cell depleted mice (\textmu MT−/− mice) (131) have their CD4+ T cell compartment depleted (CD8+ T cell compartments remained intact) they were able to clear infection, albeit in a substantially delayed fashion low pathogenic influenza virus (153) infection highlighting a critical role for CD8+ T cell immunity. Depletion of both CD8+ and CD4+ T cell subsets in mice is lethal in the presence of influenza virus infection (154) indicating a need for both subsets.

1.8 Host cellular factors in influenza virus infection

Alongside the roles of the immune response in the clearance of influenza virus infection, tools such as short interfering RNA (siRNA) and short hairpin RNA (shRNA) screens have provided a mechanism for identifying which host cellular factors are also involved in the removal of disease. siRNAs are short double stranded RNA molecules comprising 17-25 base pairs (155, 156) (Figure 1.3). Synthetic siRNAs can be introduced to silence target messenger RNA (mRNA) preventing protein expression within appropriate cell lines (156). The introduction of siRNA activates the RNA induced silencing complex (RISC) that binds to synthetic siRNA mediating the unwinding of siRNA double strands (155, 157). The siRNA and RISC composite
Figure 1.3. siRNA pathways. Double stranded RNA enters the cell and is recognised and cleaved by the Dicer enzyme, creating siRNA. siRNA combines with RISC which degrades the mRNA preventing translation, allowing silencing of the gene. siRNAs are loaded directly into RISC and then follows the normal RNAi pathway.
will then bind and cleave target mRNA. Cleaved mRNA is identified by the cell as aberrant and is destroyed, thus preventing translation of the gene (155, 156).

The silencing of genes of interest using siRNAs and shRNAs has allowed the identification of the role key host genes play in important cellular responses, such as the immune response. Indeed, RNAi screens have been readily used to assess the role of host cellular factors in influenza virus replication (158-160). Although these studies have identified many important host cellular factors involved in controlling influenza virus infection, few overlapping genes have been observed (160). This can be attributed to differences in the cell lines, viruses and methodologies used (160, 161). For example, Brass et al (2009) used a human sarcoma cell line, PR8 (H1N1) influenza virus and HA immunostaining to identify IFITM3 as an inhibitor of influenza virus infection (162). On the other hand, Hao et al (2009), using a drosophila cell line, recombinant WSN (H1N1) virus and luciferase activity identified ATP6V0D1, COX6A1 and NXF1 as host genes that play a role in influenza virus replication (158). The lack of overlap and consistent identification of host genes in these screens suggests that they are context dependent (160, 161) and can be affected by cell line variations and virus strains in any given experiment. This is supported by Karlas et al and Konig et al (2009), who both used an identical cell line and virus and showed only 3 host genes (COPI complex, PRSS31 and CAMK2B) in common (159, 163).

More recently, a siRNA screen assessed the role of human proteases in influenza virus replication virus. In this screen, all known 481 human proteases were assessed for their role in influenza virus replication (160, 161). Twenty-one proteases were identified that either increased or decreased influenza virus replication through the use of an in vitro human lung cell culture model (A549 cells) (161). The screen identified A Disintegrin-like and Metalloproteinase with Thrombospondin-1 repeats (ADAMTS) 7 as having a key role in influenza virus replication. Additionally, downstream analysis identified A Disintegrin-like
and Metalloproteinase (ADAM) 30, ADAMDEC1, matrix metalloproteinase (MMP) 13 and 14 as having key roles in influenza virus replication (161). Moreover, a related enzyme to ADAMTS7, ADAMTS5, was also identified in this screen as having a role in influenza virus replication through downstream analyses (personal communication – Ralph Tripp, University of Georgia, Georgia, USA). These targets were validated multiple times and suggest a definitive role for these enzymes in influenza virus replication.

1.9 Metalloproteinases

Recent evidence suggesting a role for metalloproteinases in influenza virus infection is attributable to their known roles in inflammation, proliferation and migration in inflamed tissues, such as in arthritis (164, 165). The metzincin superfamily of metalloproteinases comprises MMPs, ADAMs and the ADAMTS enzymes (166, 167). These enzymes cleave many substrates including extracellular matrix (ECM) components and cytokines.

1.9.1 Structure of metalloproteinases

The zinc-dependant metalloproteinase (metzincin) superfamily includes 26 MMPs (168), 40 ADAMs and 19 ADAMTS metalloproteinases (169, 170) in mammals. All metzincins are synthesized as zymogens, containing a pro-domain and a catalytic domain for enzymatic activity, with each group having a distinctive C-terminus or ancillary domain (Figure 1.4) (171, 172). MMPs can be distinguished by the presence of a haemopexin-like domain that facilitates adhesion to their various substrates (173). MMPs can be further divided into collagenases, gelatinases, stromelysins or further separated by the presence or absence of a transmembrane domain (MT-MMPs or MMPs respectively), allowing them to exist either as membrane-anchored or secreted proteinases (Figure 1.4) (174). ADAMs and ADAMTS
Figure 1.4. The basic domain organisation of members of the metalloproteinase family. Structurally, metalloproteinases contain similar N-terminal domains. Differences in structure are observed at the C-terminus or the ancillary domain: ADAMTS enzymes contain a series of Thrombospondin-1 repeats, ADAMs are membrane-anchored through a transmembrane domain and MMPs contain haemopexin-like regions.
metalloproteinases vary from MMPs due to the presence of a Disintegrin-like domain (169). These domains interact with integrins which can facilitate localisation of excreted metalloproteinases to the ECM (175). ADAMs contain a transmembrane domain in the C-terminus not present in ADAMTS enzymes. The transmembrane domain acts as a membrane anchor not unlike MT-MMPs. ADAMTS metalloproteinases contain a series of Thrombospondin-1 repeats within their ancillary domain (176). These Thrombospondin-1 repeats function as binding domains for glycosaminoglycans (GAG) found on proteoglycans (177, 178).

1.9.2 Activation of metalloproteinases

The metzincin family of metalloproteinases is named due to the conserved methionine residue at the active site and the use of a zinc ion in the enzymatic reaction (179). Soluble metalloproteinases, including ADAMTSs and MMPs, are synthesized and secreted as inactive enzymes. They remain in a latent state until activation by pro-protein convertases, such as furin (180).

Catalytic activation of MMPs allows cleavage of an array of ECM proteins including laminin (181), collagen (182, 183), proteoglycans (184) tenascin-R (185), growth factors (i.e. pro-nerve growth factor) (186) and cytokines (pro TNFα) (187, 188). Catalytic activation of ADAMs mostly occurs intra-cellular and so they are secreted in their active forms (189-191). Docking to the cellular membrane for MT-MMP’s and ADAMs provides specialised functions in cleavage of membrane-associated proteins (192). Shedding of membrane-associated proteins frees extracellular peptides into their active forms (i.e. TNFα) (193) or regulates gene expression through the generation of intracellular domains that translocate to the nucleus to act as transcription factors, such as Notch proteins (194). Like ADAMs, ADAMTS metalloproteinases are activated intracellularly and are secreted in their active forms (164,
A group of ADAMTS enzymes (ADAMTS1, 4, 5, 8, 9 and 15) can cleave aggrecan, versican, brevican, and neurocan and are termed “hyalectinases” (196-199).

Metalloproteinases collectively cleave a vast amount of ECM substrates including proteoglycans, collagens as well as membrane-associated protein substrates, such as soluble chemokines (193, 200).

1.9.3 Metalloproteinase regulation by tissue inhibitor of metalloproteinases (TIMPs)

Dysregulated, catalytically-active metalloproteinases can enhance inflammatory disorders and other pathological conditions, causing excessive ECM destruction and tissue damage (105). However, metalloproteinase-mediated catalytic activity can be regulated and inhibited. α-macroglobulin, reversion-inducing cysteine-rich protein with Kazal motifs, and TIMPs can all regulate metalloproteinase activity, although TIMP inhibitors are the predominant regulators of metalloproteinase activity (201). All four of the TIMP (TIMP1 - 4) family members display broad inhibition of MMPs (201-205), with TIMP3 having the broadest activity, inhibiting all MMPs, ADAM10, 12, 17, 28 and 33 and ADAMTS1, 2, 4 and 5 (206-208).

The regulation of metalloproteinases by the TIMP family is important in pathological and physiological processes as they can effectively control metalloproteinase activity. As such novel therapeutics involving TIMP inhibitors are currently being studied for disease, such as cancer and arthritis. Additional roles for TIMPs are also being identified using TIMP knockout mice. Timp1−/− mice display delayed neuronal death leading to significant brain injury and delayed ECM repair following myocardial infarction (202, 209). Timp2−/− mice display motor defects that have been associated with tissue destruction and unregulated metalloproteinase activity (210). Timp3−/− mice are highly susceptible to arthritis (211), due to unrestrained
ADAMTS [such as ADAMTS4 and 5 (see section 1.7.1)] degradation of aggrecan, a proteoglycan found in joints (212, 213). Finally, Timp4−/− mice are highly susceptible to myocardial infarctions caused by excessive MMP activity (214). The use of TIMP knockout mice helps identify roles for these inhibitors in development and disease pathologies, as well as allowing the identification of potential therapeutic targets.

Broad-spectrum synthetic metalloproteinase inhibitors have been used extensively to assess the effects of metalloproteinases in various disease models (215), including cancer (216-218) and arthritis (219). Studies have consistently shown however that metalloproteinase inhibitors show poor efficacy due to a lack of specificity (220). In contrast and despite the findings above, specific metalloproteinase inhibitors still remain an attractive therapeutic strategy for a variety of diseases; most notably ADAMTS5 inhibitors that are currently in Phase III trials (http://clinicaltrials.gov/show/NCT00454298) for treatment of osteoarthritis (199) as cleavage of aggrecan by proteases, such as ADAMTS5 has been shown to exacerbate osteoarthritic disease (165, 221).

In conclusion, a greater understanding of the functional mechanisms of metalloproteinases is required before therapeutic inhibition of metalloproteinases can become a viable treatment option for disease.

1.10 The role of metalloproteinases in infection and immunity

Here, I will discuss the known roles of metalloproteinases in infection and the current understanding for their role in facilitating immune responses. These responses are broadly outlined in Figure 1.5.
Figure 1.5. The role of metalloproteinases in mediating the immune response.
The release of metalloproteinases is observed in diseases such as influenza virus infection (A). These enzymes can help control disease and can enhance the inflammatory process by cleaving chemokines and generating chemokine gradients to enhance immune cell migration (B). Furthermore, metalloproteinases can cleave components of the basement membrane, such as collagen and proteoglycans, allowing immune cell infiltration into the diseased tissues (C). For ease, MMPs denotes all metalloproteinases - MMPs, ADAMs and ADAMTS enzymes.
1.10.1 Detection of metalloproteinases throughout influenza virus infection

Upon infiltration of the host, pathogens deploy several immune evasion strategies to enhance survival. This includes taking advantage of host cell machinery for replication. Metalloproteinase activity is commonly hijacked to enhance tissue destruction, cell lysis and enhance replication of progeny (222). Metalloproteinase expression and activity has been detected and described in many bacterial and viral infections [e.g. Helicobacter pylori, human immunodeficiency virus (223-225) and influenza virus infection (161, 222, 226-228)].

As discussed in section 1.8, ADAMTS7 metalloproteinase expression is critically important for influenza virus replication (161). Other studies have indicated that influenza virus infection results in a significant release of metalloproteinases, mainly MMP9 (222, 226, 227, 229-231). The release of MMP9 predominantly occurs from infiltrating neutrophils and its release is stimulated by pro-inflammatory cytokines, such as TNFα, IL6 and IL1β (230). Neutrophil numbers are found to be at their highest in the lung between 3-5 dpi. MMP9 expression levels coincide with neutrophil infiltration into the lung, peaking at 3-5 dpi (232). Influenza virus mediated enhanced expression of MMP9 is also observed in cell culture models (228). Here, MMP2 and 9 expression and activity can be stimulated by influenza virus infection of epithelial cell lines (228).

1.10.2 Metalloproteinase control of cytokine cleavage

Cytokine expression and signalling is essential for host cell immunity in response to infection (233). For the activation of immune responses cytokine signalling needs to occur, cytokines need to be released from the ECM and cleaved into their soluble forms, from here they can bind to their receptors and activate the signalling cascade. This process can be performed by metalloproteinases (193). TNFα is a pro-inflammatory cytokine that induces immune cell migration (233). A number of diseases including rheumatoid arthritis (234, 235)
and Crohn’s disease are also associated with TNFα expression (236). Cleavage of the TNFα’s C-terminus is required to release its soluble form and to induce pro-inflammatory effects (193). ADAM17, or TNFα convertase, is the major enzyme involved in this process. ADAM10, MMP1, 2, 3, 7, 9 and 12 can also cleave TNFα, but not as efficiently as ADAM17 (187, 237, 238). Once cleaved TNFα can induce the expression of a number of metalloproteinases including MMP3, 9 and 14 and ADAMTS1, 4, 5 and 7 (239). Release of these metalloproteinases can have downstream effects on inflammation and immune cell functions, such as the release of pro-inflammatory cytokines.

Chemokines, a subfamily of cytokines, attract immune cells towards damaged or infected tissue. Neutrophils are typically the first cell to respond to infection and infiltration may be supported by metalloproteinase activity. During allergic rhinitis, upregulated ADAM12 cleaves and releases neutrophil chemoattractants (CXCL1 and 8) from the ECM, resulting in recruitment of neutrophils to the nasal cavity (240). Mmp7−/− and Mmp8−/− mice have also been utilised to discover the role for these metalloproteinases in neutrophil chemotactic gradients (241, 242). These metalloproteinases are involved in the cleavage of CXCL2 and syndecan-1, respectively, which control the release of chemokines and neutrophil chemotaxis (242). Whilst the studies discussed here do not relate to influenza viruses or infectious diseases it is important to note that cleavage of these cytokines by metalloproteinases would have a definitive role in the immune response to influenza virus infection, and it is imperative that we gain a thorough understanding of these roles.

1.10.3 The role of metalloproteinases in immune cell migration

ECM components can block migration and infiltration of innate and adaptive immune cells, preventing effective clearance of infection. Immune cell infiltration into the infected tissues requires traversing the basement membrane and physical barriers imposed by the ECM.
1.10.3.1 Innate immune cell migration

Neutrophils are generally the first infiltrating cell population into infected tissue (90, 91). Neutrophil infiltration of influenza virus infected lungs of mice correlated with upregulated levels of MMP2 and 9, leading to increased pathogenesis due to MMP9 mediated tissue damage (105). In support of this study, influenza virus infected Mmp9\textsuperscript{−/−} mice have decreased neutrophil infiltration into the lung (105). Although, this reduced neutrophil infiltration affected the ability for Mmp9\textsuperscript{−/−} mice to remove influenza virus infected cells (105), highlighting the importance of neutrophils in this infection.

In several non-influenza virus infection models, it has been observed that macrophages must first traverse the basement membrane of inflamed tissues. MMP2, 9, and 14 (MT1-MMP) activity increase macrophage infiltration by degrading collagen components of the basement membrane (243-246). Depletion of plasminogen (an activator of MMP9) in mice resulted in decreased macrophage infiltration following thioglycolate-induced peritonitis, which contributed to increased aortic aneurysms (244). Moreover, Mmp2\textsuperscript{−/−} mice show decreased infiltration, resulting in enhanced renal fibrosis because of decreased ECM remodelling (247). Finally, macrophage infiltration into the dermal layer was altered in Mmp14\textsuperscript{−/−} mice with croton-oil induced contact dermatitis (245). Whilst these discussed studies are not influenza-specific studies, collectively, these studies highlight the importance for MMPs in macrophage migration. In addition, the related enzymes, ADAMs (ADAM8, 9, 15 and 19) and ADAMTS (ADAMTS1, 4, 5 and 8) are highly expressed in macrophage-rich areas of atherosclerosis (248-252), although, it is unclear if this expression confers improved infiltration capacity (239, 253).

Migration of DCs is important for activation of the adaptive immune response (72, 73). DCs recognise and capture antigen in infected tissue before trafficking to draining lymph nodes to present antigen to cells of the adaptive immune system (72, 73). The ECM has been shown to block the migration of DCs \textit{in vitro}, which could significantly impact their migration \textit{in vivo}. 
Indeed, the addition of extracellular MMPs (e.g. MMP9) enhanced the degradation of the ECM and improved DC migration in vitro (254). This result was supported in vivo, where lipopolysaccharide-induced DCs derived from Mmp9\(^{−/−}\) mice show decreased infiltration into the draining lymph node (254). Reduced DC infiltration, as observed in Mmp9\(^{−/−}\) mice, suggest that these mice might be susceptible to infection if the activation of the adaptive immune response is impeded due to delayed DC migration.

1.10.4.2 Adaptive immune cell migration

Research documenting the involvement of metalloproteinases in the migration of adaptive immune cells is relatively limited. Lymphopoiesis studies by Melamed and colleagues has shown that MMP9 secretion is altered during different stages of B cell development (255). Secretion of MMP9 is markedly increased in pro-B and pre-B cells and is rapidly reduced in immature, transitional and mature B cells (255). However, further studies are required to tease out the role for metalloproteinases in B cell development and migration.

The migration of T cells towards targeted tissues (e.g. cancerous, infected) is vital for immunosurveillance and homeostasis. T cell exodus from the blood stream involves an initial attachment of T cells, followed by degradation of the basement membrane ECM to allow entry into these targeted tissues. Basement membrane components, such as collagen, laminin, fibronectin and gelatin, can inhibit the T cell extravasation process (256-258). Metalloproteinases play a key role in the removal of these basement membrane components, particularly collagen, promoting the migration of T cells. Studies involving T cell migration have identified that T cells require the secretion of MMP2 and 9. Inhibiting these MMPs significantly reduced collagen degradation and impeded T cell movement in a transwell-migration assay (256). In support, in vivo inhibition of the Wnt signalling, the major signalling pathway for the secretion of extracellular MMP2 and 9, in mice prevents ECM degradation.
and, hence, T cell migration through the basement towards a dermally-induced cytokine (TNFα and IFNγ) signal (259).

Like the aforementioned basement membrane components (collagen, laminin, fibronectin and gelatin), versican, an ECM proteoglycan, can also restrict T cell migration (257). Versican contains an N-terminal (immunoglobulin-like) domain, followed by a GAG domain and a C-terminal domain (Figure 1.6A) (260, 261). The final structure of versican can vary based on mRNA splicing within the GAG domain. This splicing can produce 4 isoforms termed V0, V1, V2 and V3. V0 contains both GAG-α and GAG-β domains, V1 has only the GAG-β domain, V2 has the GAG-α domains, and V3 is lacking GAG domains and is this not a proteoglycan but rather a glycoprotein (260). GAG domains contain attachment site for CS chains. Interaction of these domains with different binding partners [e.g. hyaluronan (262), tenascin-R (263), L-selectin (264, 265)] leads to various functional consequences for ECM structure and cell migration (261) (Figure 1.6B). In particular, the N-terminus of versican has been shown to bind hyaluronan, a non-sulphated GAG widely distributed in connective tissue, via a link protein to maintain ECM structure (266). The interaction of versican and hyaluronan can increase the viscosity of the ECM and has been shown to inhibit the amoeboid shape formed by migrating CD4+ T cells (257). When CD4+ T cells are stimulated with the immunostimulatory molecule Poly I:C, increased versican expression prevented cell “crawling” along the ECM scaffold (257). Further evidence is provided following analysis of versican expression in human cervical cancer samples. Increased versican expression in stromal cells was shown to inhibit CD8+ T cell invasion, which may impede the removal of cancerous tissue (258). MMP1, 2, 3, 7 and 9 and ADAMTS1,
Figure 1.6. The structure and binding partners of versican.

(A). Versican (V0 isoform) contains a hyaluronan binding domain, two GAG domains (α and β domains), an epidermal growth factor receptor domain, a lectin-like domain and terminates in a complement-regulatory protein. Some of these domains can interact with extracellular factors (highlighted in the red boxes) which can influence cellular processes. Adapted from Wu et al (267). (B). A diagrammatic representation of the interaction between versican, hyaluronan and CD44. Adapted from Ween et al (268).
4, 5, 9 and 15 can remodel and cleave versican (198, 269-272), however only MMP2 and 9 mediated collagen-degradation has been associated with T cell migration (273).

1.11 ADAMTS5 and ADAMTS7

1.11.1 ADAMTS5

ADAMTS5 (Figure 1.7) was first discovered in 1999 (196, 274), and has since been found in various cells and tissues throughout the body including the heart, brain, macrophages smooth muscle cells and lung epithelium (275). ADAMTS5 contains the typical C-terminal domains, including the catalytic, disintegrin-like and thrombospondin-1 domain. Additionally, ADAMTS5 contains one C-terminal thrombospondin-1 repeat with attached molecules within its cysteine rich and spacer domain. Binding sites found within the cysteine rich domain and spacer domain help localize ADAMTS5 to the ECM. Heparin, a highly sulfated GAG, has ADAMTS5 binding sites in the cysteine rich and spacer domains (196), allowing ADAMTS5 to localize within the ECM. Hyaluronan, a non-sulfated GAG, binds to the disintegrin-like domain of ADAMTS5 (276) and localizes ADAMTS5 to the ECM through its interaction with CD44 (277), a cell-surface glycoprotein.

Catalytically active ADAMTS5 has the ability to act on and cleave its extracellular substrates, predominately the hyalectan/lectican class of chondroitin sulphate proteoglycans: aggrecan, versican, brevican and neurocan (278, 279). Cleavage of versican by ADAMTS5 is essential for the removal of webbing in between digits through the remodeling of the ECM (280). In arthritis, hyaluronan interacts with aggrecan in the synovial joint to form large aggregates. Hyaluronan serves to lubricate the joint and aggrecan gives the joint its load bearing properties, compressibility and elasticity (165, 221, 281). As previously mentioned, cleavage of aggrecan by ADAMTS5 has been shown to exacerbate osteoarthritic disease (165, 221).
*Adamts5−/−* mice have been used to understand the role this enzyme in osteoarthritis. These studies indicated that *Adamts5−/−* mice are viable and were initially characterized as phenotypically normal based on gross phenotypical analysis of histological samples (213, 221). Upon closer investigation, *Adamts5−/−* mice have been shown to have decreased interdigital web regression (280), valve maturation (269), altered formation of multinucleated myotubes for skeletal muscle development (282) and these mice are resistant to osteoarthritis (213, 221).

### 1.11.2 ADAMTS7

ADAMTS7 (Figure 1.7) is expressed in the heart, lung, bone, cartilage, meniscus, skeletal muscle and fat (195, 283, 284). ADAMTS7 contains the typical conserved structural domains (the catalytic, disintegrin-like, thrombospondin, cysteine and spacer domains) in the N-terminus but differs in the number of C-terminal thrombospondin-1 repeats. ADAMTS7 has 7 C-terminal thrombospondin-1 repeats separated by a mucin domain (195). Within the mucin domain are attachment sites for chondroitin sulphated GAGs, covalently bound to serine residues (195).

Catalytically active ADAMTS7 has been identified in arthritis (285, 286), atherosclerosis (283) and cancer (287). In arthritis, increased levels of ADAMTS7 are believed to cleave cartilage oligomeric matrix protein, compromising cartilage function (288). ADAMTS7 is also observed to promote migration of vascular smooth muscle cells leading to vessel thickening in arthritis (289). Increased ADAMTS7 expression has also been detected in the urine of patients with prostate and bladder cancers (287). Some of these processes, such as arthritis and atherosclerosis, could be prevented when ADAMTS7 is inhibited by α2 macroglobulin or granulin epithelin precursor (290, 291).
ADAMTS5 and ADAMTS7 each contain conserved structural domains at the N-terminus but differ in the number of C-terminal thrombospondin-1 repeats. ADAMTS7 C-terminal repeats are separated by a mucin domain. The mucin domain of ADAMTS7 has covalently attached chondroitin sulphate and sialic acid chains. ADAMTS5 can bind hyaluronan and heparin in its ancillary of its domains.

Figure 1.7. The structure of ADAMTS5 and ADAMTS7.
Studies in *Adams7*\(^{-/-}\) knockout mice, although limited, indicate that these mice are healthy and viable (283, 284). These studies show that *Adams7*\(^{-/-}\) mice have normal whole blood counts and gross morphology (284). It was identified that *Adams7*\(^{-/-}\) mice had decreased anxiety and increased lung function parameters (284). Phenotypic analysis has identified that these mice are less susceptible to artherogenesis and neointima formation and that ADAMTS7 promotes vascular remodelling (283, 284). More studies in these mice are required to gain a greater understanding the role of ADAMTS7 in cellular processes.

1.11.3 *Evolutionary relationships of ADAMTS5 and ADAMTS7*

Evolutionary clades formed within the ADAMTS family spark interest due to their distinct substrate specificities. There are four evolutionary related groups within the ADAMTS family (see Figure 1.8); the proteoglycanases (ADAMTS1, 4, 5, 8, 9, 15 and 20), the procollagenases (ADAMTS2, 3 and 14), an under-characterized clade (ADAMTS6, 7, 10, 12, 16, 17, 18 and 19) and the Von-Willebrand cleaving protease (ADAMTS13). Clade members can form cooperative proteolytic networks (292). For example, ADAMTS compensation has been observed in collagen formation where ADAMTS14 continues to cleave procollagen in the absence of ADAMTS2, resulting in a less penetrant form of Ehlers Danlos syndrome (293).

ADAMTS5 is closely related to ADAMTS1, 4, 8, 9, 15 and 20 (292). All six enzymes have the ability to cleave aggrecan and versican, being termed “aggrecanases” and “versicanases”, respectively (279). As aggrecan and versican make up the majority of the ECM, the removal of one ADAMTS enzyme without the compensatory effects of others may affect homeostasis including migration, differentiation and proliferation of cells (196, 261).

ADAMTS7 and ADAMTS12 are derived from a single gene duplication, and only differ in length, with ADAMTS12 having a shorter mucin domain (195). Both metalloproteinases cleave cartilage oligomeric matrix protein, which has led to their
association with arthritis (291). ADAMTS12 has a novel role in inflammation and it would be of interest if similar inflammation properties could be induced by ADAMTS7. The relationship between ADAMTS12 and inflammation was originally identified in an allergen induced inflammation model. This study used an *Adamts12*−/− knockout mouse, which displayed exacerbated asthma pathologies (compared to their WT counterparts) related to increased neutrophil influx (294). Furthermore, laboratory-induced sepsis, pancreatitis and colitis all resulted in increased inflammatory responses in *Adamts12*−/− mice (295). This result was caused by observed delays in neutrophil apoptosis in these mice, suggesting that ADAMTS12 controls neutrophil death. As ADAMTS7 and ADAMTS12 are very closely related it is likely ADAMTS7 has similar pro-inflammatory effects.
Figure 1.8. The evolutionary relationships of ADAMTS family members. The ADAMTS family contains 19 members in mammals which all contain similar N-terminal structures but differ at the C-terminal end of the protease. There are four evolutionary clades: ADAMTS1, 4, 5, 8, 9, 15 and 20 (proteoglycanases) ADAMTS7, 10, 12, 16, 17, 18 and 19 (poorly defined) ADAMTS13 (Von Willebrand factor) and ADAMTS2, 3 and 14 (pro-collagenases) (292).
1.12 Hypothesis and Aims

Given the identification of ADAMTS7 and ADAMTS5 (through downstream analysis) in the human protease siRNA screen in influenza virus infection (161), and the already established roles of related metalloproteinases in the immune response, we hypothesized that ADAMTS5 and ADAMTS7 would have dynamic roles in the outcome of influenza virus infection, through aiding cell migration and inflammatory properties.

Therefore, the aims of this project are:

1. To define the role of ADAMTS5 in vivo influenza virus infection.
2. To determine the function ADAMTS7 in influenza virus infection.
3. To understand the role of ADAMTS5 and ADAMTS7 in other models of virus infection.

This project aims to investigate the role that ADAMTS5 and ADAMTS7 in the immune response following infectious disease. We aim to assess the function of these enzymes in vivo using the well-established C57BL/6 mouse model to determine the mechanism by which these enzymes influence pathogenesis and immunity.
Chapter 2 - Materials and Methods

2.1 Reagents

2.1.1 Buffers and solutions

*Ammonium Tris Chloride:* Ammonium Tris Chloride was prepared using 0.16 M HCl and 0.17 M Tris-chloride (Amresco, Ohio, USA) dissolved in MilliQ water and the pH was adjusted to 7.65. MilliQ water was obtained using a MilliQ direct filtration device (Merck Millipore, Darmstadt, Germany).

*Bovine Serum Albumin (BSA):* Ten % BSA (Invitrogen, California, USA) was prepared following the manufacturer’s instructions by tissue culture staff at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australian Animal Health Laboratory (AAHL), Geelong, Australia.

*β-galactosidase staining solution:* β-galactosidase staining solution was prepared using 5 mM potassium ferricyanide (C₆N₆FeK₃) (Sigma-Aldrich, Missouri, USA), 5 mM potassium ferrocyanide (C₆N₆FeK₄) (Sigma-Aldrich) and 1 mg/ml β-galactosidase powder (Amresco) in dimethyl sulfoxide (275). The final solution was prepared in MilliQ water.

*β-galactosidase wash buffer:* β-galactosidase wash buffer was prepared using 0.1 M phosphate buffer (pH of 7.4), 2 mM MgCl₂ (Sigma-Aldrich), 0.02 % NP-40 (Amresco) and 0.01 % Na deoxycholate (Sigma-Aldrich) in MilliQ water (275).
**Collagenase tissue dissociation solution:** Collagenase (Sigma-Aldrich) was diluted to 2 mg/ml in Hank’s Balanced Salt Solution (HBSS) (Invitrogen). HBSS was prepared by tissue culture staff at CSIRO AAHL, Geelong.

**CXCL12 T cell migration media:** CXCL12 (BioLegend, California, USA) was diluted to 10 ng/ml working concentrations in Roswell Park Memorial Institute (RPMI) (Invitrogen) media.

**Dulbecco’s Modified Eagle Medium (DMEM):** DMEM (Invitrogen) was supplemented with 10 % heat-inactivated foetal calf serum (FCS) (HyClone Scientific, Delaware, USA), 2 mM L-glutamine (Invitrogen), 100 µM minimum essential media, 100 µM non-essential amino acids (Invitrogen), 5 mM sodium pyruvate (ThermoFisher, Massachusetts, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin (Invitrogen), 55 µM β-mercaptoethanol (Invitrogen) and 100 µg/mL 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) (MP Biomedicals, California, USA). HEPES solution was prepared using manufacturer’s guidelines by tissue culture staff at CSIRO AAHL, Geelong. FCS was heat inactivated in a 56 °C water bath by tissue culture staff at CSIRO AAHL, Geelong, Australia.

**Fluorescent Activated Cell Sorter (FACS) Buffer:** FACS buffer was prepared using 10 % BSA and 10 % sodium azide (Sigma-Aldrich) in phosphate buffered saline (PBS) (Oxoid, Hampshire, United Kingdom). PBS was prepared following the manufacturer’s instructions by tissue culture staff at CSIRO AAHL, Geelong, Australia.

**Fixing buffer:** Paraformaldehyde (Amresco) was prepared in PBS and diluted to a final concentration of 4 %.
**Genotyping Extraction buffer:** Extraction buffer was prepared using 25 mM NaOH and 0.25 mM ethylenediaminetetraacetic acid (EDTA) in MilliQ water.

**Genotyping Neutralisation buffer:** Neutralisation buffer contained 40 mM Tris-HCl in MilliQ water at a final pH of 5.

**Leibovitz-15 media:** Leibovitz-15 media was prepared by dissolving two packets of 2 x Leibovitz-15 powder (Invitrogen) in MilliQ water. The mixture was then adjusted to a pH of 6.8 and supplemented with 7 % NaCHO₃, 1 M HEPES buffer, 100 µg/ml penicillin, 100 µg/ml streptomycin and 40 mg/ml gentamycin (58).

**Laurel Broth (LB):** LB broth was prepared using 10 g/L Tryptone (BD Medical Supplies, North Ryde, Australia), 5 g/L yeast extract (Amresco), 10 g/L NaCl and 1 M NaOH in MilliQ water. In some experiments, LB was supplemented with 1 µg/ml ampicillin.

**Lysis Buffer:** Lysis buffer was prepared using a digestion buffer [50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA and 1 % sodium dodecyl sulphate (SDS)] and a 20 mg/ml proteinase K buffer (Sigma-Aldrich) prepared in MilliQ water.

**Nuclease free water:** Nuclease free water was purchased from Ambion (Burlington, Canada).

**RPMI-anti (serum-free media):** RPMI media was supplemented with 24 µg/ml gentamycin, 100 µg/ml penicillin and 100 µg/ml streptomycin.
**RPMI growth media:** RPMI media was supplemented with 10 % FCS, 2 mM L-glutamine (Invitrogen), 100 mM minimum essential media, 100 mM non-essential amino acids, 5 mM sodium pyruvate, 100 µg/mL HEPES buffer, 100 µg/mL penicillin, 100 µg/mL streptomycin and 55 µM β-mercaptoethanol.

**Trypan Blue:** 0.4 % trypan blue (Invitrogen) diluted in PBS was prepared by tissue culture staff at CSIRO AAHL, Geelong.

**Trypsin-Versene:** 0.25 % trypsin-EDTA (Invitrogen) diluted in PBS was prepared by tissue culture staff at CSIRO AAHL, Geelong.

**Trypsin Worthington:** 0.1 % L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington Biochemical Corporation (New Jersey, USA). Trypsin Worthington was prepared in PBS following the manufacturers’ instructions.

**Virus isolation buffer:** Virus isolation buffer was prepared using 10 % BSA, 100 µg/ml penicillin and 100 µg/ml streptomycin in PBS (296).

**Western Blot loading dye:** Loading dye was prepared using 0.5 M Tris-HCl (pH 6.8), 20 % (w/v) glycerol (Sigma-Aldrich), 10 % (w/v) SDS (Sigma-Aldrich), 55 mM β-mercaptoethanol and 10 % bromophenol blue (Sigma-Aldrich) in MilliQ water.

**Western Running Buffer:** Running buffer contained 30.3 g/L Tris base (Amresco), 144 g/L glycine and 10 g/L SDS prepared in MilliQ water. Running buffer was diluted in MilliQ water for immunoblotting, to a final concentration of 10 %.
**Western Transfer Buffer:** Transfer buffer contained 29 g/L glycine, 58 g/L Tris base and 3.7 g/L SDS prepared in MilliQ water. Transfer buffer was diluted in MilliQ water for immunoblotting, to a final concentration of 10 %.

2.1.2 Cell lines

All cell lines were supplied by the CSIRO AAHL tissue culture facility. Human Embryonic Kidney 293T (HEK293T) cells were maintained in DMEM growth media. MDCK cells were maintained in RPMI growth media.

2.1.3 Viruses

Mouse-adapted influenza virus strains X31 (H3N2) and PR8 (H1N1) were grown in eggs and titrated by plaque assay on MDCK cells (see section 2.5.9) (58, 59, 65). Hendra virus (HeV) (HeV/Australia/Horse/2008/Redlands) (HeV) was isolated by CSIRO AAHL staff from the spleen of a Redlands Bay HeV-infected horse (297). The isolated virus was then passaged in VERO cells by Bio-security Level (BSL) 4 staff at CSIRO AAHL three times, before titration using a Tissue culture infectious dose (TCID) $50$ assay (297).

2.1.4 Mice

WT mice, on a C57BL/6 background, were obtained from the Animal Resources Centre, Western Australia. B6.129P2-Adams5$^{tm1Dgen}$/J (Adams5$^{-/-}$) on a C57BL/6 background were obtained from Jackson Laboratories (Maine, USA). Adams7$^{-/-}$ and Versican$^{\text{heart defect}}$/+(Vcan$hdf$) mice on a C57BL/6 background were a kind donation from Suneel Apte, Cleveland Clinic, Ohio, USA. Adams7$^{-/-}$ and Vcan$hdf$ mice were generated by insertion of a LacZ cassette
insertion into the gene of interest (298). All mice strains were housed at the Deakin University animal house.

2.2 Cell Culture

2.2.1 Thawing of HEK293T and MDCK cells

Cryogenic tubes (Nunc, New York, USA) containing cells were thawed in a water bath at 37 °C. The cell suspension was then added into a T75 flask (Corning, Lowell, USA) containing RPMI or DMEM growth medium and incubated overnight as described above (see section 2.1.2).

2.2.2 Maintenance of cells

HEK293T and MDCK cells were passaged at 95 % confluence. Flask medium was aspirated and cells were washed with PBS. Cells were then treated with 0.125 % trypsin versene/5 mM EDTA at 37 °C.

Growth medium was then added to inhibit trypsin activity. HEK293T and MDCK cells were typically passaged 1:10 into a new T75 flask containing growth medium (in DMEM and RPMI, respectively). Flasks were then incubated at 37 °C, with 5 % CO₂. Growth medium was replenished every 48 hours.
2.3 Molecular methods

2.3.1 RNA extraction

Cell and tissue RNA was extracted using RNeasy (Invitrogen), following the manufacturer’s guidelines. RNA concentration was determined using the NanoVue spectrophotometer (GE Healthcare, Buckinghamshire, UK). RNA quality was determined using an \( A_{260}/A_{280} \) nm ratio. A ratio of ≥ 1.8 was used to determine RNA sample purity. RNA samples were stored at -80 °C.

2.3.2 Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA was synthesized from purified RNA (see section 2.3.1) using a SuperScript First strand cDNA synthesis kit (Invitrogen), as per the manufacturer’s instructions. cDNA concentration was determined using a NanoVue spectrophotometer. Purity was determined using an \( A_{260}/A_{280} \) nm ratio of ≥ 1.8 (see Section 2.3.1). cDNA samples were stored at -80 °C.

2.3.3 Polymerase chain reaction (PCR) Visualisation

PCR products were resolved on 1.5 % (w/v) gels containing 1 x tris-acetate EDTA buffer (Amresco), and 1 x SYBR safe deoxyribonucleic acid (DNA) gel stain (Invitrogen) at 100 volts for 35 minutes. DNA Molecular Weight Marker XIV (100 base pair ladder) (Roche, Castleville, Australia) was used to confirm the molecular weight of the expected PCR products. Gels were visualised under UV illumination on a ChemiDoc XRS (Bio-Rad, Gladesville, Australia) using Quantity ONE software for analysis.
2.3.4 Primer Design

Primers for PCR experiments were designed using the Primer3Plus website; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. DNA oligonucleotides were acquired from Sigma-Aldrich and Gene Works (Table 2.1 and 2.2). The following parameters were generally used for optimal primer design: length (18 – 27 base pairs), melting temperature (57 – 63 °C) and Guanine-Cytosine base pair concentration (20 - 40 %).

2.3.5 Plasmid DNA transformation

Plasmids (2 µg) containing versican constructs were added to DH5α E. coli cells (2 x 10^6 cells) (Promega, Wisconsin, USA) and incubated for 20 minutes on ice, followed by heat shock at 42 °C for 45 seconds and a further incubation on ice for 2 minutes. Under sterile conditions, LB was added and cells were incubated at 37 °C for 1 hour with agitation at 250 revolutions per minute (rpm). Transformation mix was then plated onto LB agar plates containing 100 µg/mL ampicillin and incubated overnight at 37 °C. Under sterile conditions, single colonies were picked from the plates and grown overnight in 6 mL LB containing Ampicillin (50 µg/mL) at 37 °C and 250 rpm to amplify the selected colonies.

2.3.6 Plasmid DNA minipreps

DH5α bacterial cell cultures containing expression constructs were centrifuged at 3000 rpm for 10 minutes to obtain bacterial cell pellets. A plasmid DNA miniprep kit (Qiagen, Hilden, Germany) was used to elute DNA following the manufacturer’s instructions. DNA was eluted in DNase-free water, and quantitated using the NanoVue spectrophotometer (see section 2.3.1). DNA constructs were then stored at -20 °C.
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<tr>
<th>Mouse</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| Adamts5+/− | *Adamts5*/* allele*  
Forward primer, 5’-GGG CCA GCT CAT TCC TCC CAC TCA T  
*Common allele*  
Reverse primer, 5’-CGC AGC TGA CTG CTC TTG TGC TTG  
*WT allele*  
Forward primer, 5’-GCA TAC CAC TCC AAA CTT AGA GAG G |
| Adamts7+/− | *WT allele*  
Forward primer, 5’-AAC TCC TTT CTG GAA TAC CCC ATC  
Reverse primer, 5’-GAG AGG AAT CAG GTT ACA ACT AGG  
*β-galactosidase allele*  
Forward primer, 5’-AAA CCG ACT ACA CAA ATC AG  
Reverse primer, 5’-GCT TCA TCC ACC ACA TAC AG-5’ |
| Vcan^{hv}/+ | Forward primer, 5’-AAC TCC TTT CTG GAA TAC CCC ATC  
Reverse primer, 5’-CCA TAA AGC CTG TGT GAA ATG CC |

*Table 2.1. Mouse genotyping primers used in this study.*
<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| ADAMTS1  | Forward primer, 5’-CCT GTG AAG CCA AAG GCA TTG  
            Reverse primer, 5’-TGC ACA CAG ACA GAG GTA GAG T |
| ADAMTS4  | Forward primer, 5’-CAA GCA GTC GGG CTC CTT  
            Reverse primer, 5’-GAT CGT GAC CAC ATC GCT GTA |
| ADAMTS5  | Forward primer, 5’-GGC AGG ACA CCT GCA TAT TT  
            Reverse primer, 5’-GCT ACT GCA CAG GGA AGA GG |
| ADAMTS7  | Forward primer, 5’-CTG GTT GAT AAT GGG GGT TG  
            Reverse primer, 5’-GGG CTA GGA GAC CAA GAT CC |
| ADAMTS8  | Forward primer, 5’-GAC AGA AGA CTC CCG CAA AG  
            Reverse primer, 5’-TTT CAC CAC CAC AAG GTT GA |
| ADAMTS9  | Forward primer, 5’-TTG TTG TCT CCA TGT CCA AAA G  
            Reverse primer, 5’-ATT GCT GAG GTT TG TGT CCA CAA T |
| ADAMTS12 | Forward primer, 5’-CCA GGG TTA GTC ACC TGG AA  
            Reverse primer, 5’-TTT CCT GGC CAT TAG GAG TG |
| ADAMTS15 | Forward primer, 5’-GCT CAT CTG CCG AGC CAA T  
            Reverse primer, 5’-CAG CCA GCC TTG ATG CAC TT |
| ADAMTS20 | Forward primer, 5’-TTG AAA TCC TTC AGC ACA GCT A  
            Reverse primer, 5’-GGT CCG TGC TGT TAA TTC TTT C |

*Table 1.2. SYBR green quantitate PCR (qPCR) primers used in this study.*
2.3.7 Transfections of plasmid constructs

HEK293T cells were seeded in 6 well plates at a density of 3 x 10^5 cells/well in DMEM growth medium. The following day, flasks were individually transfected with versican or with an empty vector control using Lipofectamine RNAi 2000 (Invitrogen) (198, 282, 299), as per the manufacturer’s instructions. After 5 hours, transfection medium was replaced with serum free medium (RPMI-anti) for 48 hours at 5 % CO2 and 37 °C. Conditioned media was collected after 48 hours of incubation (see section 2.3.8).

2.3.8 Collection of conditioned media from transfections

Transfected cells (section 2.3.7) were harvested and the suspension deposited into a falcon tube (Corning), followed by centrifugation at 3000 rpm for 5 minutes (198, 282). Supernatant containing the conditioned media was then removed and stored at -80 °C.

2.3.9 Immunoblotting

Immunoblotting was used to confirm the presence of constructs in conditioned media of transfected HEK293T cells (section 2.3.7). Prior to immunoblotting, western blot loading dye was added to conditioned media (section 2.3.8) and samples were boiled for 5 minutes and then placed on ice for 2 minutes. Samples were then separated using 8 % SDS – Polyacrylamide Gel Electrophoresis at 35 milli-amps for 60 minutes. Proteins were then transferred onto a nitrocellulose membrane at 150 milli-amps for 60 minutes and then detected using primary α-GAG antibody (versican) or α-DPEAAE (versikine - the versican cleavage fragment) in 1 % skim milk in Tris Buffered Saline Triton X (1 ng/µL) (clone 9E10) (Sigma-Aldrich). Blots were rinsed in 5 % skim milk in Tris Buffered Saline Triton X and then a goat-α-rabbit secondary antibody conjugated to horse radish peroxidase added for detection (2 ng/µL).
(Sigma-Aldrich). Immunoblots were visualised using enhanced chemiluminescence reagent (GE Healthcare) on a ChemicDoc XRS (Bio-Rad, Gladesville, Australia) using Quantity ONE software for analysis. Immunoblots were analysed using ImageJ software.

2.4 Animal maintenance

2.4.1 Ethics Statement

All influenza virus animal experiments were conducted following Deakin University Animal Welfare Committee approval (A80-2011, G38-2013 and G35-2014: see section 2.5). HeV studies were approved by the CSIRO AAHL Animal Ethics Committee under AEC1765 and the Deakin University Animal Welfare Committee AEX09-2015 (see section 2.6). All procedures were conducted according to the guidelines of the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes. For all infection models, a weight loss of 20% initial body weight was considered a humane endpoint.

2.4.2 Maintenance of mice

Mouse cages were changed weekly by Deakin animal house staff. Cages (Tecniplast, Sydney, Australia) were sterilised by autoclaving in a Chipmunk autoclave (Atherton, Melbourne, Australia) at 121 °C. Tap water used for drinking was also sterilised. Food and water were provided ad libitum.
2.4.3 Breeding of Adamts5<sup>−/−</sup> mice

Three 1st generation Adamts5<sup>−/−</sup> mouse breeding pairs (obtained at backcross 8) were set up and mated. Second generation pups were weaned from their parents at 3 weeks of age. Breeding pairs from 2nd generation mice were set up at 6 weeks of age. Third generation mice were weaned at 3 weeks of age and grown to 8 – 10 weeks of age prior to experimental analysis.

2.4.4 Breeding of Adamts7<sup>−/−</sup> mice

Three 1st generation Adamts7<sup>+/−</sup> mouse breeding pairs (obtained at backcross 8) were obtained and mated. Second generation pups were weaned from their parents at 3 weeks of age. Homozygote (Adamts7<sup>−/−</sup>) breeding pairs from 2nd generation mice were set up at 8 weeks of age. Third generation mice were weaned at 4 weeks of age and grown to 8 - 10 weeks of age prior to experimental analysis.

2.4.5 Breeding of Vcan<sup>hdf/+</sup> deficient mice

Three Vcan<sup>hdf/+</sup> mouse breeding pairs (backcrossed 8 times) were mated and weaned from their parents at 3 weeks of age. At 8 - 10 weeks of age mice were used in experimental analyses.

2.4.6 Breeding of Adamts5<sup>−/−</sup>xVcan<sup>hdf/+</sup> mice

Adamts5<sup>−/−</sup>xVcan<sup>+/+</sup> and Adamts5<sup>+/−</sup>xVcan<sup>hdf/+</sup> breeding pairs were obtained and mated. Second generation pups were then weaned from their parents at 3 weeks of age. Second generation Adamts5<sup>+/−</sup>xVcan<sup>+/+</sup> and Adamts5<sup>+/−</sup>xVcan<sup>hdf/+</sup> mice were set up at 6 weeks of age. Third generation Adamts5<sup>−/−</sup>xVcan<sup>hdf/+</sup> and Adamts5<sup>−/−</sup>xVcan<sup>+/+</sup> mice were mated at 6 weeks of
age and offspring weaned at 3 weeks of age. Mice were then genotyped and maintained to 8 weeks of age prior to influenza virus infection.

2.4.7 Genotyping of mice

To genotype mice, genomic DNA from ear tissue was placed in genotyping extraction buffer and incubated at 100 °C for 30 minutes. Samples were then placed on ice and allowed to cool, before the addition of equal volumes of genotyping neutralisation buffer. A PCR reaction was performed on genomic DNA using genotyping primers found in Table 2.1. A negative RNase-free water control was used to detect for potential DNA contamination. The PCR reaction was performed in the Bio-Rad thermocycler under the parameters found in Table 2.3 and bands were visualised as described in section 2.3.3. Additional details to identify and analyse PCR products from the genomic targets in Adamt5<sup>−/−</sup> mice can be found at [http://jaxmice.ax.org/strain/005772.html](http://jaxmice.ax.org/strain/005772.html).

2.4.8 Euthanasia

Mice were euthanized using slow-fill inhalation of CO<sub>2</sub> at various time points post infection or for colony maintenance.

2.5 Influenza virus infection and analysis

2.5.1 Intranasal influenza virus infection of mice

Adamts5<sup>−/−</sup>, Adamts7<sup>−/−</sup>, Vcan<sup>hdf/+</sup>, Adamts5<sup>−/−</sup>xVcan<sup>hdf/+</sup> and WT mice were individually numbered and assigned to groups for influenza virus infection. Mice were anaesthetised by inhalation of 1 - 3 % isoflurane in oxygen. X31 (H3N2) influenza virus (1x10<sup>4</sup> plaque forming
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>PCR Parameters</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adamts5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>15 minutes at 95 °C</td>
<td>1</td>
</tr>
</tbody>
</table>
| Adamts5<sup>−/−</sup> | 94 °C for 30 seconds  
   60 °C for 90 seconds  
   72 °C for 30 seconds | 35 |
| Adamts5<sup>−/−</sup> | 72 °C for 10 minutes | 1 |
| Adamts7<sup>−/−</sup> | 1 minute at 95 °C | 1 |
| Adamts7<sup>−/−</sup> | 95 °C for 30 seconds  
   58 °C for 1 minute  
   72 °C for 1 minute | 35 |
| Adamts7<sup>−/−</sup> | 72 °C for 10 minutes | 1 |
| Vcan<sup>hdf/+</sup> | 5 minutes at 95 °C | 1 |
| Vcan<sup>hdf/+</sup> | 95 °C for 30 seconds  
   56 °C for 30 seconds  
   72 °C for 1 minute | 35 |
| Vcan<sup>hdf/+</sup> | 72 °C for 10 minutes | 1 |

Table 2.3. PCR parameters followed for mouse genotyping.
Figure 2.1. Influenza virus infection models used throughout this study. Mice were either infected intranasally for primary acute responses or intraperitoneally for secondary challenge analyses. Seven weeks after intraperitoneal injection with PR8 (H1N1) influenza virus (1.5x10^7 pfu/mouse), mice were intranasally challenged with X31 (H3N2) influenza virus (1x10^4 pfu/mouse) and secondary challenge responses were measured at day 7 post challenge. *Secondary memory was not analysed in this study.
units (pfu/mouse) in PBS was then administered intranasally to each mouse (58, 65, 300). Body weights were recorded daily. Figure 2.1 details the influenza virus infection regime for in vivo influenza experiments undertaken in this study.

2.5.2 Intraperitoneal influenza injection of mice

Adamts5−/−, Adamts7−/− and WT mice were individually numbered and assigned to groups for influenza virus priming by intraperitoneal injection. Each mouse was intraperitoneally administered PR8 (H1N1) influenza virus (1.5 x 10⁷ pfu/mouse) in PBS (65). Figure 2.1 demonstrates the experimental plan established for intraperitoneal influenza infection studies.

2.5.3 Preparation of mouse tissue-single cell suspensions

Mice were euthanized and lungs, spleen and MLN were removed from naïve or influenza virus infected mice and placed in HBSS. Figure 2.2 details the experimental assays performed on each tissue at various time points post infection.

Lung tissue was weighed and disrupted using a variable speed homogeniser (Dremel, Melbourne, Victoria). The homogenised lung was then centrifuged and the supernatant removed and stored at -80 °C for use in plaque assays (see section 2.5.9). Any remaining lung tissue was used for RNA extraction (see section 2.3.1) and qPCR reactions (see section 2.5.7). Lung was also collagenase digested (2 mg/ml in HBSS) and incubated at 37 °C for 30 minutes to generate single cell suspensions. Cells were then filtered through a 70 µM strainer into a 50 ml tube and washed with HBSS. Red blood cells were lysed using ammonium tris chloride buffer for 3 min at room temperature, washed twice in HBSS and resuspended in 1 mL RPMI growth medium for flow cytometric analysis (see section 2.5.4).
Figure 2.2. Experimental regimes following influenza virus infection. Tissues were removed from influenza virus infected mice at 3, 7, 10 and 30 dpi or at day 7 post-secondary challenge. Virus titres in the lung were assessed using plaque assays. Immune cell responses were characterised using qPCR and flow cytometric analysis of immune cell populations using antibodies, tetramers and intracellular cytokine staining assays.
Spleen and MLN were pushed through a 70 µM strainer to generate single cell suspensions. Spleen cell suspensions were further enriched for T cells following B cell panning on plates coated with goat anti-mouse IgG and IgM antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour at 37 °C. The enriched suspension was then centrifuged at 1600 rpm for 5 minutes and the supernatant removed. Five mL ammonium tris chloride buffer was added to the cell pellet for 3 minutes at room temperature to lyse red blood cells and the reaction neutralised through the addition of HBSS. Cell suspensions were washed and the cell pellet was resuspended in 3 mL RPMI growth medium.

2.5.4 Immune cell phenotyping

Single cell suspensions (see section 2.5.3) were added to a 96-well U-bottomed plate (Nunc), centrifuged (1500 rpm for 5 minutes) and then resuspended in 50 µL FACS buffer containing diluted antibodies (Table 2.4) for 30 minutes at 4 °C and analysed on a BD-LSRII (BD-USA). Results were analysed using Flowjo software version 7 (Flowjo LLC, Ashland, USA). Gating strategies for phenotyping analyses are displayed in Figure 2.3.

2.5.5 Tetramer and intracellular cytokine staining (ICS)

CD8+ lymphocyte populations from the spleen, lung and MLN were enumerated using fluorescently labelled tetrameric complexes directed against two immunodominant influenza-specific CD8+ T cells epitopes (NP<sub>366-374</sub>-PE or PA<sub>224-233</sub>-PE) (purchased from University of Melbourne, Australia) for 1 hour at room temperature in FACS buffer, as previously described (68, 149). Cells were washed and stained with anti-mouse CD8α-PERCP and analysed on a BD-LSRII (BD Biosciences). In some cases, CD62L-FITC and CD44-APC antibodies were also added. NP<sub>366-374</sub> and PA<sub>224-233</sub> CD8+ T cell function was further assessed using the ICS assay. Briefly, cells were cultured for 5 hours in a 96-well round bottom plates with influenza
<table>
<thead>
<tr>
<th>Target (Mouse)</th>
<th>Fluoro chrome</th>
<th>Clone</th>
<th>Company</th>
<th>Isotype</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>PERCP</td>
<td>RAB3-6B2</td>
<td>BD</td>
<td>Rat</td>
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</tr>
<tr>
<td>CD3</td>
<td>APC</td>
<td>145-2C11</td>
<td>BD</td>
<td>Hamster</td>
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</tr>
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<td>CD4α</td>
<td>FITC</td>
<td>RM4-4</td>
<td>BD</td>
<td>Rat</td>
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<tr>
<td>CD8α</td>
<td>PE</td>
<td>53-6.7</td>
<td>BD</td>
<td>Rat</td>
<td>1/400</td>
</tr>
<tr>
<td>CD8α</td>
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<td>53-6.7</td>
<td>BD</td>
<td>Rat</td>
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<tr>
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<td>M1/70</td>
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<td>BD</td>
<td>Hamster</td>
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<tr>
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<td>APC</td>
<td>IM7</td>
<td>BD</td>
<td>Rat</td>
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<td>CD62L</td>
<td>FITC</td>
<td>MEL-14</td>
<td>BD</td>
<td>Rat</td>
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</tr>
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<td>Rat</td>
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<tr>
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<td>BM8</td>
<td>BioLegend</td>
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<tr>
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<td>BD</td>
<td>Rat</td>
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</tr>
</tbody>
</table>

Table 2.4. Flow cytometry antibodies used in this study.
Figure 2.3. Gating strategy for immune cell phenotyping.
(A) Flow cytometric analysis showing the gating strategy used to distinguish CD4+ and CD8+ T and B220+ B cells. (B) Flow cytometric analysis depicting the gating strategy used to distinguish CD11c+ DCs, CD11b+ macrophages and neutrophils. APCs = antigen presenting cells.
NP<sub>366-374</sub> (ASNENMETM) or PA<sub>224-233</sub> (SSLENFRAYV) peptides in the presence of Golgi-plug (BD Biosciences) and IL2. Cells were then permeabilised and stained with anti- mouse CD8α-PERCP, anti-mouse IFNγ-FITC and TNFα-APC (BD Biosciences) as previously described (300). Data was acquired on a BD-LSRII and analysed using Flowjo software. Gating strategies for tetramer and ICS staining are displayed in Figure 2.4.

2.5.6 Magnetic enrichment of cells

CD8<sup>+</sup> and CD11c<sup>+</sup> splenocytes (see section 2.5.3) were isolated using negative selection kits and magnetic beads (Stem Cell Technologies, Vancouver, Canada) on a fully automated cell separator (Stem cell Technologies).

2.5.7 qPCR analysis for cytokine expression

Lung cDNA, 1 µL probe and primers (TaqMan, Mulgrave, Australia) were added to detect cytokine gene expression (TNFα and, IFNγ) (Table 2.5). qPCR samples were prepared in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems, Austin, Texas, USA). Each reaction contained 10 µM forward primer, 10 µM reverse primer and 10 µM probe (see Table 2.5), 10 µL of TaqMan 2X master mix and 100 ng of sample cDNA. Adhesive film was applied to the plates prior to centrifugation at 1600 RPM for 1 minute. The plates were placed on a 7500 Fast Real-Time PCR system (Applied Biosystems) under the following PCR cycle conditions; 10 minutes at 95 °C, and 40 repeating cycle of 95 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 30 seconds. Ct values were measured and compared to cDNA concentrations determined using the Oligreen protocol (see section 2.5.8).
Figure 2.4. Gating strategy used for tetramer and ICS analyses.
Flow cytometric gating strategy used to enumerate (A) tetramer$^+$CD8$^+$ T cells and (B) IFN$\gamma^+$CD8$^+$ T cells.
Table 2.5. *TaqMan* primers purchased for this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Product Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Mm00443528_m1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Mm01168134_ml</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mm99999915_g1</td>
</tr>
</tbody>
</table>
2.5.8 *OliGreen protocol*

A known 20 x DNA standard (Invitrogen) was diluted to in 1 x elution buffer and a series of dilutions followed to prepare 8 known DNA standards. Five µl of the DNA standards and cDNA samples used in qPCRs (see section 2.5.7) were loaded into their respective wells in a black 96 well plate (Nunc). Ninety-five µL of 1 x elution buffer was added to each well as was a further 100 µL of 1 x Quant-iT™ OliGreen® single stranded DNA reagent (Invitrogen). The plate was then read on a Perkin Elmer plate reader to determine cDNA concentrations for normalisation of Ct values obtained from RT-qPCR reactions.

2.5.9 *Plaque assay*

MDCK cells (1.2 x 10⁶ cells/well) were seeded in 6-well plates. The following day, confluent cell monolayers were washed with RPMI-anti. Ten-fold dilutions (10⁻¹-10⁻⁶) of infected lung supernatant (see section 2.5.3) were prepared in RPMI-anti. The RPMI-anti on the 6-well plates was removed and replaced with diluted (10⁻¹-10⁻⁶) virus supernatant and incubated at 5 % CO₂ and 37 °C for 45 minutes (rocking each plate every 15 minutes). Leibovitz-15 medium containing Trypsin Worthington and 1.8 % agarose in water were placed in a pre-heated 45 °C water bath. Following the 45 minute incubation, Leibovitz-15 agarose overlay was added to the MDCK monolayer and left to set at room temperature (301). Plates were then incubated at 5 % CO₂ and 37 °C for 3 days. Visible plaques formed in the agarose overlay were counted and virus titres determined.
2.6. **HeV infection and analysis**

2.6.1. **HeV inoculation in mice**

HeV infections were performed at BSL4, where CSIRO staff wore fully encapsulating suits with an external air supply. Eight-week-old *Adamts5*<sup>−/−</sup>, *Adamts7*<sup>−/−</sup> and WT female mice were anaesthetised through intraperitoneal injection with ketamine (75 mg/kg; Ketamil; Ilium, Smithfield, Australia) and medetomidine (1 mg/kg; Domitor; Novartis). Once anaesthetised, mice were intranasally inoculated with 50,000 TCID<sub>50</sub> HeV (HeV Australia/Horse/2008/Redlands) in saline (296). Mice were euthanized at 6 dpi.

Due to the nature of BSL4 agents and the detailed training required, these experiments were only performed twice, with 4 biological replicates.

2.6.2 **Tissue collection and processing**

At 6 dpi, mice were anaesthetised by CSIRO AAHL staff and blood was collected via cardiac puncture, placed into EDTA and serum separator tubes. Following cardiac puncture, brain, kidney, liver, lung and spleens were collected for virus isolation. Samples were then placed into virus isolation buffer containing aluminium silicate beads (Biospec Products Inc., USA) (296). Samples were homogenised for 30 seconds in a bead beater (Biospec Products Inc.), centrifuged for 1 minute at 1600 rpm and stored at -80 °C for subsequent processing (see section 2.6.3). The remaining tissues were processed following protocols outlined in section 2.6.4 for flow cytometric analysis. Aliquots for flow cytometry were stored in liquid nitrogen.
2.6.3 RNA extraction and virus quantification of HeV samples by qPCR

MagMAX lysis/binding solution (Ambion) was added to homogenised tissue samples (see section 6.2) by CSIRO AAHL staff for 2 hours and samples removed from BSL4 to BSL3. RNA was then extracted using the MagMAX-96 viral RNA isolation kit (Ambion). HeV Nucleocapsid (N) gene levels were assessed by qPCR (Table 2.6), following AgPath-ID one-step reverse transcription-PCR kit instructions (Applied Biosystems, Victoria, Australia). Cycle threshold (C\text{t}) values below 39.6 were considered positive (296). Samples were normalised against the 18S housekeeping gene.

2.6.4 Fixing of HeV infected cells for flow cytometry analysis

Frozen cells (see section 2.6.2) were removed from liquid nitrogen at BSL4 and were immediately thawed at 37 °C in a water bath. Once thawed, CSIRO AAHL BSL4 staff aliquoted cells into a 96-well plate, centrifuged, then stained with conjugated anti-mouse antibodies (see Table 2.4) for 30 minutes at 4 °C (see section 2.5.4). Cells were then washed in FACS buffer and resuspended in 2 % paraformaldehyde at room temperature for 3 hours. After this time cells were no longer infectious and were removed to BSL3 laboratories for flow cytometric analysis. Cells were then washed twice and resuspended in 200 µL FACS buffer. Samples were run on the BD-Fortessa (BD, Biosciences) HTS system. Data was then analysed using Flowjo software.
<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
</table>
| Hendra N Gene | Forward primer, GATATITTTGAGMAGGCGGCTAGTT  
|             | Reverse primer, 5’-CCCATCTCTAGTGCTGGGCTATTAG  
|             | Probe, 6FAM-CTACTTTGACTACTAAGATAAGA-MGB                                           |
| 18S         | Forward primer, 5’-GGG AGG TAG TGA CGA AAA ATA ACA AT  
|             | Reverse primer, 5’-TTG CCC TCC AAT GGA TCC T                                        |

*Table 2.6. TAQMAN primers designed for this study*
2.7 Mouse tissue experiments

2.7.1 β-galactosidase staining of tissues

MLN and lungs were removed from naïve and infected WT, Adamts5<sup>-/-</sup> and Adamts7<sup>-/-</sup> mice and fixed in 4% paraformaldehyde prepared in β-galactosidase wash buffer. Tissues were then washed in this buffer and incubated overnight in β-galactosidase staining solution at 37 °C. The next day, MLN and lungs were rinsed in wash buffer and fixed in 4% paraformaldehyde (prepared in wash buffer) at 4 °C overnight (275). After a brief rinse in wash buffer tissues were imaged then embedded in PBS before processing (see section 2.7.2).

2.7.2 Histology

Tissues from WT and Adamts5<sup>-/-</sup> mice were fixed in 4% paraformaldehyde at 4 °C overnight. Following processing (2 hours in 30% EtOH, 2 hours in 70% EtOH, 2 hours in 90% EtOH, 4 hours in histolene (Grale, Victoria, Australia) and 8 hours in paraffin (Sigma-Aldrich), tissues were paraffin-embedded and sectioned. Seven µM sections were mounted on a slide and left to dry at 37 °C overnight. Sections were then used for immunohistochemistry (see section 2.7.3).

2.7.3 Immunohistochemistry

Seven µM sections were stained with anti-GAGβ (versican) (1/1000) (Merck-Millipore) or anti-DPEAAE (versikine) (1/500) (ThermoFisher) antibodies in Tris Buffered Saline Tween 20 at 4 °C overnight. The following day, tissues were washed with 10% Tris Buffered Saline Tween 20 to remove excess antibody and incubated with Alexa-Fluor594 goat-α-mouse antibody (Life-Technologies, California, USA) (198, 275, 282). Sections were re-washed in 10% Tris Buffered Saline Tween 20 (3 x 10 min) and nuclei stained with DAPI
Sections were viewed under a confocal microscope at 60X magnification (Leica, Wetzlar, Germany).

2.7.4 *Ex vivo* migration assays

Migration assays were performed in 12-well 5 µm chamber inserts (Corning), as previously described (256). Inserts were coated with versican conditioned medium (see section 2.3.8) and recombinant mouse CXCL12 T cell migration medium (10 ng/ml) was added to the lower chamber of the transwell to promote migration. *Adamts5*−/− or WT enriched (section 2.5.8) CD8+ T cells (10^5) were loaded into the upper chamber. The cells were allowed to migrate for 4 hours at 37 °C and 5 % CO₂ before the removal of non-migrating cells in the upper chamber. The transwell membrane was then stained with haematoxylin (Sigma-Aldrich) to enumerate the number of migrated cells.

2.7.5 DC proliferation assays

DCs (CD11c+) and CD8+ T cells were isolated from the spleen of naïve WT and *Adamts7*−/− mice as previously described (see Section 2.5.6). DCs (1x10^4 cells) were then infected with PR8 (H1N1) influenza virus (multiplicity of infection (MOI) 0.6) and incubated at 37°C for 6 hours (68, 72, 154, 302). After 6 hours, CD8+ T cells (5x10^3 cells) were added to infected DCs and activation of both cell subsets was assessed 48 hours later via flow cytometry and results were analysed using Flowjo software version 7.
2.8 Statistical analyses

Normal distribution data is expressed as mean ± standard error of the mean (SEM); \( n \) represents the number of mice per group. Statistical differences between two groups were analysed by Student's \( t \)-test. Statistical differences between multiple groups were analysed by one-way ANOVA with Bonferroni post-test analysis. All statistical analyses were performed using GraphPad Prism 5 for Windows. In all cases, probability levels less than 0.05 (*\( p < 0.05 \)) were indicative of statistical significance.
Chapter 3 – ADAMTS5 and the clearance of influenza virus infection

3.1 Overview

ADAMTS5 is one of the most highly-characterised and well-known metalloproteinases in the ADAMTS family. ADAMTS5 has been shown to cleave the hyalectan class of chondroitin sulphate proteoglycans, including aggrecan, brevican, neurocan and versican (299, 303-305). Hyalectans/chondroitin sulphate proteoglycans are large aggregating macromolecules that hydrate tissue by attracting water molecules and confer rigidity to the extracellular space. Cleavage of these ECM components by ADAMTS5 has been associated with arthritis and developmental defects in vitro (281). The role of ADAMTS5 has been further defined in vivo by taking advantage of ADAMTS5 knockout mice (Adams5^−/− mice). These mice were previously generated through the insertion of a LacZ cassette into the ADAMTS5 gene (275), thereby preventing the translation of the enzymatically active ADAMTS5 protein. In these mice, the LacZ cassette, which contains β-galactosidase, can be used as a surrogate reporter of ADAMTS5 expression. β-galactosidase staining in tissues from Adams5^−/− mice has helped characterise tissue expression patterns of ADAMTS5 (275). Expression of ADAMTS5 has been detected in the cartilage, joints lungs, heart and spleen (275, 306).

The presence of ADAMTS5 in the embryonic lung, and more importantly within the alveoli (284), suggests that ADAMTS5 may have an unidentified role in the control of lung homeostasis. In support of this unknown role, personal communication from data obtained in a siRNA screen assessing proteinase activity in influenza virus infection, identified ADAMTS5 as a downstream modulator of influenza virus pathogenesis (Prof Ralph Tripp, University of Georgia, Georgia, USA). The presence of ADAMTS5 in the alveolar space and its identification in a downstream analysis of the siRNA screen indicates that ADAMTS5 may be
required for the clearance of influenza virus infection, possibly through its matrix remodelling capabilities.

Aside from determining the expression pattern of ADAMTS5, research has focused on ADAMTS5’s role in the development of arthritis. *Adams5<sup>−/−</sup>* mice are resistant to aggrecan cleavage in articular cartilage, and are thus protected from experimentally induced arthritis (213, 221). These observations have led to ADAMTS5 becoming a major drug target for arthritis therapy. Moreover the *Adams5<sup>−/−</sup>* mouse model has also been used to identify ADAMTS5’s key role in embryonic and skeletal muscle development, including limb and cardiac morphogenesis (269, 282). In these studies, *Adams5<sup>−/−</sup>* mice were observed to have reduced myoblast fusion resulting in weakened muscles (282). Additionally, *Adams5<sup>−/−</sup>* mice were found to have enlarged heart valves due to reduced proteoglycan turnover, and hence ECM accumulation occurs in these vessels (269).

In this current study, we utilise the *Adams5<sup>−/−</sup>* mouse model to assess the role of ADAMTS5 in the immune response to influenza virus infection. Herein, we describe a novel association between the ADAMTS family, influenza virus pathogenesis and immunity. To characterise these results *Adams5<sup>−/−</sup>* mice were intranasally infected with X31 (H3N2) influenza virus to assess primary infection or primed with PR8 (H1N1) and challenged with X31 (H3N2) to characterise secondary challenge in the absence of ADAMTS5.

### 3.2 *Adams5<sup>−/−</sup>* mice are immunologically "normal"

ADAMTS enzymes are widely distributed in human adult tissues and play a key role in normal cellular function (275, 279, 306). Although *Adams5<sup>−/−</sup>* mice are viable and phenotypically normal, based on gross analysis (213, 221), detailed characterisation has revealed decreased interdigital web regression leading to fused digits (299), irregular cardiac
valve maturation (269) and abnormal formation of multinucleated myotubes required for skeletal muscle development in these mice (282). In contrast, the immune cell populations in 

Adams5−/− mice are yet to be analysed. To determine this, we removed spleen and lung from Adams5−/− and WT mice and enumerated immune cell populations in these tissues as these would be used as points of comparison following influenza virus infection. In the spleen, we observed no differences in innate immune cells neutrophils (Ly6GhiGR1hi) (Figure 3.1A), NK cells (CD314+CD3−) (Figure 3.1B), macrophages (CD11bhiF4/80hi) (Figure 3.1C), DCs (CD11cMHCIIhi) (Figure 3.1D), B cells (B220+) (Figure 3.1E) and CD4+ T cells (CD4+CD3+) and CD8+ T cells (CD8+CD3+) (Figure 3.1F). Similar results were observed between WT and Adams5−/− mice in the lung (Figure 3.2). These data support previously published evidence suggesting that Adams5−/− mice develop normally and indicates that Adams5−/− mice develop normal immune cell populations. As such, we conclude that Adams5−/− mice are immunologically “normal”.

3.3 Adams5−/− mice are more susceptible to influenza virus infection

To investigate the role of ADAMTS5 during influenza virus infection, we initially examined in vivo weight loss and viral burden following primary infection with X31 (H3N2) influenza virus. Influenza virus replication in C57.BL6 mice normally peaks at 3 dpi, and virus is cleared by 7-10 dpi (70, 72). In these experiments, we intranasally infected Adams5−/− mice and WT controls with 10^4 pfu/mouse adapted X31 (H3N2) influenza virus, and observed enhanced weight loss in Adams5−/− mice across two experimental time points (p < 0.05 on 8 and 9 dpi) period when compared to WT controls (Figure 3.3A). At the peak of virus replication (3 dpi), Adams5−/− mice had higher virus titres in the lung when compared to WT controls (p = 0.045) (Figure 3.3B). Delayed virus clearance was observed in Adams5−/− mice
Figure 3.1. Naïve *Adamts5*°/° mice have normal immune cell subsets in the spleen.
Spleens were removed from naïve WT and *Adamts5*°/° mice and immune cell subsets were characterized by flow cytometry. (A) Neutrophils (Ly6G<sup>hi</sup>GR1<sup>hi</sup>), (B) NK cells (CD314<sup>+</sup>CD3<sup>-</sup>), (C) macrophages (CD11b<sup>hi</sup>F4/80<sup>hi</sup>), (D) DCs (CD11c<sup>hi</sup>MHCII<sup>hi</sup>), (E) B cells (B220<sup>+</sup>) and (F) T cells (CD4<sup>+</sup>CD3<sup>+</sup> or CD8<sup>+</sup>CD3<sup>+</sup>). The results are expressed as means ± SEM (n = 5. Figures are representative of 3 independent experiments).
Figure 3.2. Naïve Adamts5<sup>−/−</sup> mice have normal immune cell subsets in the lung.
Lungs were removed from naïve WT and Adamts5<sup>−/−</sup> mice and immune cell subsets were enumerated by flow cytometry. (A) Neutrophils (Ly6<sup>hi</sup>G<sup>ri</sup>hi), (B) NK cells (CD314<sup>+</sup>CD3<sup>−</sup>), (C) macrophages (CD11b<sup>hi</sup>F4/80<sup>hi</sup>), (D) DCs (CD11c<sup>hi</sup>MHCII<sup>hi</sup>), (E) B cells (B220<sup>+</sup>) and (F) T cells (CD4<sup>+</sup>CD3<sup>+</sup> or CD8<sup>+</sup>CD3<sup>+</sup>). The results are expressed as means ± SEM (n = 5 representing 3 experiments).
Figure 3.3. Adamts5−/− mice are more susceptible to influenza virus infection.

Adamts5−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus. (A) Percent of initial body weight loss was measured throughout the time course of infection. Virus titres in lung homogenates were determined at (B) 3 and (C) 7 dpi. The results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, n = 5 representing 3 experiments).
at 7 dpi ($p = 0.013$) when compared to WT controls (Figure 3.3C). These data clearly suggest that $Adamts5^{-/-}$ mice do not clear influenza virus infection as effectively as WT controls.

### 3.4 $Adamts5^{-/-}$ mice have similar APC numbers in the lung

Increased lung virus titres observed in $Adamts5^{-/-}$ mice at 3 and 7 dpi suggests that defective innate immune cell responses may be influencing pathogenesis, leading to enhanced virus titres and weight loss (Figure 3.3). Initially, we chose to enumerate macrophages and DCs (APCs) in the lung, the primary site of infection and a key site for antigen uptake (73). We originally hypothesized that a defect in APC numbers in the lung could result in enhanced virus titres (as observed at 3 dpi) and increased weight loss at later time points (Figure 3.3), as there may have been reduced or delayed antigen presentation and priming of T cells in $Adamts5^{-/-}$ mice. Our initial observations indicated that there were no differences in APCs (macrophages and DCs) in the lung of WT and $Adamts5^{-/-}$ mice at 3 and 7 dpi (Figure 3.4), although there was a trend for reduced macrophages at 3 and 7 dpi, $p = 0.06$ and $p = 0.08$ respectively. This data suggests that APC numbers were not directly contributing to increased weight loss and virus titres.

### 3.5 Lack of ADAMTS5 expression results in the accumulation of T cells in the MLN

As there was evidence of delayed viral clearance in $Adamts5^{-/-}$ knockout mice (Figure 3.3) and no differences in the numbers of APCs (Figure 3.4), we wanted to determine if perturbations in the adaptive immune response contributed to disease. B and T cells are required for efficient clearance of influenza virus infection (see section 1.7). Disruption in
either population may contribute to delayed viral clearance observed in Adamts5−/− mice. We initially enumerated total T and B cells numbers in the spleen and lung at 7 and 10 dpi as these time points correspond to key time points in adaptive immunity following influenza virus infection. At 7 dpi, reduced numbers of total CD4+ (p = 0.004) and CD8+ (p = 0.001) T cells were observed in the spleen of Adamts5−/− mice when compared to WT controls (Figure 3.5A). Similar findings were observed in the lung, where total B cells (p = 0.043), CD4+ (p = 0.032) and CD8+ (p = 0.04) T cell numbers were lower in Adamts5−/− mice when compared to WT controls (Figure 3.5C and D). Interestingly there was a striking difference and contrasting accumulation of total B cells, CD4+ and CD8+ T cell numbers in pooled MLN in Adamts5−/− mice when compared to WT mice (Figure 3.5E and F).

We then continued our analysis of adaptive immune cell populations at 10 dpi, the peak of influenza-specific CD8+ T cell immunity. Results at this time point were similar to those observed at 7 dpi (Figure 3.5). Adamts5−/− mice had fewer B cells (p = 0.07), CD4+ (p = 0.012) and CD8+ (p = 0.008) T cells in the spleen when compared to WT controls (Figure 3.6A and B). Enumeration of total adaptive immune cell populations in the lung suggested that Adamts5−/− mice had fewer B cells (p = 0.11) and CD4+ (p = 0.007) and CD8+ (p = 0.006) T cells when compared to WT controls (Figure 3.6C and D). In contrast Adamts5−/− mice had higher numbers of total B cell, CD4+ and CD8+ T cells in pooled MLN samples when compared to WT controls (Figure 3.6E and F).

These experimental observations suggest that Adamts5−/− mice have fewer total adaptive immune cells in peripheral tissue following infection, but accumulate cells in the MLN. These results link ADAMTS5 expression with altered adaptive immunity in the MLN, and suggests the presence of ADAMTS5 aids the egress of cells from the MLN to the lung and spleen.
Figure 3.4. APCs numbers are comparable in influenza virus infected Adamts5⁻/⁻ and WT mice.

Adamts5⁻/⁻ and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus and APC numbers were assessed in the lung at 3 and 7 dpi. Macrophages (CD11b⁺ F4/80⁺) in the lung at (A) 3 and (B) 7 dpi. DCs (CD11c⁺ MHCII⁺) in the lung at (C) 3 and (D) 7 dpi. The results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (n = 5 representing 3 experiments).
Figure 3.5. CD8+ T cells accumulate in the MLN of Adamts5−/− mice 7 dpi. Adamts5−/− and WT mice were intranasally infected with 10^4 pfu/mouse X31 (H3N2) influenza virus and total adaptive immune cell populations were assessed in the spleen, lung and MLN 7 dpi. Total B cells (B220+) in the (A) spleen, (C) lung and (E) MLN. Total T (CD4+ or CD8+ CD3+) cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, *** = p ≤ 0.005, n = 5 representing 3 experiments). MLN data are expressed as pooled means (n = 5 mice/group).
Figure 3.6. CD8+ T cells continue to accumulate in the MLN of Adamts5−/− mice 10 dpi.
Adamts5−/− and WT mice were intranasally infected with 10^4 pfu/mouse X31 (H3N2) influenza virus and total adaptive immune cell populations were enumerated in the spleen, lung and MLN 10 dpi. Total B cells (B220+) in the (A) spleen, (C) lung and (E) MLN. Total T (CD4+ or CD8+ CD3+) cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung data are expressed as means ± SEM. Statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.001, n = 5 representing 3 experiments). MLN data are expressed as pooled means (n = 5 mice/group).
3.6 Influenza-specific CD8+ T cell responses in the periphery of Adamts5−/− mice

Our data clearly demonstrated the accumulation of total CD8+ T cells in the MLN of Adamts5−/− knockout mice (Figure 3.5 and 3.6). We wanted to further confirm that this accumulation also included influenza-specific CD8+ T cells.

To assess influenza-specific CD8+ T responses we enumerated antigen specific CD8+ T cells using tetrameric complexes that recognise the immunodominant NP366-374 (ASNENMETM) or PA224-233 (SSLENFRAYV) CD8+ T cell epitopes at 7 and 10 dpi (65). At 7 dpi, fewer NP366-374 (p < 0.005) and PA224-233 (p = 0.13) CD8+ T cells were detected in the spleen of Adamts5−/− mice when compared to WT controls (Figure 3.7A), although the differences in PA224-233+CD8+ T cells was not statistically significant. Similarly, in the lung, fewer NP366-374 (p = 0.019) and PA224-233 (p = 0.006) CD8+ T cells were found in Adamts5−/− mice when compared to WT controls (Figure 3.7C). Furthermore, and as suspected, NP366-374 and PA224-233 CD8+ T cells accumulated in the pooled MLN of Adamts5−/− mice (Figure 3.7E). These results were recapitulated at 10 dpi, where fewer NP366-374 and PA224-233 CD8+ T cell numbers were detected in the spleen and lung of Adamts5−/− mice when compared to WT controls (Figure 3.8A and C). In pooled MLN samples, pooled samples suggested that NP366-374 CD8+ and PA224-233 CD8+ T cells were increased in Adamts5−/− mice when compared to WT controls (Figure 3.8E).

The ICS assay was used to assess the functionality of influenza-specific CD8+ T cells in the spleen, lung and MLN at 7 and 10 dpi where CD8+ NP366-374 or PA224-233 T cells expressed IFNγ (300). Using this assay, we identified that Adamts5−/− mice had reduced numbers of NP366-374*IFNγ+ (p = 0.044) and PA224-233*IFNγ+ (p = 0.03) CD8+ T cells in the spleen at 7 dpi (Figure 3.7B). Similarly, Adamts5−/− mice had reduced numbers of NP366-374*IFNγ+ and PA224-233*IFNγ+ CD8+ T cells in the lung at 7 dpi (Figure 3.7D), although this was not statistically significant.
In pooled MLN samples, *Adams5*−/− mice had increased numbers of NP<sub>366-374</sub>IFNγ<sup>+</sup> and PA<sub>224-233</sub>IFNγ<sup>+</sup> CD8<sup>+</sup> T cells 7 dpi (Figure 3.7F). At 10 dpi, the peak of influenza-specific CD8<sup>+</sup> T cell immunity, *Adams5*−/− mice had fewer NP<sub>366-374</sub>IFNγ<sup>+</sup> and PA<sub>224-233</sub>IFNγ<sup>+</sup> CD8<sup>+</sup> T cells in the spleen and lung when compared to WT controls (Figure 3.8B and D). Pooled MLN samples indicated an increase in the numbers of NP<sub>366-374</sub>IFNγ<sup>+</sup> and PA<sub>224-233</sub>IFNγ<sup>+</sup> CD8<sup>+</sup> T cells when compared to their WT counterparts (Figure 3.8F).

The data presented herein suggests that influenza-specific CD8<sup>+</sup> T cell immunity was significantly impaired in the spleen and lung of *Adams5*−/− mice at 10 dpi and provides a possible explanation for the increased viral titres and weight loss observed in *Adams5*−/− mice (Figure 3.3).

### 3.7 *Adams5*−/− mice have reduced numbers of influenza-specific memory CD8<sup>+</sup> T cells

Activation of adaptive immune cells during acute influenza virus infection results in the expansion and eventual contraction of cells following elimination of a pathogen (307, 308). During this process a small subset of adaptive immune cells will become memory T cells (68) that will be available to rapidly respond to re-infection (307, 308). We hypothesised that the reduced numbers of influenza-specific CD8<sup>+</sup> T cells observed in the peripheral tissue during acute infection of *Adams5*−/− mice may also reflect the influenza-specific CD8<sup>+</sup> T cell memory pool. Our observations in influenza virus infected *Adams5*−/− mice indicated that effector CD8<sup>+</sup> T cells accumulated in the MLN (Figure 3.5 – 3.8). Hence, we wanted to assess if this defect affected memory responses. Here, we assessed influenza-specific memory CD8<sup>+</sup> T cells in the spleen of *Adams5*−/− mice that had fully recovered from virus infection (30 dpi).
Figure 3.7. Influenza-specific CD8+ T cell immunity in Adamts5−/− mice 7 dpi.
Spleen, lung and MLN from influenza virus infected Adamts5−/− and WT mice were assessed for influenza-specific immunity 7 dpi. Tetramer+CD8+ T cells in the (A) spleen, (C) lung and (E) MLN. IFNγ+CD8+ T cells in the (B) spleen, (D) lung and (F) MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 5 representing 3 experiments). MLN data are expressed as pooled means (n = 5 mice/group).
Figure 3.8. Influenza-specific CD8⁺ T cell immunity in Adamts5⁻/- mice 10 dpi.
Spleen, lung and MLN from influenza virus infected Adamts5⁻/- and WT mice were assessed for influenza-specific immunity 10 dpi. Tetramer⁺ CD8⁺ T cells in the (A) spleen, (C) lung and (E) MLN. IFNγ⁺ CD8⁺ T cells in the (B) spleen, (D) lung and (F) MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, n = 5 representing 3 experiments). MLN data are expressed as pooled means (n = 5 mice/group).
Our data suggest that Adamts5−/− mice and WT controls had similar numbers of total CD4+ and CD8+ T cells in the spleen (Figure 3.9A). However, fewer NP366-374 (p < 0.005) and PA224-233 (p < 0.005) tetramer+ CD8+ T cells were quantified in Adamts5−/− mice when compared to WT controls (Figure 3.9B). Similarly, fewer NP366-374+IFNγ+ (p = 0.023) and PA224-233+IFNγ+ (p = 0.008) CD8+ T cells were observed in Adamts5−/− mice when compared to WT mice (Figure 3.9C). Detailed subsetting of influenza-specific CD8+ memory populations revealed that fewer NP366-374+CD8+ effector memory (CD62LhiCD44hi) (p = 0.037) cells were isolated from the spleens of Adamts5−/− mice when compared to WT counterparts (Figure 3.10). Although, there was no statistical significance between PA244-233+CD8+ memory cells in Adamts5−/− and WT mice (Figure 3.10). This data suggests that only NP366-374+ memory CD8+ T cells populations are significantly affected by the lack of ADAMTS5 expression.

3.8 Reduced influenza-specific NP366-374+CD8+ T cell responses in Adamts5−/− mice following secondary challenge

Due to the lower number of NP366+CD8+ T memory cells observed in Adamts5−/− mice (Figure 3.10) following primary infection, we hypothesized that this would significantly impact the ability of Adamts5−/− mice to mount an effective recall response. To determine if this was indeed true, we intraperitoneally primed Adamts5−/− and WT mice with PR8 (H1N1) influenza virus (1.5 x 10^7 pfu/mouse) and then intranasally challenged these mice with the serologically distinct X31 (H3N2) (10^4 pfu/mouse) influenza virus 6 weeks later. The X31 (H3N2) and PR8 (H1N1) influenza viruses are laboratory adapted influenza virus strains that contain the same internal gene segments, but differ in external HA and NA proteins (H3N2 and H1N1, respectively). Priming with PR8 (H1N1) and challenging with X31 (H3N2) facilitates boosting
**Figure 3.9. Influenza-specific CD8+ T cell immunity in Adamts5−/− mice 30 dpi.**

Adamts5−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus. Spleens were removed and immunity assessed 30 dpi. (A) Total CD4+ or CD8+ CD3+ T cells, (B) tetramer+ CD8+ T cells and (C) functional (IFNγ+) CD8+ T cells were enumerated. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 5 representing 3 experiments).
Figure 3.10. Adamts5−/− mice have fewer NP336-374+ influenza-specific memory CD8+ T cells. Adamts5−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus and influenza-specific NP366-374+ or PA224-233+ effector memory CD8+ T cells (CD62LhiCD44hi) were enumerated in the spleen. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, n = 5 representing 3 experiments).
of CD8+ T responses during secondary infection, as the immunodominant CD8+ T cell responses are encoded within the NP_{366-374} and PA_{224-233} internal gene segments. Using this challenge model, weight data indicated that Adamts5−/− mice lost more weight when compared to WT controls, with significant differences occurring at 5, 6 and 7 dpi (p = 0.045, p = 0.031 and p = 0.047, respectively) (Figure 3.11A).

Next we enumerated adaptive immune cells, where we found comparable total B cell and CD4+ T cell numbers in Adamts5−/− and WT mice in the spleen (Figure 3.12A and B). However, total CD8+ T cell numbers (p = 0.045) were lower in the spleens of Adamts5−/− mice when compared to WT controls (Figure 3.12B). In the lung, B cells (p = 0.031), CD4+ T cells (p = 0.101) and CD8+ T cells (p = 0.03) were all reduced in Adamts5−/− mice when compared to WT mice (Figure 3.12C and D). As with primary infection, increased numbers of B cells (p = 0.007), CD4+ (p = 0.036) and CD8+ (p = 0.013) T cells were observed in the MLN of challenged Adamts5−/− mice when compared to WT controls (Figure 3.12E and F).

Besides the expansion of CD8+ T cells, the C57BL/6 mouse model also displays a typical secondary challenge responses dominated by influenza-specific NP_{366-374}CD8+ T cells, where a 6-fold increase is observed when compared to PA_{224-233} CD8+ T cells (66, 67, 149). Given that total CD8+ T cell numbers were reduced in the periphery (lung and spleen) of Adamts5−/− mice during secondary challenge (Figure 3.12B and D), we also expected influenza-specific immunity in Adamts5−/− mice to be compromised. Indeed, NP_{366-374}tetramer+ T cell numbers were reduced in the spleen (p = 0.049) and lung (p = 0.048) of Adamts5−/− mice (Figure 3.13A and C). No differences in PA_{224-233} tetramer+ (p = 0.11) and PA_{224-233}IFNγ+ (p = 0.63) CD8+ T cell numbers were observed in the spleen (Figure 3.13B), although these cell numbers were reduced in the lung of Adamts5−/− mice when compared to WT controls (Figure 3.13D).

As with primary infections, NP_{366-374} (p < 0.005) and PA_{224-233} (p < 0.005) CD8+ T cell numbers were increased in the MLN of Adamts5−/− mice when compared to WT controls
during secondary challenge (Figure 3.13E). However, functional NP\textsubscript{366-374}^{+}IFN\textgamma^{+}CD8\textsuperscript{+} (p = 0.03) T cells were reduced in the MLN of \textit{Adams}5^{−/−} mice (Figure 3.13F). This was peculiar, as numbers obtained in tetramer and ICS assays are normally similar (66, 67, 149). Finally, no differences in PA\textsubscript{224-233}^{+}IFN\textgamma^{+}CD8\textsuperscript{+} T cell numbers were detected between \textit{Adams}5^{−/−} and WT mice in the MLN (Figure 3.13F), suggesting that ADAMTS5 is required for normal NP\textsubscript{366-374}^{+}CD8\textsuperscript{+} secondary challenge responses.

This mechanism was confirmed by analysing differences between the staining profiles of NP\textsubscript{366-374}^{+}CD8\textsuperscript{+} secondary challenge responses. Staining suggested that increased NP\textsubscript{366-374}^{+}CD8\textsuperscript{+} T cells were found in the MLN of \textit{Adams}5^{−/−} mice (Figure 3.14A) and mean fluorescent intensity (MFI) staining further supported this (Figure 3.14B). However, IFN\textgamma staining was reduced in this tissue (Figure 3.14C) and was again supported by MFI (Figure 3.14D).
Figure 3.11. *Adams5*<sup>−/−</sup> mice show increased weight loss following secondary challenge. *Adams5*<sup>−/−</sup> and WT mice were intraperitoneally primed with 1.5 x 10<sup>7</sup> pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10<sup>4</sup> pfu/mouse X31 (H3N2) influenza virus. Weight loss in (A) percent initial body weight and (B) grams lost was measured throughout the time course of secondary challenge. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, n = 4 representing 3 experiments).
Figure 3.12. B and T cell responses in influenza virus infected Adamts5−/− mice following secondary infection.

Adamts5−/− and WT mice were intraperitoneally primed with 1.5 x 10⁷ pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10⁴ pfu/mouse X31 (H3N2) influenza virus. Total B cells (B220⁺) in the (A) spleen, (C) lung and (E) MLN. Total T cells (CD3⁺CD4⁺ or CD3⁺CD8⁺) in the (B) spleen, (D) lung and (F) MLN. Data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, n = 4 representing 3 experiments).
Figure 3.13. Influenza-specific immunity in Adams5−/− mice following secondary acute infection.

Adams5−/− and WT mice were intraperitoneally primed with 1.5 x 10^7 pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10^4 pfu/mouse X31 (H3N2) influenza virus. Spleen, lung and MLNs were removed and influenza-specific immunity assessed 7 dpi. Tetramer^+CD8^+ T cells in the (A) spleen, (C) lung and (E) MLN. IFNγ^+CD8^+ T cells in the (B) spleen, (D) lung and (F) MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 4 representing 3 experiments).
Figure 3.14. Influenza-specific immunity staining in the MLN of Adamts5−/− mice following secondary acute infection.
Adamts5−/− and WT mice were intraperitoneally primed with 1.5 × 10⁷ pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10⁵ pfu/mouse X31 (H3N2) influenza virus. MLNs were removed and influenza-specific immunity assessed 7 dpi. (A) NP366-374+CD8+ T cells and (B) MFI of tetramer staining. (C) IFNγ+CD8+ T cells in the MLN and (D) MFI of ICS staining. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (n = 4 representing 3 experiments).
3.9 Discussion

Increasing evidence in the literature highlights the importance of zinc-dependent metzincins in the regulation of immune responses. Dysregulated MMPs and ADAMs have been strongly associated with perturbations in the immune response, such as cytokine expression and immune cell infiltration (256, 309, 310). Here, we show for the first time that ADAMTS5, a member of the ADAMTS family, plays a key role in the immune response to influenza virus infection. Our research suggests that ADAMTS5 activity is required for effective CD8\(^+\) T cell responses in influenza virus infected \(Adamts5^{-/-}\) mice during acute infection, as these mice displayed fewer CD8\(^+\) T cell populations in the periphery (Figure 3.5 – 3.8), yet, increased CD8\(^+\) T cells were observed in the MLN at 7 and 10 dpi (Figure 3.5 – 3.8). ADAMTS5 may be required for effective migration of CD8\(^+\) T cells, given that CD8\(^+\) T cell egress from the MLN seemed to be delayed (Figure 3.5 – 3.8). In support of this hypothesis, other studies have suggested that the related zinc-dependent metzincins, the MMPs, are essential for immune cell trafficking (244, 256, 310). Like ADAMTs enzymes, MMPs contain a catalytic domain that utilises a conserved zinc binding sequence (HEXXHXXXGXX) for catalysing reactions (167) and have a broad range of cleavage substrates [collagen, laminin, proteoglycan etc. (224, 311, 312)]. This contrasts with the highly specific cleavage moieties associated with ADAMTS enzyme activity (proteoglycans). It is therefore not surprising that the MMPs have been identified in a vast number of physiological processes (311), including those of the immune responses. Indeed, MMP9 is highly intertwined with the trafficking of immune cells (105, 244, 259). These studies have utilised the highly-characterised \(Mmp9^{-/-}\) mouse. In fact, influenza virus infected \(Mmp9^{-/-}\) mice have decreased neutrophil infiltration into the lung (105). This is caused by reduced collagen degradation and infiltration through the basement membrane. Additionally, the ECM has been shown to block the migration of DCs \textit{in vivo}. In this study, lipopolysaccharide-induced DCs derived from \(Mmp9^{-/-}\) mice show decreased infiltration into
the draining lymph node (254). Here ECM components, such as collagen and elastin, blocked
the crawling of CD4+ T cells along the ECM, inhibiting their migration. Collagen and elastin
form key ECM components of basement membranes and so reduced MMP enzymatic activity
would, in turn, lead to the accumulation of these components and the inhibition of immune cell
migration and tissue infiltration. Versican, a key ADAMTS5 substrate, is widely expressed in
tissues and is not predominately associated with the basement membrane (unlike MMP
substrates). ECM components, such as versican, provide a “sticky” surface for T cells allowing
cell surface adhesion (257). We therefore speculate that the cleavage and removal of versican
blockades via the action of proteoglycanases, such as ADAMTS5, is required to prevent
excessive T cell adhesion and facilitate their migration towards the peripheral tissue.

Lymph node accumulation studies using the FTY720 inhibitor (an agonist of
sphingosine receptors) indicate that accumulation of CD8+ T cells in the lymph node may
inhibit the functionality and availability of memory CD8+ T cells (313, 314). In these studies,
mice were treated orally with FTY720 and found that FTY720-treated mice had reduced T cell
infiltration towards lymphocytic choriomeningitis infection (314). It was found that T cells
accumulated in the lymph node of these mice. Our studies, showed a similar result to those
observed with FTY720 inhibitor studies, where T cells accumulated in the MLN in the absence
of ADAMTS5 in the MLN during acute infection (Figure 3.5 and 3.6), although we did not
assess if this was linked to sphingosine receptors Unlike the FTY720 studies, which found
similar numbers of memory T cells in the periphery (314), we found fewer memory NP366-
374+CD8+ T cell numbers in the spleen of Adamts5+/− mice (Figure 3.10). It would be of interest
to further understand memory immune cell populations (e.g. central and effector memory cell
subsets) in Adamts5+/− mice and if increased apoptosis is specific for NP366-374+CD8+ T cells
(given that memory PA224-233+CD8+ T cells were not affected) in the MLN through techniques
such as propidium iodide and annexin V staining (apoptosis markers) (315, 316).
Differential epitope-specific responses have been documented between CD8+NP<sub>366-374</sub> and CD8+PA<sub>224-233</sub> T cells of WT C57/BL.6 mice based on antigen processing and presentation as DCs have been shown to favour expansion of NP<sub>366-374</sub>+CD8+ T cells during secondary infection (317). Our observations show that CD8+NP<sub>366-374</sub> T cells are impacted in mice lacking ADAMTS5, however CD8+PA<sub>224-233</sub> T cells remain unchanged (Figure 3.13). Because of this, it may be beneficial to assess DC processing and presentation of the in Adams5<sup>−/−</sup> mice for both epitopes to try to pinpoint a reason for this discrepancy.

Two interesting results were observed during secondary challenge responses in Adams5<sup>−/−</sup> mice. Briefly, in a normal secondary responses, NP<sub>366-374</sub>+CD8+ T cells are the immunodominant influenza-specific CD8+ T cell type, their number being 6 times larger than the PA<sub>224-233</sub> influenza-specific CD8+ T cells in the MLN and spleen (66, 67, 149). Although our ICS and tetramer numbers reflect those in previous studies (65, 66, 318), our study we found that NP<sub>366-374</sub>+CD8+ T cells were increased in the MLN of Adams5<sup>−/−</sup> mice (Figure 3.13E), but the ability of these cells to produce IFNγ<sup>+</sup> was significantly altered in the MLN of Adams5<sup>−/−</sup> mice (Figure 3.13F). Some metalloproteinases act as transcription factors for signalling pathways to regulate the expression and extracellular release of both cytokines and chemokines, enhancing the immune response. For example, MMP12 can regulate the expression of IFNα in mice infected with cocksackie virus (309). In these studies, Mmp12<sup>−/−</sup> mice showed delayed inflammation (309). IFNα is known to inhibit viral replication by inducing antiviral activity in surrounding cells. In this study it was found that macrophage-secreted MMP12 enters cocksackie virus infected cells, where it then enters in the nucleus (309). In the nucleus, intracellular MMP12 binds to the NF-κB promoter allowing secretion of IFNα (309). Additionally, extracellular MMP12 cleaved the IFNα receptor, preventing IFNα binding. Regulation of IFNα production by MMP12 following infection could not only limit pathogen replication, but also prevent excessive inflammation. In support of this study,
ADAMTS7 has been shown to govern TNFα expression via a positive-feedback loop through modulation of the NF-κb pathway in an osteoarthritis-induced mouse model (285). In this study, ADAMTS7 transgenic mice showed increased cartilage destruction due to elevated TNFα expression. Given ADAMTS7’s control in TNFα expression, it is likely that related family members, such as ADAMTS5, may also be controlling cytokine/chemokine expression/release. Further, ADAMTS5 may be involved in regulating IFNγ production and secretion in the rapid and heightened memory recall response, hence promoting functional CD8+ T cell responses in influenza virus infected mice, however more studies are required to determine this.

The second interesting result for discussion is the unchanged PA224-233 responses in the spleen of WT and Adamts5−/− mice following secondary challenge. This is in contrast to NP366-374 responses that were consistently reduced in the spleen and lung of Adamts5−/− mice during memory and secondary challenge (Figure 3.10 and 3.13). This result indicates that ADAMTS5 is involved in sustaining memory NP366–374+CD8+ T cell responses (but not PA224–233+CD8+ T cell responses) (Figure 3.9 and 3.10) and may be contributing to the reduced numbers of NP366–374+CD8+ T cell responses observed in secondary challenge

### 3.10 Conclusion

These data indicate that Adamts5−/− mice are more susceptible to influenza virus infection and CD8+ T cell immunity is critically compromised in these mice. The next chapter will assess the mechanism behind immune cell accumulation in the MLN of Adamts5−/− mice.
Chapter 4 - ADAMTS5-mediated cleavage of versican enhances CD8\(^+\) T cell migration

4.1 Overview

Chapter 3 described how the lack of ADAMTS5 in mice resulted in decreased influenza-specific CD8\(^+\) T cells in the periphery (spleen and lung) following influenza virus infection. Importantly, we observed that these cells accumulated in the MLN of Adamts5\(^{-/-}\) mice. Given these findings we hypothesized that ADAMTS5 degradation of the ECM facilitates migration of CD8\(^+\) T cells from the lymph node to the periphery.

ADAMTS5 cleaves a number of ECM proteoglycans, including brevican, neurocan, aggrecan and versican (221, 269). Of these proteoglycans, versican has been detected within most tissues (261), whilst the other ECM proteoglycans show selective tissue expression [e.g. the joints, brains and the central nervous system, respectively (319, 320)]. Importantly, versican has a role in the normal functioning of the immune response. Its GAG chains interact with receptors on the surface of immune cells, including CD44, TLR2 and selectins, and providing essential signals to influence inflammation and effector function, such as cytokine release (264, 265, 267, 321-323). Versican also interacts with other ECM components, such as hyaluronan, fibronectin and tenascin, which are important in inflammatory responses (Figure 1.6) (324-326). Interaction between these ECM components promotes/inhibits leukocyte migration (257, 327-329). Interestingly, versican has been shown to inhibit the attachment of CD4\(^+\) T cells to hyaluronan, disrupting migration and immune responses (257). A similar result has also been observed with CD8\(^+\) T cells, whereby versican inhibited cells from entering stromal tissue to attack cervical cancer cells in human patients (258, 330).

The remodeling and proteolysis of versican has been carried out by MMP1, 2, 3, 7 and 9 and ADAMTS1, 4, 5, 8, 9, 15 and 20 physiological processes such as wound healing and
embryonic development (198, 269-272). Versican proteolysis, however, has never been associated with the migration of immune cells. This chapter examines versican expression in influenza virus infected $Adamts5^{-/-}$ mice and highlights the importance of the ADAMTS5/versican interaction for CD8$^+$ T cell migration. Additionally, this chapter utilizes a versican reduced ($Vcan^{hld/+}$) mouse model to understand the role of ADAMTS5 and versican in the immune response to influenza virus infection.

### 4.2 Versican accumulation in the MLN of $Adamts5^{-/-}$ mice

Versican, a key ECM proteoglycan and cleavage substrate of ADAMTS5, impedes the trafficking of immune cell populations, such as T cells (257, 258). We hypothesised that a lack of versican degradation to versikine (the versican cleavage fragment) in the MLN of $Adamts5^{-/-}$ mice could result in the accumulation of versican, a large ECM proteoglycan, in the MLN, resulting in inhibition of T cell movement to effector sites and the periphery. We therefore assessed versican and versikine expression in the MLN and lung of influenza virus-infected $Adamts5^{-/-}$ and WT mice at 10 dpi. MLN tissue from influenza virus infected mice was paraffin embedded, sectioned and stained with anti-GAG-β (versican) and anti-versikine antibodies. Fluorescence imagery of sections revealed increased levels of versican in the MLN of $Adamts5^{-/-}$ mice when compared to WT controls (Figure 4.1A) and correlated with decreased versikine levels in $Adamts5^{-/-}$ mice (Figure 4.1C), suggesting reduced cleavage of versican into versikine in the MLN. qPCR analysis (Figure 4.1B and D) also confirmed that versican expression was increased in the pooled MLN of $Adamts5^{-/-}$ mice.
Figure 4.1. Versican accumulation in the MLN of Adamts5−/− mice.
WT and Adamts5−/− mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus and MLNs were removed 10 dpi. MLNs were sectioned and stained for the expression of versican and versikine. (A) Versican staining in the MLN of influenza virus infected WT and Adamts5−/− mice. (B) Fold change data (vs WT) of versican expression in the MLN of influenza virus infected Adamts5−/− mice. (C) Versikine staining in the MLN of WT and Adamts5−/− mice. (D) Fold change data (vs WT) of versikine expression in the MLN of influenza virus infected Adamts5−/− mice. Blue = nucleus, Red = versican/versikine (n = 5 mice/group). B and D are expressed as pooled data from 5 mice.
Immunofluorescence and qPCR data of the lung tissue showed no differences in the levels of versican (Figure 4.2A and B) and versikine (Figure 4.2C and D) observed between WT and Adamts5\(^{-/-}\) mice.

Because of these contrasting differences in versican accumulation in the MLN and lung, we suspected that the level of ADAMTS5 expression in these tissues varied. To gain a better understanding of ADAMTS5 expression in the MLN and lung, we performed β-galactosidase staining of whole MLN and found that β-galactosidase was highly expressed throughout the MLN (Figure 4.3A), but localised expressed was observed in the lung (Figure 4.3B), of Adamts5\(^{-/-}\) mice. β-galactosidase staining was used here because Adamts5\(^{-/-}\) mice were generated through the insertion of a LacZ cassette into the ADAMTS5 gene (275), thereby preventing the translation of the enzymatically active ADAMTS5 protein. In these mice, the LacZ cassette, which contains β-galactosidase, can be used as a surrogate reporter of ADAMTS5 expression. Interestingly, the lung displayed β-galactosidase staining within the trachea of both WT and Adamts5\(^{-/-}\) mice (Figure 4.3B). We believed this expression is because of bacterial colonisation (which express β-galactosidase) within the trachea, hence why both mice have staining. This data suggests that the enzymatic activity of ADAMTS5 on its substrate versican is critically important in the MLN, but not in the lung.
Figure 4.2. Versican and versikine expression is similar in the lungs of WT and Adamts5<sup>−/−</sup> mice.

WT and Adamts5<sup>−/−</sup> mice were infected intranasally with 10<sup>4</sup> pfu/mouse X31 (H3N2) influenza virus and lungs removed 10 dpi. Lungs were sectioned and stained for the expression of versican and versikine. (A) Versican staining in the lung of influenza virus infected WT and Adamts5<sup>−/−</sup> mice. (B) Fold change data (vs WT) of versican expression in the lung of influenza virus infected Adamts5<sup>−/−</sup> mice. (C) Versikine staining in the lung of WT and Adamts5<sup>−/−</sup> mice. (D) Fold change data (vs WT) of versican expression in the lung of influenza virus infected Adamts5<sup>−/−</sup> mice. Blue = nucleus, Red = versican/versikine. The results are expressed as means ± SEM (n = 5 mice/group).
Figure 4.3. ADAMTS5 is highly expressed in the MLN.
MLN and lungs were removed from influenza virus infected WT and Adamts5−/− mice 10 dpi and stained for β-galactosidase (ADAMTS5) expression. β-galactosidase staining in the (A) MLN and (B) lung. n = 5 mice/group.
4.3 Versican inhibits ex vivo CD8+ T cell migration

Accumulation of versican in the MLN of influenza virus infected Adamts5−/− mice highlighted a potential role for versican-mediated impediment of immune cell trafficking out of this tissue. As such, we examined if the absence of ECM remodelling by ADAMTS5 was linked to impaired CD8+ T cell migration. Ex vivo transwell assays were employed to assess the migration of CD8+ T cells as previously described (244, 256). In our assay, the surface of the upper transwell chamber was coated with versican-enriched conditioned medium from transfected HEK293T cells (198, 282) prior to the addition of a T cell chemoattractant (CXCL12) to the lower transwell chamber. CD8+ T cells isolated from the spleens of influenza virus infected Adamts5−/− or WT mice were then added to the upper chamber of the transwell and migration through the versican-overlay was assessed. The data demonstrates that CD8+ T cells isolated from Adamts5−/− influenza-infected mice showed impaired migratory capacity through the versican overlay (p < 0.005) when compared to WT controls (that express ADAMTS5) (Figure 4.4A).

Our transwell studies suggested that in the absence of ADAMTS5, CD8+ T cell migration was perturbed. We next decided to assess ADAMTS expression levels in CD8+ T cells. This data indicates that WT CD8+ T cells express ADAMTS5 (Figure 4.4B) and that enzymatic activity may be a key requirement for CD8+ T cell migration through versican. We confirmed this by determining the ability of CD8+ T cells to cleave versican. In this study, splenic CD8+ T cells from WT and Adamts5−/− mice were incubated with HEK293T versican conditioned medium and versican cleavage by CD8+ T cells was assessed via western blot (299). Higher levels of versikine (p < 0.005) were detected in samples from WT CD8+ T cells when compared to Adamts5−/− CD8+ T cells (Figure 4.4C and D).
Figure 4.4. ADAMTS5 degradation of versican facilitates ex vivo CD8$^+$ T cell migration. Splenic CD8$^+$ T cells were isolated from WT and Adamts5$^{-/-}$ mice and migration and versican degradation assessed. (A) Migration of CD8$^+$ T cells through a versican overlay is shown by graphical representation. (B) Expression of ADAMTS1, 4, 5, 9, 15 and 7 in CD8$^+$ T cells by qPCR analysis. (C) Western blot analysis of versican cleavage fragments generated by WT and Adamts5$^{-/-}$ mice. (D) Densitometric quantification of Figure 4.4C using ImageJ software. The results are expressed as means ± SEM, and statistical significance (relative to WT) was determined by a student’s t test (*$p<0.05$, ***$p<0.005$ relative to WT; $n=3$ mice/group representing 3 individual experiments).
4.4 Immunity in influenza virus infected $Vcan^{hdf/+}$ mice is similar to WT mice

Results in Figure 4.4 suggest that ADAMTS5 is required for CD8$^+$ T cell migration. However, the transwell assays used represent an *ex vivo* system and so to further define the effect of versican reduction on CD8$^+$ T cell migration we utilised an *in vivo* mouse model. The role of versican in embryonic development, specifically heart ventricle formation, has been previously described using a versican reduced mouse model (298). In this model, a complete knockout of both versican alleles causes a hdf and results in embryonic lethality. Because of this, a heterozygote mouse model [$Vcan^{hdf/+}$ (versican reduced)] has been used previously to describe versican’s role in embryonic heart development (298, 331). To assess the impact of versican reduction on influenza-specific immunity we intranasally infected these $Vcan^{hdf/+}$ and WT control mice with X31 (H3N2) influenza-virus (10$^4$ pfu/mouse). Given that versican is reduced in $Vcan^{hdf/+}$ mice and ADAMTS5 enzymatic activity is still intact, we anticipated that CD8$^+$ T cell egress from the MLN would be increased, due to decreased levels of versican that inhibit migration, resulting in increased numbers of these cells in the periphery.

Weight loss was documented throughout the time course of infection and immune cell numbers enumerated in these mice 10 dpi in the spleen, lung and MLN. No differences in weight loss were observed between influenza virus infected $Vcan^{hdf/+}$ and WT control mice (Figure 4.5). Enumeration of total T cell populations in the spleen showed that influenza-infected $Vcan^{hdf/+}$ mice had fewer total CD4$^+$ T cells ($p = 0.03$) and CD8$^+$ T cells ($p = 0.007$) when compared to WT mice (Figure 4.6A). However, CD4$^+$ T cells and CD8$^+$ T cells were comparable between $Vcan^{hdf/+}$ and WT mice in the lung (Figure 4.6B). In the pooled MLN samples T cell numbers were reduced in $Vcan^{hdf/+}$ mice when compared to WT controls (Figure 4.6C).
Figure 4.5. Weight loss in influenza virus infected Vcan<sup>hdhf/+</sup> mice.
WT and Vcan<sup>+/hdhf</sup> mice were infected intranasally with $10^4$ pfu/mouse X31 (H3N2) influenza virus and % initial body weight measured throughout infection. Results are expressed as means ± SEM ($n = 5$ mice/group representing 3 experiments).
Figure 4.6. Influenza virus infected Vcan^{hdf/+} mice have fewer total T cells in the spleen and MLN.

WT and Vcan^{hdf/+} mice were infected intranasally with 10^4 pfu/mouse X31 (H3N2) influenza virus. Spleens, lungs and MLNs were removed 10 dpi and total T cell numbers enumerated. Total T cells (CD3+CD4+ and CD3+CD8+) in the (A) spleen (B) lung and (C) MLN. Spleen and lung results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined using a Student’s t test (\(*p ≤ 0.05, \,**p ≤ 0.01, n = 5\) mice/group representing 3 experiments). MLN results are expressed as pooled MLNs from 5 mice.
Although a reduction in total T cell numbers in the spleen and MLN of influenza-infected $Vcan^{hdf/+}$ mice was noted (Figure 4.6), the number of influenza-specific CD8$^+$ T cells was still to be analysed. To assess this, we enumerated NP$_{366-374}^+$ and PA$_{224-233}^+$ influenza-specific CD8$^+$ T cells using tetramer technology and quantified functional CD8$^+$ T cells (IFN$\gamma^+$ cells) in the spleen, lung and MLN using the ICS assay.

Our results indicated that fewer influenza-specific NP$_{366-374}^+$ ($p = 0.045$) and PA$_{224-233}^+$ ($p = 0.035$) CD8$^+$ T cells were detected in the spleen of $Vcan^{hdf/+}$ mice when compared to WT controls (Figure 4.7A). However, no statistical differences in functional CD8$^+$ T cells were observed in the spleen (Figure 4.7B). In the lung, we observed an increased number of in NP$_{366-374}$ ($p = 0.009$) and PA$_{224-233}$ ($p = 0.056$) tetramer$^+$ CD8$^+$ T cells (Figure 4.7C), although no differences in functional NP$_{366-374}$ ($p = 0.057$) and PA$_{224-233}$ (IFN$\gamma^+$) CD8$^+$ T cells were detected (Figure 4.7D). In the MLN, fewer tetramer$^+$ and IFN$\gamma^+$ cells were found in $Vcan^{hdf/+}$ mice (Figure 4.7E and F), suggesting that reducing versican in mice leads to reduced influenza virus-specific responses in the spleen and MLN, with comparable numbers in the lung.
Figure 4.7. Influenza-specific CD8$^+$ T cells in Vcan$^{hdf/+}$ mice.

Spleens, lungs and MLNs were removed from influenza virus infected WT and Vcan$^{hdf/+}$ mice and influenza-specific immunity enumerated 10 dpi. Tetramer$^+$CD8$^+$ T cells in the (A) spleen, (C) lung and (E) MLN. IFN$\gamma^+$CD8$^+$ T cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (*p ≤ 0.05, **p ≤ 0.01, n = 5 mice/group representing 3 experiments). MLN results are expressed as pooled MLNs from 5 mice.
4.5 Immunity in influenza infected \textit{Adamts5}\textsuperscript{-/-} mice with reduced versican

Influenza virus infection of \textit{Vcan}\textsuperscript{hdf/+} mice demonstrated that reduction in one allele of versican did not fully result in increased immune cell migration to the periphery, i.e. fewer cells in the MLN and increased numbers in the lung and spleen, as initially suspected. To further validate the interaction between enzyme and substrate, and in an attempt to rescue the WT phenotype, we crossed \textit{Adamts5}\textsuperscript{-/-} mice with \textit{Vcan}\textsuperscript{hdf/+} mice to produce \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} offspring (see section 2.4.6). We then intranasally infected \textit{Adamts5}\textsuperscript{-/-} mice, \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} mice and WT controls with X31 (H3N2) influenza virus (10\textsuperscript{4} pfu/mouse) and assessed weight loss and immune responses in spleen, lung and MLN at 10 dpi. Our weight loss data indicates that \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} mice had a similar % initial body weight profile as WT controls (Figure 4.8), whereas \textit{Adamts5}\textsuperscript{-/-} mice lost significantly more weight. Enumeration of immune cell populations in the spleen indicated that \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} and WT mice had similar numbers of total T cells (Figure 4.9A). This correlated with data in the lung (Figure 4.9B) and pooled MLN (Figure 4.9C).

We also enumerated influenza-specific CD8\textsuperscript{+} T cells and found increased NP\textsubscript{366-374} and PA\textsubscript{224-233} CD8\textsuperscript{+} T cell numbers following tetramer staining in the spleens of \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} mice at 10 dpi when compared to \textit{Adamts5}\textsuperscript{-/-} mice (Figure 4.10A), partially restoring the WT phenotype. These results correlated with IFN\textgammaproduction in the spleen (Figure 4.10B). Comparable results were found in the lung (Figure 4.10C and D). We also assessed influenza-specific CD8\textsuperscript{+} T cell numbers in pooled MLN to determine if CD8\textsuperscript{+} T cell egress towards the lung could be restored. Careful analysis revealed comparable numbers of influenza-specific CD8\textsuperscript{+} NP\textsubscript{366-374} and PA\textsubscript{224-233} (by tetramer and ICS) in the MLN of \textit{Adamts5}\textsuperscript{-/-}x\textit{Vcan}\textsuperscript{hdf/+} (versican reduced) and WT control mice (Figure 4.10E and F). These important and highly novel findings highlight the importance of the ADAMTS5 enzyme-versican substrate interaction as a key process in the regulation of virus-specific immunity.
WT, Adamts5−/− and Adamts5−/− x Vcan<sup>hdf+/+</sup> mice were infected intranasally with 10<sup>4</sup> pfu/mouse X31 (H3N2) influenza virus and % of initial body weight was measured. The results are expressed as means ± SEM. Statistical significance was determined by a One-way ANOVA (p ≤ 0.05 relative to WT mice, #p ≤ 0.05 relative to Adamts5−/− x Vcan<sup>hdf+/+</sup> mice, n = 5 mice/group representing 3 experiments).
Figure 4.9. Influenza virus infected WT and Adamts5<sup>-/-</sup>xVcan<sup>hdf/+</sup> mice have similar numbers of total T cells.

WT, Adamts5<sup>-/-</sup> and Adamts5<sup>-/-</sup>xVcan<sup>hdf/+</sup> mice were infected intranasally with 10<sup>4</sup> pfu/mouse X31 (H3N2) influenza virus. Spleens, lungs and MLNs were removed from infected mice 10 dpi and total T cell numbers were enumerated. Total T cells (CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>) in the (A) spleen (B) lung and (C) MLN. Spleen and lung results are expressed as means ± SEM, and statistical significance was determined by One-way ANOVA (*<sup>p</sup> ≤ 0.05, **<sup>p</sup> ≤ 0.01, n = 5 mice/group representing 3 experiments). MLN results are expressed as pooled MLNs from 5 mice.
Figure 4.10. WT and Adamts5−/−xVcan<sup>hdf/+</sup> mice have comparable influenza-specific CD8<sup>+</sup> T cell numbers

Spleens, lungs and MLNs were removed from influenza virus infected WT, Adamts5<sup>−/−</sup> and Adamts5<sup>−/−</sup>xVcan<sup>hdf/+</sup> mice and influenza-specific immunity enumerated 10 dpi. Tetramer<sup>+</sup>CD8<sup>+</sup> T cells in the (A) spleen, (C) lung and (E) MLN. IFNγ<sup>+</sup>CD8<sup>+</sup> T cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung results are expressed as means ± SEM, and statistical significance was determined by a One-way ANOVA (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005 n = 5 mice/group representing 3 experiments). MLN results are expressed as pooled MLNs from 5 mice.
4.6 Discussion

Migration of CD8\(^+\) T cells from draining lymph nodes to the periphery is critically important for elimination of pathogens. Inhibition of immune cell migration results in increased susceptibility to influenza virus infection, as observed in our \textit{Adams}5\(^{-/}\) mice. We have demonstrated that the accumulation of versican in the MLN of \textit{Adams}5\(^{-/}\) mice prevents the establishment of normal T cell effector function in the periphery and results in the exacerbation of influenza virus infection.

Versican GAG chains interact directly or indirectly with molecules on the surface of T cell lymphocytes, such as CD62L and CD44 (257, 264, 265, 332), providing attachment points for T cell rolling and trafficking. Previous studies have shown that the N-terminal globular domain of versican prevents CD4\(^+\) T cell migration and lymphocyte rolling (257). Moreover, versican overexpression has been associated with decreased infiltration of CD8\(^+\) T cells into the stromal compartments of cervical cancer patients (258). ECM components, such as versican, provide a “sticky” surface for T cell adherence and antigen presentation. However, inhibited degradation of “sticky” surfaces leads to unwanted accumulation of these cells in lymph nodes, compromising effector function in the periphery. We therefore propose that cleavage and removal of versican blockades via the action of proteoglycanases, such as ADAMTS5, is critical for efficient T cell/ECM interaction to encourage migration to effector sites in the periphery and for the subsequent resolution of infection.

Our studies suggest that CD8\(^+\) T cells in \textit{Adams}5\(^{-/}\) mice become trapped in the MLN after infection. We attribute this phenomenon to the accumulation of versican, because of reduced degradation of the substrate itself. ADAMTS5 expression is required for the cleavage of versican and migration through this proteoglycan, as demonstrated by our \textit{in vitro} migration assays (Figure 4.4A). Our studies in the \textit{Adams}5\(^{-/}\;xVcan^{hdf/+}\) mice support the notion that versican accumulation in \textit{Adams}5\(^{-/}\) mice is preventing the migration of CD8\(^+\) T cells.
suggesting that a reduction in versican could rescue the phenotypes observed in \textit{Adams5}^{-/-} mice (Figure 4.8 - 4.10). However, it is important to note that the migration of influenza-specific CD8\(^{+}\) T cells, and other immune cells (e.g. CD4\(^{+}\) T cells), was not completely impeded in \textit{Adams5}^{-/-} mice. ADAMTS5 may therefore be working in concert with other metalloproteinases to facilitate T cell migration. The proteoglycanases, ADAMTS1, 4, 8, 9, 15 and 20, as well as MMP1, 2, 3, 7 and 9, are capable of producing versican fragments in a similar fashion to ADAMTS5 (164, 184, 198, 282, 331). It is reasonable to suspect that there is redundancy built into the trafficking system as related family members may be providing some compensatory function in the absence of ADAMTS5 allowing some (although highly restricted) T cell migration into the peripheral tissue (Figure 3.5 - 3.8). Indeed, Figure 4.4B indicates that ADAMTS4, 9 and 15 expression was increased in CD8\(^{+}\) T cells from \textit{Adams5}^{-/-} mice. Cooperative versican cleavage has been observed in embryogenesis, where ADAMTS1, 5, 9 and 20 are required for interdigital web regression (299), and so their compensatory presence in the regulation of immune cell migration cannot be discounted. This is also the case for collagen degradation, where MMP2 and 9 both contribute to T cell migration (256, 273). This is similar for macrophage migration, where MMP10 degrades collagen to facilitate its migration through the basement membrane (333). Further studies with related family members are required to ascertain their specific contribution to versican cleavage and influenza-specific immunity in the \textit{Adams5}^{-/-} mouse.

The effect of other ECM components on T cell activation and migration in influenza virus infected \textit{Adams5}^{-/-} mice are not described in this chapter. Versican was chosen for closer analysis because of its global tissue expression pattern (334). It would not be surprising if other proteoglycans targeted by ADAMTS5 influence viral pathogenesis and immunity in other systems. An altered ADAMTS5/versican/hyaluronan complexes within the \textit{Adams5}^{-/-} mouse model may be affected. Hyaluronan is a large polysaccharide and a key structural component
of the ECM, contributing to normal tissue hydrodynamics, including proliferation and migration (335). Versican binds to hyaluronan through its G1 domain, allowing versican localisation to the ECM (336). Recent studies indicate that hyaluronan can act as a signalling molecule for inflammatory cells, such as macrophages (337-339). Hyaluronan also binds molecules on the surface of T cells (340), including CD44 and CD62L, both of which are important for migration, proliferation and activation of T cells (341). In addition, hyaluronan can affect the inflammatory properties of activated T cells. High molecular weight hyaluronan displays anti-inflammatory properties that suppress the functionality and proliferation capacity of regulatory T cells, preventing excessive inflammation (342). Importantly, hyaluronan released on the surface of DCs contributes to the activation of naïve T cells by helping cell attachment during the antigen presentation (343, 344). Whilst we did not fully address hyaluronan expression in the Adams5⁻/⁻ mice, preliminary qPCR data in our laboratory indicates that hyaluronan synthases (HAS1, 2 and 3 - enzymes involved in the synthesis of hyaluronan), were significantly altered in influenza-specific CD8⁺ T cells isolated from Adams5⁻/⁻ mice.

4.7 Conclusion

In summary, our data show that the ADAMTS5 ECM enzymatic activity is important for lymphocyte trafficking following influenza virus infection (especially for CD8⁺ T cell immunity). In conclusion, interventions that facilitate increased ADAMTS5 expression used in conjunction with current approved antivirals and or vaccines offer a new approach for combating unexpected emerging influenza virus pandemic threats.
Chapter 5 - ADAMTS7 and the immune response to influenza virus infection

5.1 Overview

ADAMTS7 remains a relatively uncharacterised member of the ADAMTS family. ADAMTS7’s functional role as an ECM enzyme is limited to cleavage of the cartilage oligomeric matrix protein and thrombospondin-1 in arteries (284, 291). Degradation of these substrates in vitro has given ADAMTS7 a causal role in atherosclerosis and arthritis (286, 288, 345). The limited number of in vivo studies dissecting the functional role of ADAMTS7 have utilised both Adamts7-/- mice and ADAMTS7 transgenic mice (283-285). Adamts7-/- mice generated through the insertion of a LacZ cassette into the ADAMTS7 gene, thereby preventing the translation of the enzymatically active ADAMTS7 protein (283). Staining for the β-galactosidase reporter gene expression in Adamts7-/- mouse tissue identified the presence of high levels of ADAMTS7 in the heart, brain, lung, intestine and adrenal glands (283). Moderate expression of ADAMTS7 was observed in the kidney, brown fat and skeletal muscle (283). Additional phenotypic screens, (morphology, behaviour, haematology, energy consumption, neurological and clinical chemistry) were performed on Adamts7-/- mice with no overt differences detected between WT and Adamts7-/- mice (284). As such, Adamts7-/- mice are thought to be phenotypically normal (283, 284). Whereas removing ADAMTS7 resulted in no overt phenotypes, overexpressing ADAMTS7 within the chondrocytes of WT mice (for the assessment of ADAMTS7’s role in arthritis) resulted in decreased weight, bone volume and skeletal length in the first 4 weeks of life in these mice (285).

The presence of ADAMTS7 in the lung, and more importantly within the bronchioles (284), suggests that ADAMTS7 may have an unidentified role in lung homeostasis. Many respiratory tract infections, such as influenza virus modify the integrity of the lung following
virus replication. The expression and function of many host cellular factors, including ADAMTS7, may be altered, leading to increased/decreased susceptibility to infection. Indeed, a siRNA screen assessing human protease activity following influenza virus infection established ADAMTS7 as an important contributor to influenza virus pathogenesis with its actions modulated through the NF-κB pathway (161). In this screen, all known 481 human proteases were assessed for their role in influenza virus replication (160, 161). Twenty-one proteases were identified that either increased or decreased influenza virus replication through the use of an in vitro human lung cell culture model (A549 cells) (161). These targets were confirmed multiple times through downstream analysis (M gene qPCR, NP expression, haemagglutination assays) and suggest a definitive role for ADAMTS7 in influenza virus replication.

Inflammatory cytokines, including TNFα, have been shown to induce the expression of ADAMTS7 in inflammatory diseases, such as arthritis (291). This expression is caused by TNFα activating the NF-κB pathway, whose components bind to the promoter region of the ADAMTS7 gene, allowing protein translation. Further, this study identified that when ADAMTS7 was overexpressed in chondrocytes, elevated levels of TNFα occur, suggesting a positive feedback loop for TNFα and ADAMTS7 (285). This is important as TNFα expression is critical in the control of influenza virus infection. TNFα creates an anti-viral state surrounding epithelial cells and encourages the development and proliferation of immune cells, such as DCs and macrophages (152). Additionally, TNFα is involved in maintaining the viability and functionality of immune cells, such as DCs (117).

The relationship identified between TNFα and ADAMTS7 is of particular interest given that ADAMTS7’s structural homolog, ADAMTS12, has been shown to play a significant role in inflammation (295, 346). Adamts12−/− mice show signs of severe neutrophilia and cytokinemia (e.g. IL6) in inflammatory disease; i.e. colitis, pancreatitis and sepsis, resulting in
delays in recovery (295). Given the association of ADAMTS12 with inflammation and the previously identified role of ADAMTS7 in the control of TNFα expression and influenza virus replication (285), we hypothesised that ADAMTS7 may have an unidentified role in viral immunity. The results in this chapter describe a novel association between influenza virus infection, immunity and ADAMTS7 expression. Here we found that Adams7⁻/⁻ mice were more susceptible to intranasal X31 (H3N2) influenza virus infection due to perturbations in the immune response described herein.

5.2 Adms7⁻/⁻ mice are immunologically “normal”

Adams7⁻/⁻ mice were generated through the insertion of a LacZ cassette within the ADAMTS7 allele (283, 284), preventing expression of protein. Previously published observations using Adms7⁻/⁻ mice indicate that these mice are phenotypically normal based on gross morphological and histological analysis (284). The immune cell populations in Adms7⁻/⁻ mice however are yet to be characterised. To define immune cell populations in uninfected mice, we removed spleens and lungs and generated single cell suspensions for comparison with WT controls using flow cytometry. These tissues were chosen as these would be used as points of comparison in future influenza virus infection experiments. In the spleen, we observed no differences in neutrophils (Ly6GhiGR1hi) (Figure 5.1A), NK cells (CD314⁺CD3⁻) (Figure 5.1B), macrophages (CD11bhiF4/80hi) (Figure 5.1C), DCs (CD11chMHCIIhi) (Figure 5.1D), B cells (B220⁺) (Figure 5.1E) and total CD4⁺ T (CD4⁺CD3⁺) and CD8⁺ T cells (CD8⁺CD3⁺) (Figure 5.1F) between WT and Adms7⁻/⁻ mice. Similar results were observed between WT and Adms7⁻/⁻ mice in the lung (Figure 5.2). This data supports previously published data suggesting that Adms7⁻/⁻ mice develop normally.
Figure 5.1. Naïve Adamts7−/− mice have normal immune cell subsets in the spleen.
Spleens were removed from naïve WT and Adamts7−/− mice and immune cell subsets were characterized by flow cytometry. (A) Neutrophils (Ly6GhiGR1hi), (B) NK cells (CD314+CD3−), (C) macrophages (CD11bhiF4/80hi), (D) DCs (CD11chiMHCI1hi), (E) B cells (B220+) and (F) T cells (CD4+CD3+ or CD8+CD3+). The results are expressed as means ± SEM (n = 5, Figures are representative of 3 independent experiments).
Figure 5.2. Naïve Adamts7−/− mice have normal immune cell subsets in the lung.

Lungs were removed from naïve WT and Adamts7−/− mice and immune cell subsets were enumerated by flow cytometry. (A) Neutrophils (Ly6GhiGR1hi), (B) NK cells (CD314+CD3−), (C) macrophages (CD11bhiF4/80hi), (D) DCs (CD11cMHCIIhi), (E) B cells (B220+) and (F) T cells (CD4+CD3+ or CD8+CD3+). The results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (n = 5, Figures are representative of 3 separate experiments).
5.3 *Adams7*−/− mice are more susceptible to influenza virus infection

The identification of ADAMTS7 as a key contributor to influenza virus infection *in vitro* (161) justifies further investigation of its role *in vivo*. To study the role of ADAMTS7 we examined *in vivo* weight loss and determined lung virus titres following X31 (H3N2) intranasal infection (10^4 pfu/mouse) of *Adams7*−/− and WT control mice. *Adams7*−/− mice lost more weight than WT controls (*p* < 0.05 on 5, 6 and 10 dpi) (Figure 5.3A). Moreover, at the known peak of virus replication in this model (3 dpi), *Adams7*−/− mice showed higher lung virus titres when compared to WT controls (*p* < 0.005) (Fig 5.3B). Delayed virus clearance was also observed in *Adams7*−/− mice at 7 dpi (*p* < 0.005) when compared to WT controls (Figure 5.3C). These data clearly demonstrate that *Adams7*−/− mice do not clear influenza virus as effectively at WT controls.

5.4 *Adams7*−/− mice have reduced DCs in the lung following influenza virus infection

Increased lung virus titres and weight loss in *Adams7*−/− mice suggested the possibility of defective immune cell responses. To further understand the mechanism involved we enumerated innate immune cell populations in the lung, the primary site of virus replication (73). We hypothesized that a defect in innate immune cell numbers in the lung could result in enhanced virus titres (as observed at 3 dpi) and increased weight loss at later time points (Figure 5.3), resulting in delayed clearance of infection. Our data indicated that there were no differences in neutrophils, NK cells and macrophages in the lung of WT and *Adams7*−/− mice (Figure 5.4A-C). Upon assessment of DC numbers in the lung, we found that DC numbers
Figure 5.3. *Adams7−/−* mice are more susceptible to influenza virus infection.
*Adams7−/−* and WT mice were intranasally infected with 10^4 pfu/mouse X31 (H3N2) influenza virus. (A) Percent of initial body weight was measured throughout the time course of infection. Virus titres in lung homogenates were determined at (B) 3 and (C) 7 dpi. The results are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, *** = p ≤ 0.005, n = 5, Figures are representative of 3 separate experiments).
Figure 5.4. **DC numbers are reduced in the lung of influenza virus infected Adamts7−/− mice.** Adamts7−/− and WT mice were intranasally infected with $10^4$ pfu/mouse X31 (H3N2) influenza virus and innate immune cell numbers were assessed 3 dpi in the lung. (A) Neutrophils (Ly6GhiGR1hi), (B) NK cells (CD314+CD3−), (C) macrophages (CD11bhiF4/80hi) and (D) DCs (CD11cMHCIIhi) in the lung. Data are expressed as means ± SEM (* = p ≤ 0.05, n = 5, Figures are representative of 3 separate experiments).
were significantly lower \((p = 0.017)\) in \(\text{Adamts}7^-\) mice when compared to WT controls (Figure 5.4D).

**5.5 Lack of ADAMTS7 reduces adaptive immunity**

Given evidence of delayed viral clearance in \(\text{Adamts}7^-\) knockout mice (Figure 5.3) and fewer DCs in the lung (Figure 5.4), we wanted to determine how this would impact on adaptive immunity. B and T cells are required for efficient clearance of influenza virus infection (see section 1.7). Disruption in either population has been shown to contribute to delayed viral clearance. We initially enumerated total B and T cell numbers in the spleen and lung at 7 and 10 dpi as these time points correspond to key time points in adaptive immunity following influenza virus infection. At 7 dpi, fewer total B cells \((p = 0.017)\) were observed in the spleen of \(\text{Adamts}7^-\) mice (Figure 5.5A). No differences were observed in total T cell numbers between WT and \(\text{Adamts}7^-\) mice in the spleen (Figure 5.5B). In the lung, we found no difference in total B cell and CD4\(^+\) numbers but saw a reduction in total CD8\(^+\) T cell numbers \((p = 0.045)\) in \(\text{Adamts}7^-\) mice when compared to WT controls (Figure 5.5C and D). When assessing adaptive immune cell populations in pooled MLN, we identified a trend for fewer total B and T cells in pooled MLN samples of \(\text{Adamts}7^-\) mice when compared to WT controls (Figure 5.5E and F).

We then continued our analysis at 10 dpi, the peak of influenza virus-specific T cell immunity. At this time point \(\text{Adamts}7^-\) mice had fewer total B and T cells in the spleen and fewer total T cells in the lung when compared to WT controls (Figure 5.6A-D). In pooled MLN samples \(\text{Adamts}7^-\) mice had similar numbers of total B cell and a trend higher for CD4\(^+\) and CD8\(^+\) T cells when compared to WT controls (Fig 5.6E and F). These experimental
Figure 5.5. Reduced total T cells 7 dpi in influenza virus infected Adamts7−/− mice. Adamts7−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus and total adaptive immune cell populations were assessed in the spleen, lung and MLN 7 dpi. B cells (B220+) in the (A) spleen, (C) lung and (E) MLN. T (CD4+ or CD8+) CD3+ cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, n = 5, Figures are representative of 3 separate experiments). MLN data are expressed as pooled means (n = 5 mice/group).
Figure 5.6. Reduced total T cells 10 dpi in influenza virus infected Adamts7−/− mice. Adamts7−/− and WT mice were intranasally infected with $10^4$ pfu/mouse X31 (H3N2) influenza virus and total adaptive immune cell populations were assessed in the spleen, lung and MLN 10 dpi. B cells (B220+) in the (A) spleen, (C) lung and (E) MLN. T (CD4+ or CD8+) CD3+ cells in the (B) spleen, (D) lung and (F) MLN. Spleen and lung data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test ($* = p \leq 0.05$, $** = p \leq 0.005$, $n = 5$, Figures are representative of 3 separate experiments). MLN data are expressed as pooled means ($n = 5$ mice/group).
observations suggest that Adamts7−/− mice have fewer total adaptive immune cells in the periphery following influenza virus infection.

5.6 Influenza-specific CD8+ T cell immunity in Adamts7−/− mice following primary influenza virus infection

Our data suggests that Adamts7−/− mice have reduced DC numbers in the lung (Figure 4.4D) that may contribute to the observed CD8+ T cells discrepancies in the lung and spleen (Figure 5.5 and 5.6). Fewer DC numbers will compromise influenza antigen uptake and antigen presentation in the MLN, culminating in reduced T cell activation and clonal expansion of influenza-specific CD8+ T cells (72). Hence, we anticipated that influenza-specific CD8+ T cell responses would be heavily impacted in Adamts7−/− mice, due to the reductions in DC numbers. To assess influenza-specific CD8+ T responses in Adamts7−/− mice we enumerated antigen specific CD8+ T cells using tetrameric complexes that recognise the immunodominant NP366-374 (ASNENMETM) or PA224-233 (SSLENFRAYV) CD8+ T cell epitopes at 7 and 10 dpi (65). At 7 dpi, a trend for fewer NP366-374 and PA224-233 CD8+ T cells were detected in the spleen (Figure 5.7A) and significantly fewer influenza-specific CD8+ T cells in the lung of Adamts7−/− mice when compared to WT controls (Figure 5.7C). A trend for higher NP366-374 and PA224-233 CD8+ T cells was found in pooled MLN samples in Adamts7−/− and WT mice (Figure 5.7E). These results were further supported at 10 dpi, where statistically fewer NP366-374 and PA224-233 CD8+ T cell numbers were noted in the spleen and lung of Adamts7−/− mice when compared to WT controls (Figure 5.8A and C). NP366-374 and PA224-233 CD8+ T cell numbers in pooled MLN samples mirrored data at 7 dpi suggesting an accumulation of cells (Figure 5.8E).
Figure 5.7. Influenza-specific CD8+ T cell immunity in Adamts7−/− mice 7 dpi.
Spleen, lung and MLN from influenza virus infected Adamts7−/− and WT mice were assessed for influenza-specific immunity 7 dpi. Tetramer+CD8+ T cells in the (A) spleen, (C) lung and (E) pooled MLN. IFNγ+CD8+ T cells in the (B) spleen, (D) lung and (F) pooled MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, n = 5, Figures are representative of 3 separate experiments). MLN data are expressed as pooled means (n = 5 mice/group).
Figure 5.8. Influenza-specific CD8+ T cell immunity in Adamts7−/− mice 10 dpi.
Spleen, lung and MLN from influenza virus infected Adamts7−/− and WT mice were assessed for influenza-specific immunity 10 dpi. Tetramer+CD8+ T cells in the (A) spleen, (C) lung and (E) pooled MLN. IFNγ+CD8+ T cells in the (B) spleen, (D) lung and (F) pooled MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 5, Figures are representative of 3 separate experiments). MLN data are expressed as pooled means (n = 5 mice/group).
The ICS assay was used to assess functionality (IFNγ expression) of influenza-specific NP\textsubscript{366-374} or PA\textsubscript{224-233} CD8\textsuperscript{+} T cells in the spleen, lung and pooled MLN at 7 and 10 dpi (300). As expected, the data obtained mostly mirrored the tetramer results where fewer IFNγ\textsuperscript{+}CD8\textsuperscript{+} T cells were observed in the spleen and lungs of Adamts7\textsuperscript{-/-} mice when compared to WT controls (Figure 5.7 and 5.8). The data presented herein suggests that influenza-specific CD8\textsuperscript{+} T cell immunity was significantly impaired in the spleen and lung of Adamts7\textsuperscript{-/-} mice at 10 dpi and provides a possible explanation for increased weight loss and delayed viral clearance observed in Adamts7\textsuperscript{-/-} mice (Figure 5.3).

5.7 ADAMTS7 is required for efficient NP\textsubscript{366-374} CD8\textsuperscript{+} T cell memory

We suspected that fewer influenza-specific effector CD8\textsuperscript{+} T cells during primary infection would impact the number of memory CD8\textsuperscript{+} T cells and downstream recall/secondary responses in Adamts7\textsuperscript{-/-} mice. We therefore assessed NP\textsubscript{366-374} and PA\textsubscript{224-233} CD8\textsuperscript{+} T cell numbers at 30 dpi in Adamts7\textsuperscript{-/-} mice. Fewer NP\textsubscript{366-374} and PA\textsubscript{224-233} tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells were found in the spleen of Adamts7\textsuperscript{-/-} mice when compared to WT controls (Figure 5.9A) and a strong but similar trend for NP\textsubscript{366-374}IFNγ\textsuperscript{+} and PA\textsubscript{224-233}IFNγ\textsuperscript{+} CD8\textsuperscript{+} T cells although this was not statistically significant (Figure 5.9B). Further analysis of influenza-specific CD8\textsuperscript{+} T cell memory indicated that Adamts7\textsuperscript{-/-} mice had fewer memory (CD62L\textsuperscript{hi}CD44\textsuperscript{hi}) NP\textsubscript{366-374} CD8\textsuperscript{+} T cells (p = 0.013) in the spleens of Adamts7\textsuperscript{-/-} mice when compared to WT controls (Figure 5.10). Although there was no difference in memory (CD62L\textsuperscript{hi}CD44\textsuperscript{hi}) PA\textsubscript{224-233} CD8\textsuperscript{+} T cells between WT and Adamts7\textsuperscript{-/-} mice.
Figure 5.9. Influenza-specific CD8+ T cell immunity in Adamts7−/− mice 30 dpi. Adamts7−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus. Spleens were removed and influenza-specific immunity was assessed 30 dpi. (A) Tetramer+ CD8+ T cells and (B) functional (IFNγ+) CD8+ T cells are displayed. These results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, n = 5, Figures are representative of 3 separate experiments).
Figure 5.10. Adamts7−/− mice have reduced numbers of influenza-specific memory CD8+ T cells.

Adamts7−/− and WT mice were intranasally infected with 10⁴ pfu/mouse X31 (H3N2) influenza virus and influenza-specific memory (CD62LhiCD44hi) CD8+ T cells were enumerated in the spleen 30 dpi. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, n = 5, Figures are representative of 3 separate experiments).
5.8 Lack of ADAMTS7 impacts secondary CD8\(^+\) T cell responses in mice

Due to perturbed CD8\(^+\) T cell memory responses in *Adams7\(^-\)/\(^-\)* mice, we speculated that recall following secondary influenza virus infection may also be affected. To study this, we intraperitoneally primed *Adams7\(^-\)/\(^-\)* and WT control mice with \(1.5 \times 10^7\) pfu PR8 (H1N1) influenza virus and 6 weeks later we intranasally challenged the same mice with \(10^4\) pfu/mouse X31 (H3N2) influenza virus as previously described (300). Both viruses share the same NP and PA internal gene segments to enable boosting of NP\(_{366-374}\) and PA\(_{224-233}\) CD8\(^+\) T cell responses. Using this challenge model, we identified that *Adams7\(^-\)/\(^-\)* mice lost more weight than WT controls (in grams lost and % initial body weight) at 6 and 7 dpi \((p = 0.043\) and 0.0047, respectively) (Figure 5.11A and 5.11B). Weight, in grams, throughout the time course of infection is also shown in Figure 5.11C.

To see if this weight loss correlated with poor immunity we assessed total T cell numbers in the spleen, lung and MLN of *Adams7\(^-\)/\(^-\)* and WT mice using flow cytometry. Our studies indicate that total CD4\(^+\) and CD8\(^+\) T cell numbers were significantly lower in the spleens and lungs of *Adams7\(^-\)/\(^-\)* mice when compared to WT controls (Figure 5.12A and B). No differences in these cell populations were detected in MLN samples (Figure 5.12C).

In the C57BL/6 influenza virus mouse model, a typical secondary recall response shows 6 fold higher numbers of NP\(_{366-374}\) CD8\(^+\) T cells when compared to PA\(_{224-233}\) CD8\(^+\) T cells (66, 67, 149). Given total CD8\(^+\) T cell numbers in the spleen and lung were reduced in *Adams7\(^-\)/\(^-\)* mice following secondary challenge (Figure 5.12), we predicted influenza-specific CD8\(^+\) T cell immunity in *Adams7\(^-\)/\(^-\)* mice to be compromised. However, tetramer and ICS staining showed that only CD8\(^+\) NP\(_{366-374}\) T cell responses in the spleen and lung were reduced, with no differences detected for PA\(_{224-233}\) tetramer\(^+\) and PA\(_{224-233}\)\(^+\)IFN\(\gamma\) CD8\(^+\) T cell numbers when compared to WT controls in these tissues (Figure 5.13A - D).
Figure 5.11. *Adams7^{-/-}* mice show increased weight loss following secondary challenge. *Adams7^{-/-}* and WT mice were intraperitoneally primed with $1.5 \times 10^7$ pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with $10^4$ pfu/mouse X31 (H3N2) influenza virus. Weight loss in (A) percent initial starting weight, (B) grams lost and (C) weight was measured throughout the time course of secondary challenge. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = $p \leq 0.05$, ** = $p \leq 0.01$, n = 5, Figures are representative of 3 separate experiments).
Figure 5.12. T cell responses in influenza virus infected Adamts7−/− mice following secondary challenge.
Adamts7−/− and WT mice were intraperitoneally primed with 1.5 x 10^7 pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10^4 pfu/mouse X31 (H3N2) influenza virus. Total CD4+ T cells and CD8+ T cells were enumerated in the (A) spleen, (B) lung and (C) MLN 7 dpi. Data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 5, Figures are representative of 3 separate experiments).
In the MLN, no differences in antigen specific and functional CD8+ T cells were found following tetramer staining, but CD8+NP366-374 were reduced in Adamts7−/− mice (Figure 5.13E and F).

5.9 ADAMTS7 is required for DC priming of CD8+ T cells

Experimental results in Adams7−/− mice following influenza virus infection suggest that ADAMTS7 may play a role in regulating DC numbers that could influence reduced CD8+ T cell numbers as observed in Figure 5.4, 5.5 and 5.6. To further clarify this observation and understand the underlying mechanism controlling activation of CD8+ T cells, splenic DCs from Adams7−/− mice and WT controls were magnetically enriched (98% population purity) and infected with PR8 (H1N1) influenza virus (MOI 0.06). After 6 hours of infection we co-cultured infected DCs with CD8+ T cells from either Adams7−/− and WT mice. After 48 hours, we assessed the activation of all cell types using flow cytometry. As expected, we detected comparable percentages of DCs in all samples (Figure 5.14A), indicating that a similar number of DCs were loaded into each sample.

When we examined how influenza virus infection affected the activation of DCs we found that activated DCs (CD11chiCD86hiMHCIIhi cells) were reduced in Adams7−/− DCs when compared to WT DC (Figure 5.14B). Additionally, although the same number of CD8+ T cells (10,000 cells) were added to the DC/T cell co-culture, a reduced proportion of total CD8+ T cells (p < 0.005) were found in the samples containing Adams7−/− DCs after the 48 hours of influenza virus infection (Figure 5.14C). This culminated in reductions in the percentage of activated CD8+ T cells (CD8+CD69hiCD44hi) (p < 0.005) in samples containing Adams7−/− DCs (Figure 5.14D).
Figure 5.13. Influenza-specific immunity in Adamts7−/− mice following secondary challenge. Adamts7−/− and WT mice were intraperitoneally primed with 1.5 x 10⁷ pfu/mouse PR8 (H1N1) influenza virus and then intranasally challenged 6 weeks later with 10⁴ pfu/mouse X31 (H3N2) influenza virus. Spleen, lung and MLNs were removed and influenza-specific immunity assessed 7 dpi. Tetramer+CD8+ T cells in the (A) spleen, (C) lung and (E) MLN. IFNγ+CD8+ T cells in the (B) spleen, (D) lung and (F) MLN. The results are expressed as means ± SEM, and statistical significance (relative to WT mice) was determined by Student’s t test (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.005, n = 5, Figures are representative of 3 separate experiments).
Figure 5.14. The removal of ADAMTS7 inhibits DC activation.
Splenic DCs were stimulated with PR8 (H1N1) influenza virus (MOI 0.06) and CD8+ T cells added 6 hours later. After 48 hours of co-culture, DC and CD8+ T cell activation was assessed by flow cytometry. Percentage of (A) DCs (CD11chi) and (B) activated DCs (CD11cMHCIIhiCD86hi). Percentage of (C) CD8+ T cells (CD8+CD69hiCD44hi) and (D) activated CD8+ T cells (CD8+CD69hiCD44hi). The data are expressed as means ± SEM, and statistical significance was determined by a One-way ANOVA (** = p ≤ 0.01, *** = p ≤ 0.005 relative to WT DC: WT T, ## = p ≤ 0.01, ### = p ≤ 0.005 relative to WT DC: TS7 T, n = 5). Figures are representative of 3 individual experiments. TS7 denotes ADAMTS7. T denotes CD8+ T cell.
The final question we addressed in this chapter was to ask why \textit{Adams7\textsuperscript{-/-}} DCs were not effectively activating CD8\textsuperscript{+} T cells (Figure 5.14D). We suspected this was related to ADAMTS7’s ability to regulate TNF\(\alpha\) expression (285), as previously described. TNF\(\alpha\) is an integral cytokine for DC maturation and maintenance (347, 348). Indeed, \textit{TNF\(\alpha\)-/-} mice have more immature DCs upon adenovirus infection (347). We compared lung cytokine levels in naïve and influenza virus infected mice. We could not detect basal levels of TNF\(\alpha\) expression by qPCR in the lungs of \textit{Adams7\textsuperscript{-/-}} mice (Figure 5.15A). Following influenza virus infection, the lungs of \textit{Adams7\textsuperscript{-/-}} mice showed lower TNF\(\alpha\) expression when compared to WT counterparts (Figure 5.15A). Interestingly, other cytokines, such as IFN\(\gamma\), were not affected by the lack of ADAMTS7 (Figure 5.15B). This data suggests that a reduction of TNF\(\alpha\) expression in the lungs of \textit{Adams7\textsuperscript{-/-}} during influenza virus infection, could be contributing to reductions in DC numbers observed in these mice. However, the cells expressing TNF\(\alpha\) needs to be further confirmed.
Figure 5.15. TNFα expression is reduced in Adamts7−/− lung tissue.
Lungs were removed from influenza virus-infected Adamts7−/− and WT mice at 0 and 3 dpi. Expression of (A) TNFα and (B) IFNγ was assessed by qPCR. The data are expressed as means ± SEM, and statistical significance (relative to WT controls) was determined by a Student’s T test (*** = p ≤ 0.005, n = 5, Figures are representative of 3 separate experiments).
5.10 Discussion

DC mediated-activation of naïve CD8$^+$ T cells is critically important for the establishment of full effector function and the eventual clearance of pathogens (72, 74, 75, 302). Our research suggests that the absence of ADAMTS7 disrupts DC maturation and CD8$^+$ T cell activation following primary influenza virus infection (Figure 5.5, 5.7 and 5.8) and results in poor memory and secondary responses (Figure 5.10- 5.13).

Decreased DC numbers observed in the lungs of naïve and influenza virus infected lungs of *Adams7*−/− mice may be a causal factor for reduced influenza-specific CD8$^+$ T cell numbers in the periphery at later dpi, and suggests that there is perturbed priming of CD8$^+$ T cells in *Adams7*−/− mice. Unfortunately, our study only enumerated DC populations in the lung, and future work should assess DC populations in other tissues, such as the thymus, of naïve and infected *Adams7*−/− mice to determine if there are differences in these populations. This is particularly important in naïve mice, as differences in DC populations in a naïve setting may contribute to defects in infected mice for both DC populations and T cell priming. Additionally, only total DC populated were assessed, i.e. CD11c$^{hi}$MHCII$^{hi}$ cells, and it would be of interest to further identify the DC subsets thought to be involved in antigen presentation in the influenza virus mouse model. Currently there is ongoing debate surrounding which DC subset is critical for antigen presentation following influenza virus infection. CD11b$^{hi}$CD103$^{lo}$ and CD11b$^{lo}$CD103$^{hi}$ migratory DCs (DCs that migrate from the influenza virus infected lung to the MLN) were identified in separate studies as the sole DC subset involved in antigen presentation in the C57BL/6 mouse model (349, 350). This confusion is further compounded by studies that suggest both CD11b$^{hi}$CD103$^{lo}$ and CD11b$^{lo}$CD103$^{hi}$ migratory DC subsets preferentially promote CD8$^+$ T cell differentiation, excluding all others (119, 120). Lymph node resident CD8$^+$ DCs are also involved in antigen presentation (72, 73, 351). This subset is thought to receive antigen from the migratory DCs for presentation to naïve CD8$^+$ T cells in
the lymph node (352, 353). Because of the lack of clarity surrounding DC subsetting and CD8+ T cell activation, it would be of benefit to assess all DC subsets suspected to be involved in antigen presentation (CD11bhiCD103lo, CD11bhiCD103hi and CD8+). We suspect that it is unlikely that only one DC subset is involved in antigen presentation and that they work together to activate naïve CD8+ T cells.

Moreover, memory CD8+ T cells do not respond to all antigen-presenting DC subsets after influenza virus infection (75). Unlike naïve CD8+ T cells, which respond to antigen presentation from CD8+, CD11bhi and CD103hi DCs, memory CD8+ T cell undergo clonal expansion following interactions with the CD8+ DC subset following challenge (75). Because of this it would be beneficial to assess if CD8+ DCs numbers were lower in Adams7−/− mice following secondary challenge, given that immune cell responses were compromised during secondary challenge (Figure 5.13).

In this chapter, we have identified a key defect in Adams7−/− mice with respect to total DC numbers. Additionally, we identified a defect in DC activation when these cells lack ADAMTS7 (Figure 5.14B). However, it would be beneficial to further characterise this activation model. In this model, we used splenic DCs at 48 hours’ post influenza virus infection. It may be better to assess activation of DCs from MLN tissues, given that this is the site of antigen presentation in the influenza virus infection model. Though, this would be very difficult given the size of the MLN. Additionally, assessing earlier time points may allow us to assess at which time point activation is occurring in WT and Adams7−/− DCs, respectively.

It is also important to discuss that TNFα expression, a key cytokine in the immune response to influenza virus infection, was perturbed in Adams7−/− mice (Figure 5.15A). Basal levels of TNFα were not detectable in Adams7−/− mice and low expression only observed at 3 dpi. Although DCs are not the main cell type responsible for TNFα production in influenza-infected lungs, TNFα, produced by surrounding cells (such as NK and T cells), is involved in
the maintenance, maturation, activation and functionality of DCs. With lower levels of TNFα in Adamts7−/− mice it is unsurprising that DC numbers were also reduced (Figure 5.4C), although this requires further analysis and would greatly benefit from assessing TNFα expression in cells which highly express TNFα (i.e. NK and T cells).

A key theme in Chapter 3 and 4 was the role ADAMTS5 played in the migration of CD8+ T cells from the MLN into the periphery. ADAMTS5 potently degrades ECM proteoglycans (versican, aggrecan, brevican and neurocan) (176, 213, 282), but knowledge surrounding which ECM components ADAMTS7 could be cleaving to facilitate immune cell migration is unknown in the matrix biology field. ADAMTS7 does not cleave versican (personal communication, Dr Daniel McCulloch, Deakin University) and the only known ECM components that ADAMTS7 cleaves is the cartilage oligomeric matrix protein and thrombospondin-1 proteins (284). Given these proteins are unlikely to be involved in migration of immune cells (due to selective expression in cartilage tissues), ADAMTS7 may be cleaving alternative ECM components to facilitate immune cell migration. Additionally, reduced TNFα expression in the lung of Adamts7−/− mice, as previously mentioned, could be the reason DC migration is inhibited (354). In vivo tracking of DC migration would provide an invaluable tool for assessing ADAMTS7’s role in this process. Ho et al. has labelled the lipid coat of influenza virus with a fluorescent dye (DiD) to assess infection, replication and uptake by DCs. Alongside intranasal infection of mice with this fluorescent virus, they intranasally inoculated mice with CFSE dye to track the migration of DCs that had taken up DiD influenza antigen (350). Alongside this, to further assess TNFα’s role in DC migration in Adamts7−/− mice, we could administer exogenous TNFα to Adamts7−/− mice during influenza virus infection to see if we could rescue the WT phenotype.

Finally, although Adamts7−/− mice has been previously characterised as “normal”, it is important to gain a further understanding of naïve and pathogen induced-induced immunity in
Adams7−/− mice. Some of the parameters measured in the original phenotypic characterisation of Adams7−/− mice by Bauer et al. were statistically significant (e.g. oxygen consumption and white blood cells in the blood) in specific sexes, whilst other measures were bordering on statistical significance (e.g. metabolic rate) (284). “Normality” in this study was established using 6 WT and 6 Adams7−/− mice from each sex. In our current study “immunological normality” was established using 15 WT and 15 Adams7−/− mice. Using this number of mice, we found that there was a trend for reduced DCs in the lung ($p = 0.09$) (Figure 5.2D) and reduced CD8+ T cells ($p = 0.06$) and CD4+ T cells ($p = 0.12$) in the spleen (Figure 5.1F). To fully establish normality of these immune cell types a greater number of WT and Adams7−/− mice could be characterised. Increasing the number of mice used in naïve phenotyping would ascertain conclusive evidence that these mice are truly “immunologically normal”.

5.11 Conclusion

In summary, our data show that ADAMTS7 is important for normal DC function during influenza virus infection. In the absence of ADAMTS7, influenza-specific CD8+ T cell responses are compromised. In conclusion, interventions that facilitate increased ADAMTS7 expression used in conjunction with current approved antivirals and/or vaccines offer a new approach to combating unexpected emerging influenza virus pandemic threats.
Chapter 6 - The role of ADAMTS enzymes in HeV infection

6.1 Overview

Our studies presented herein used the well-characterised influenza virus infection model to assess the role of ADAMTS5 and ADAMTS7 in influenza virus immunity. Given the increased morbidity observed in influenza virus infected Adamts5\(^{-/-}\) and Adamts7\(^{-/-}\) mice, we wanted to further assess the role of these enzymes in the immune response to other viral diseases. This would allow us to determine if ADAMTS enzymes contribute to viral immunity in a broader context.

Our research group has set up a collaboration with CSIRO AAHL investigators (Deborah Middleton and Linfa Wang) to establish and improve a mouse model for HeV infection. HeV is a RNA virus belonging to the paromyxoviridea family and was originally isolated from infected horses in Hendra, Queensland, Australia. Severe cases of HeV infection result in systemic disease, multiple organ failure and death (296, 297). Susceptibility to HeV infection has been observed in bats, ferrets, guinea pigs, pigs, cats, horses, dogs and humans (355-358). Infected horses display acute, febrile respiratory disease. Other symptoms include facial ataxia, swelling and frothing from the mouth. Horses display respiratory distress, neurological symptoms and vascular disease upon HeV infection (297). These symptoms are also observed in humans with the fatality rate of 57 % (4/7 human deaths) (359). Although most susceptible animals display clinical signs there is a paucity of immunological reagents (i.e. antibodies for flow cytometry) to define disease pathogenesis. Other small animal models, such as guinea pigs, require high dose inoculation to establish HeV infection but only develop severe encephalitis (360) and not fulminating disease. A robust small animal model for HeV infection that displays both respiratory and neurological symptoms, does not currently exist. Development of a consistent small animal model for the study of HeV or other henipavirus
infections, such as Nipah virus, would be an invaluable research tool. Unfortunately, mice are not readily susceptible to HeV as they are not a natural host and currently there are no mouse-adapted HeV strains available to ensure consistent infection. Recently our CSIRO AAHL collaborators, demonstrated that C57BL/6 and BALB/C mice could be infected with HeV via the intranasal route (296). In these studies, HeV infected mice displayed acute, transient and asymptomatic virus replication in the respiratory tract along with clear encephalitis (although this did not occur consistently in all HeV infected mice) with older mice more susceptible than younger counterparts.

In this results chapter we investigated the susceptibility of Adamts5⁻/⁻ and Adamts7⁻/⁻ mice to HeV in the context of establishing a model that fully reflects disease pathogenesis in humans and horses, as these mice were more susceptible to influenza virus infection as outlined in Chapters 3, 4 and 5.

### 6.2 HeV infection of Adamts5⁻/⁻ and Adamts7⁻/⁻ mice

WT, Adamts5⁻/⁻ and Adamts7⁻/⁻ mice were intranasally infected with 50,000 TCID₅₀ HEV (296). At 6 dpi, tissues (blood, brain, liver, lung, kidney and spleen) were harvested and RNA extracted for qPCR analysis to establish the presence of HeV N gene. Detection of HeV N gene in multiple organs would be indicative of a systemic infection. Table 6.1 demonstrates that the HeV N gene was detected in the lungs of all Adamts5⁻/⁻ (8/8 infected mice) and Adamts7⁻/⁻ mice (8/8 infected mice) and 7/8 WT mice. Adamts5⁻/⁻ mice had a greater number of mice with increased HeV N gene expression in the brain (4/8) when compared to WT (1/8) and Adamts7⁻/⁻ (1/8) mice. Adamts5⁻/⁻ mice also had increased HeV N gene expression in the kidney (2/8) when compared to WT (0/8) and Adamts7⁻/⁻ (1/8) mice. Adamts7⁻/⁻ mice were the only mice group with confirmed expression of the HeV N gene in the spleen (1/8) and liver
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**Table 6.1. HeV N gene expression in tissues.**

WT, Adamts5⁻/⁻ and Adamts7⁻/⁻ mice were intranasally infected with 50,000 TCID₅₀ HeV. Tissues were removed at dpi and expression of HeV N gene assessed by qPCR. n = 8 mice/group.
This data suggests that Adamts5\(^{-}\) and Adamts7\(^{-}\) mice are generally more susceptible to HeV infection, although further confirmation is required.

### 6.3 Immune responses in HeV infected Adamts5\(^{-}\) and Adamts7\(^{-}\) mice

Given the increased expression of the HeV N gene observed in Adamts5\(^{-}\) and Adamts7\(^{-}\) tissues when compared to WT mice (Table 6.1), we suspected that deficiencies in innate immune responses could be responsible for the increased presence of HeV N gene in these mice. We therefore enumerated neutrophils (Ly6G\(^{hi}\)GR1\(^{hi}\)), NK cells (CD3\(^{-}\)CD314\(^{+}\)), DCs (CD11b\(^{hi}\)F4/80\(^{hi}\)) and macrophages (CD11c\(^{hi}\)MHCII\(^{hi}\)) in the lungs and spleens of HeV infected WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) mice under PC3/4 conditions. Overall, no statistically significant differences were observed in these cell types in WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) in the spleen (Figure 6.1) or lung (Figure 6.2). Interestingly, small trends were observed in mice which were qPCR positive for the HeV N gene. Adamts7\(^{-}\) mice had a higher average percentage of neutrophils, NK cells, DCs and macrophages in the spleen (Figure 6.1). In the lung, 4/8 Adamts5\(^{-}\) mice showed a trend for higher percentages of neutrophils, (Figure 6.2A) but lower percentages for NK cells, macrophages and DCs (Figure 6.2B-D).

We also assessed adaptive immune cell populations in these mice. Six days after HeV infection, the spleen and lung were removed, fixed, and stained with monoclonal antibodies targeting B cells (B220\(^{+}\)), CD4\(^{+}\) T cells (CD3\(^{+}\)CD4\(^{+}\)) and CD8\(^{+}\) T cells (CD3\(^{+}\)CD8\(^{+}\)) in WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) mice. In the spleen, no differences were observed in percentages of total B cells, CD4\(^{+}\) and CD8\(^{+}\) T cells in HeV infected WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) mice (Figure 6.3). In the lung, the percentage of B cells and CD4\(^{+}\) and CD8\(^{+}\) T cells were comparable across all groups (Figure 6.4). These results suggest that there are no overt differences in adaptive immune cell populations in HeV infected WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) mice.
Figure 6.1. Innate immunity in the spleen of HeV infected mice.
WT, Adamts5<sup>−/−</sup> and Adamts7<sup>−/−</sup> mice were intranasally infected with 50,000 TCID<sub>50</sub> HeV and spleens removed 6 dpi. Percentages of (A) neutrophils (Ly6<sup>G</sup>hiGR1<sup>hi</sup>), (B) NK cells (CD3<sup>+</sup>CD314<sup>+</sup>), (C) macrophages (CD11b<sup>hi</sup>F4/80<sup>hi</sup>) and (D) DCs (CD11c<sup>hi</sup>MHCII<sup>hi</sup>) were enumerated. The results are expressed as means ± SEM (n = 8 mice/group).
Figure 6.2. Innate immunity in the lung of HeV infected mice.

WT, Adams5−/− and Adams7−/− mice were intranasally infected with 50,000 TCID₅₀ HeV and lung removed 6 dpi. Percentages of (A) neutrophils (Ly6G⁺GR1⁺), (B) NK cells (CD3⁻ CD314⁺), (C) macrophages (CD11b⁺F4/80⁺) and (D) DCs (CD11c⁺MHCII⁺) were enumerated. The results are expressed as means ± SEM (n = 8 mice/group).
Figure 6.3. Adaptive immunity in the spleen of HeV infected mice. WT, Adamts5\(^{-}\) and Adamts7\(^{-}\) mice were intranasally infected with 50,000 TCID\(_{50}\) HeV and spleens were removed 6 dpi. Percentages of total (A) B cells (B220\(^{+}\)), (B) CD4\(^{+}\) T cells (CD4\(^{+}\)CD3\(^{+}\)) and (C) CD8\(^{+}\) T cells (CD8\(^{+}\)CD3\(^{+}\)) were measured. The results are expressed as means ± SEM (n = 8 mice/group).
Figure 6.4. Adaptive immunity in the lung of HeV infected mice. WT, Adamts5−/− and Adamts7−/− mice were intranasally infected with 50,000 TCID<sub>50</sub> HeV and lungs removed 6 dpi. Percentages of total (A) B cells (B220<sup>+</sup>), (B) CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD3<sup>+</sup>) and (C) CD8<sup>+</sup> T cells (CD8<sup>+</sup>CD3<sup>+</sup>) were measured. The results are expressed as means ± SEM (n = 8 mice/group).
6.4 Discussion

Our studies, and those of others have identified that the mouse may not be the most efficient model for HeV infection or that it indeed requires further development (296). This is similar for viral diseases such as Ebola virus, where knockout models (e.g. Ifnar-/- mice) display increased susceptibility to infection compared to WT mice (361, 362). Because of this our study endeavoured to find a knockout mouse model that promoted systemic HeV infection that represented disease pathogenesis as observed in humans and horses. We proposed that modulating ECM enzymes would lead to increased HeV susceptibility in mice, as the ECM a) contains receptors that bind viruses to enhance replication or b) given its role in immune cell responses and functions may influence the overall immunity to HeV infection. However, our preliminary studies require further detailed analysis to conclusively determine if Adamts5-/- and Adamts7-/- mice are more susceptible to HeV infection via virus isolation in cell culture.

Our studies were not conclusive in highlighting differences in the immune responses between WT, Adamts5-/- and Adamts7-/- mice. This may have been because of the time point chosen to assess the immune response. Six dpi is generally considered to be an early time point in HeV infection (296). In the mouse model of HeV, encephalitis occurs at around 14 dpi (296), hence, it would be interesting to discover if encephalitis was more pronounced in Adamts5-/- and Adamts7-/- mice (given our observations in influenza virus infection) and if the immune response in these mice differed to that of WT controls at later dpi. We were however constrained by the need to handle the pathogen under PC4 conditions and had limited flexibility in terms of resourcing for this work.

Finally, this study assessing the immune response to HeV infection using flow cytometry, whilst preliminary, is novel, because studies of BSL3/4 agents are generally limited to analyses involving molecular biology. To our knowledge flow cytometric analysis of this magnitude has never been performed previously on this BSL4 agent. Because of the strict
safety requirements for BSL4 agents, our studies involved antibody staining and fixing protocols at BSL4 (performed by staff in fully encapsulated suits) and flow cytometric analysis occurred at BSL3. In this study, we only determined percentages and did not use cell counts as in previous chapters. Future studies would benefit from determining cell numbers to establish whether true differences in these HeV infected mice exist, however this was not possible in this current study due to the time constraints and occupational health and safety requirements for individuals working under BSL4 conditions.

6.5 Conclusion

The data presented in this chapter suggests that ADAMTS5 and ADAMTS7 may potentially be required for the control of systemic spread of HeV infection, although in depth studies need to be completed to validate these preliminary observations.
Chapter 7 - Discussion and Future Directions

7.1 Discussion

Since their initial discovery in 1997, the ADAMTS family of enzymes have been found to regulate or contribute to a vast array of diseases (363) including arthritis (213, 221, 364). Despite this knowledge, ADAMTS enzymes remain relatively uncharacterised compared to their MMP counterparts. Additionally, the inhibition of ADAMTS5 remains a key focus for the fields of rheumatology and matrix biology. Indeed, ADAMTS aggrecanase-specific inhibitors for arthritis are likely to be introduced to patients in the next few years following successful clinical trials which limited arthritis pathogenesis (365-367). Given the development of novel ADAMTS5 inhibitors for arthritis, it is important to understand the role ADAMTS5 (and other ADAMTS family members) plays in other disease states before therapeutic inhibition is undertaken, especially as these arthritis interventions will generally target susceptible elderly populations. Despite a significant body of knowledge describing the role of ADAMTS5 in arthritis, very little information is available regarding infection or immunity.

The research described in Chapters 3 and 4 has generated a substantial body of data outlining the role of ADAMTS5 in influenza specific immunity. We demonstrated that ADAMTS5 was required for efficient primary and secondary immune responses. These responses were linked to the accumulation of influenza-specific CD8+ T cells in the MLN of Adamts5−/− mice during primary influenza virus infection. We believe that poor egress of CD8+ T cells from the MLN is associated with reduced enzymatic degradation of versican as depicted in Figure 7.1. In our model, ADAMTS5 must cleave versican in the MLN to facilitate egress from this tissue (Figure 7.1). Due to its localization, large size and substrate binding characteristics, versican significantly effects the outcome of many cellular processes, including normal immune cell migration (as described in this thesis). Although versican is beneficial in
a homeostatic environment (e.g. tissue stabilization), inflammation often results in the upregulation of versican (322, 330). This upregulation supports wound repair in the dermis and lung (277) and contributes to normal immune responses (e.g. IFNγ and TLR2 signaling) (322, 368). However, without effective ECM turnover via enzymes, such as those in the ADAMTS family, the ECM can become an entangling web that prevents normal cell function.

Unlike ADAMTS5, ADAMTS7 does not cleave versican and so its role in influenza virus immunity may not be closely associated with ADAMTS5 function. Our data in Chapter 5 suggests that the total number of DCs (CD11c<sup>hi</sup>MHCII<sup>hi</sup>) in Adamts7<sup>−/−</sup> mice is perturbed leading to poor influenza-specific CD8<sup>+</sup> T cell immunity (as depicted in Figure 7.2). As previously discussed, ADAMTS7 has been shown to form a positive feedback loop with TNFα. TNFα then drives expression of ADAMTS5. In our own studies, we observed increased ADAMTS7 expression in CD8<sup>+</sup> T cells isolated from influenza virus-infected Adamts5<sup>−/−</sup> mice. This was not detected in CD8<sup>+</sup> T cells from WT control mice (Figure 4.4B). We believe increased expression of ADAMTS7 in CD8<sup>+</sup> T cells from Adamts5<sup>−/−</sup> mice may be a direct response to the lack of ADAMTS5. We propose in our model that ADAMTS7 is trying to promote ADAMTS5, which would in turn promote versican cleavage and egress of CD8<sup>+</sup> T cells from the MLN to the lung. Further analysis is required to determine if this is indeed the case, however the study by Lai et al suggests that a therapeutic intervention that enhances ADAMTS7 may also enhance ADAMTS5 activity (285), which in our model would support DC activation (ADAMTS7 mediated) and T cell migration (ADAMTS5 mediated).

Understanding a mechanism such as this would firstly benefit from analysing the immune responses in an Adamts5<sup>−/−</sup>xAdams7<sup>−/−</sup> mouse model before any therapeutic studies were undertaken.
Figure 7.1. T cell migration through the lymph node.
APCs enter the MLN through the afferent lymphatics. In the cortex of the MLN they present naïve T cells with influenza antigen. T cells then proliferate and become activated. Activated T cells then exit the MLN through the efferent lymphatics. T cells express versicanases, such as ADAMTS5, which cleave versican to facilitate their migration through the MLN.
Figure 7.2. ADAMTS7 in the control of DC functionality.
ADAMTS7 has previously been shown to form a positive feedback loop with TNFα through the NF-κB pathway. TNFα is an important cytokine that regulates the expression of ADAMTS5 and controls T cell migration. TNFα is also involved in DC viability and functionality. Without TNFα, DC mediated activation of CD8+ T cells cannot occur, and may explain why Adams7−/− mice have lowered CD8+ T cell responses up in influenza virus infection.
This dissertation also identified that the absence of ADAMTS5 and ADAMTS7 not only impacts influenza virus immunity but may also alter HeV pathogenesis. Unfortunately, it was outside the feasibility of this Ph.D. project to assess HeV infection in Adamts5\(^{-/-}\) and Adamts7\(^{-/-}\) mice in greater detail given the BSL4 nature of these experiments. However, these studies provide preliminary evidence supporting a generalised role for ADAMTS5 and ADAMTS7 in overall virus immunity and suggests that these knockout mice may be susceptible to several pathogens. Infection of Adamts5\(^{-/-}\) and Adamts7\(^{-/-}\) mice with viruses such as Herpes Simplex Virus (HSV) may also provide additional evidence given the importance of CD8\(^{+}\) T cells in controlling infection. HSV is a BSL2 agent with a well-characterised murine model (369, 370). As with influenza virus infection of Adamts5\(^{-/-}\) and Adamts7\(^{-/-}\) mice, we suspect HSV infection could lead to increased susceptibility, poor CD8\(^{+}\) T cell immunity and delayed healing of HSV dermal lesions, highlighting the broader significance of these ECM enzymes in viral immunity.

Virus infections, such as influenza virus, may therefore benefit from increases in ADAMTS expression to improve immune cell migration and clearance of pathogens. Our laboratory has previously developed recombinant influenza viruses expressing microRNA’s (miR) to modulate host immunity. In these studies, miR-155 was incorporated into the NS gene segment (371). In vitro infection of MDCK’s cells with recombinant viruses resulted in increased cytokine expression (IL6 and IL1\(\beta\)) (371), whilst in vivo studies show enhanced CD8\(^{+}\) T cell immunity (personal communication – Leonard Izzard, Stambas Laboratory). This technology can be modified to modulate ADAMTS expression. Incorporating a miR that negatively regulates ADAMTS5 [miR-195 (372, 373)] or ADAMTS7 [miR-106b (161)] into the influenza virus NS segment could potentially improve vaccine efficiency when used as a LAIV. Alternatively, exogenous delivery of ADAMTS enzymes may also be beneficial as
ADAMTS13 has already been used in mice to prevent thrombotic thrombocytic purpura and reduced the severity of the disease (374).

Some infectious diseases are characterised by uncontrolled infiltration of immune cells leading to exacerbated disease and increased mortality. For example, H5N1 highly pathogenic avian influenza viruses have a mortality rate of ~60% in infected individuals. Death is usually associated with a cytokine storm, dysregulated immune cell infiltration and increased lung pathology (19, 27, 375). Cerebral malaria, triggered by *Plasmodium* parasite, is responsible for 500,000 deaths each year (376). Cerebral malaria manifests following sequestration of the parasite in the brain and the excessive release of pro-inflammatory cytokines (e.g. IFNγ) (377-379), resulting in the infiltration of antigen-specific CD8+ T cells and brain pathology (380). Hence, inhibition of ADAMTS enzyme expression in these circumstances may be appropriate. The inhibition of ADAMTS4 and ADAMTS5 aggrecanases has been a key focus in the field of rheumatology. Two selective ADAMTS5 antibodies have already been developed: a recombinant monoclonal antibody directed towards the ancillary domain of ADAMTS5 (CRB0017) and a humanised ADAMTS5 monoclonal antibody (GSK2394002) (366, 367). Additionally, a selective aggrecanase inhibitor is currently in clinical trials (365). The generation of ADAMTS5 specific inhibitors has provided a strong foundation from which other MMP and ADAMTS inhibitors might be generated in the future to assist not only in the treatment of arthritis, but also in infectious disease. However, the inhibition of ADAMTS7 is slightly more challenging given the dearth of information regarding its structure and function.

Our study would suggest that inhibiting ADAMTS5 for arthritis treatment may make these individuals more susceptible to influenza virus infection. It is particularly important to understand this as it is generally the elderly who are afflicted with arthritis, and it is this population who is more likely to succumb to influenza virus infection. Assessing ADAMTS5 inhibitors in an influenza virus/arthritis model would aid in our understanding of this effect, as
the inhibition of ADAMTS5 in arthritis may not affect the immune response to influenza virus infection given the different tissues. This could initially be tested in an arthritis and influenza virus mouse model with further studies undertaken to compare immune responses in influenza infected arthritis patients and non-arthritis patients.

This study highlights a necessity for metalloproteinase activity in the immune response and suggests enhancing the expression of these enzymes would increase the immune response to influenza virus infection, resulting in the timely removal of disease. However, I believe this study establishes paradoxical and conflicting views in the ECM field. We know that dysregulation of metalloproteinases has a detrimental effect on ECM remodelling in arthritis. We also know that metalloproteinases in general cause exhaustive immune cell infiltration, excessive inflammation and unwarranted tissue destruction (227, 381-383). For example, excessive ECM remodelling by MMP9 in asthmatic patients can result in further tissue degradation, worsening the disease (384) suggesting that inhibition of MMP9 would provide a reliable therapeutic target to reduce asthma complications. Yet lack of MMP9 in Mmp9−/− mice results in delayed fracture repair indicating that MMP9 is required for cellular processes (385). Further, neutrophil infiltration into the lung and tissue destruction during influenza infection is directly correlated to MMP9 expression, hence inhibiting MMP9 seems necessary to prevent tissue destruction (91, 105). Following removal of MMP9 (Mmp9−/− mice) influenza infection was increased, due to reduced neutrophil infiltration and control of infection (105). Indeed, our study would suggest that presence of ADAMTS5 and ADAMTS7 is required for a functional immune response, although in other disease models the presence of ADAMTS5 and ADAMTS7 can have detrimental effects. If therapeutics were created that could enhance the expression of ADAMTS5 (as described above) we would have to assess the longevity of increased ADAMTS5 expression in the body. Ideally, increased expression of ADAMTS5 would be short-lived. However, if expression is enhanced over a longer period following the
removal of influenza virus infection one would expect tissue damage to occur, including the knee joint.

These studies, and our current study, provide key indications that understanding the multiple and complex role metalloproteinases play in disease is essential before assessing their potential applications as pharmacological targets.

7.2 Future Directions

As previously discussed, ADAMTS5 and ADAMTS7 regulate influenza-specific immunity via different mechanisms, hence the future directions in this section will be addressed separately

7.2.1 ADAMTS5

Chapters 3 and 4 identified a key role for ADAMTS5 in influenza virus infection, whereby our results indicate that ADAMTS5 mediated cleavage of versican is involved in CD8+ T cell migration. To further assess the migration of CD8+ T cells from Adamts5−/− mice, we could use adoptive transfer experiments to determine if this phenomenon was T cell intrinsic or in some way associated with the MLN microenvironment (65, 386). In these studies, we would transfer CD8+ T cells from Adamts5−/− mice to WT recipients. Upon influenza virus infection, one would expect these cells to be at a migratory disadvantage given that they do not express ADAMTS5. Alternatively, CD8+ T cells from WT mice would be adoptively transferred into Adamts5−/− mice to confirm our hypothesis. In this scenario versican accumulation would still occur in the MLN of Adamts5−/− mice, but one would anticipate that ADAMTS5 expression from WT CD8+ T cells would facilitate versican cleavage in the MLN, and egress from this tissue.
Combinatorial mutant mice have been used previously to characterise the role of ADAMTS enzymes in versican cleavage, as single mutants have not always shown a fully penetrant phenotype. Adamts\(5^+/x\)Adamts9\(+/-\) and Adamts\(20^+/x\)Adamts9\(+/-\) mutant mice [Adamts9\(+/-\) mice were used in this study as Adamts9\(-/-\) mice are embryonic lethal (387)] have been used previously to identify the importance of versican cleavage in interdigital web regression (299). It would be of interest to examine the impact of influenza immunity in this model i.e. if a more severe phenotype is observed. Adamts\(+/-\), Adamts4\(+/-\) and Adamts15\(+/-\) mice are available and it would be of significant interest to assess the role these enzymes in influenza virus pathogenesis (alone or in combination) given that they all cleave versican.

Finally, a topic that was not addressed fully in this study but is important in the matrix biology field, is the impact of versikine (the versican cleavage fragment) on overall immunity. Versikine acts as a damage associated molecular pattern, that triggers TLR2 signalling and activation of the NF-κB pathway that supports T cell activation through the release of type I cytokines (e.g. IL6) (258, 322, 388). We also know that the presence of versikine has been associated with a higher frequency of activated CD8\(^+\) T cells in myeloma patient samples (330). This suggests that versikine may have a crucial role in chemotactic signalling for CD8\(^+\) T cell migration and tissue infiltration. To further assess this our transwell assays used in this study can be modified by adding versikine to the lower chamber of the transwell in place of CXCL12 chemoattractant medium. Inhibiting versikine signalling in vivo would be difficult, as it is not present until versican is cleaved, hence why Adamts5\(+/-\) mouse models, and other versicanase mouse models (e.g. Adamts4\(+/-\) mice), are assessed for versican degradation. It would be of interest to further extend this study towards how removing versikine affects cellular processes in the Adamts5\(+/-\) mouse through an in-depth immunohistochemical analysis.
7.2.2 ADAMTS7

The matrix biology field would greatly benefit from understanding the enzymatic or non-enzymatic role of ADAMTS7 in cellular processes. ADAMTS7 is an extracellularly secreted, catalytically active enzyme, with very little information currently available about its substrates (195, 284). A highly novel study by Marchant et al. helped clarify the role of MMP12 during cocksackie virus infection in mice (309). In this study chromatin immunoprecipitation DNA sequencing technology was used to identify the interactions of MMP12 with DNA intracellularly. In this study it was found that macrophage-secreted MMP12 enters cocksackie virus infected cells, where it then enters in the nucleus (309). In the nucleus, intracellular MMP12 binds to the NF-κB promoter allowing secretion of IFNα (309). Regulation of IFNα production by MMP12 following infection could not only limit pathogen replication, but also prevent excessive inflammation. Our study would benefit substantially from utilising this technology to further characterise ADAMTS7’s role in the intracellular environment.

Chapter 5 described immunity in influenza virus infected $ADamts7^{-/-}$ mice. In these studies, we observed an increase in influenza virus lung titres and higher weight loss associated with perturbed influenza-specific CD8$^+$ T cell responses. We speculated that this was caused by fewer activated DC populations in influenza virus infected $ADamts7^{-/-}$ mice resulting in impaired antigen presentation. We did not however fully establish defective antigen uptake by CD8$^+$ T cells in $ADamts7^{-/-}$ mice. The use of endogenous influenza-specific CD8$^+$ T cells may be beneficial in assessing antigen uptake in $ADamts7^{-/-}$ mice, although this is technically difficult due to the very low numbers of influenza-specific CD8$^+$ T cells that can be isolated. Transgenic animals offer the ability to easily track pathogen-specific responses, although, transgenic animal models for influenza virus research are not available or are inappropriate. However, we have identified transgenic animals that could be used to test the migration of DCs and activation of T cells in the context of ADAMTS7/influenza virus infection. Transgenic
mice that express T cell receptors specific for glycoprotein B from HSV (369, 370) and modified to express allelic markers (e.g. CD45.1) or green fluorescent protein (to facilitate their detection in recipient mice) exist allowing efficient tracking of pathogen-specific responses. By crossing the Adams7⁻/⁻ mice with glycoprotein B HSV-specific transgenic mice we could generate transgenic T cells deficient in ADAMTS7 for use in transfer experiments with wild-type mice as recipients. The use of these mice in transfer experiments would also allow us to better understand the role of ADAMTS7 in memory CD8⁺ T cell responses, given the deficiency in memory CD8⁺ T cell numbers observed in Adams7⁻/⁻ mice (Figure 5.10).

Interestingly we also observed that older uninfected Adams7⁻/⁻ mice (+16 weeks of age) were heavier than their WT counterparts (Figure 5.11C). Although it has been reported that Adams7⁻/⁻ mice show no differences in weight when compared to WT littermates, the knockout mice used in these previous studies only reached 14 weeks of age (283, 284), whereas our studies assessed mice up to 20 weeks of ages. Additionally, ADAMTS7 expression has been detected in white fat tissue (283), suggesting a novel role for ADAMTS7 in fat storage or metabolism. It is important to note that obesity in humans has been associated with enhanced susceptibility to H1N1 infection (389-391). Similarly, obese mice show enhanced lung pathology, virus titres and mortality following infection (391, 392) that correlated with poor CD8⁺ T cell memory (391, 392). Future studies could investigate the effects obesity has on influenza immunity in Adams7⁻/⁻ mice through the analysis immune cell metabolism (metabolomics) during infection (393, 394).

Finally, this dissertation has provided a thorough analysis of influenza-specific CD8⁺ T cell immunity in both Adams5⁻/⁻ and Adams7⁻/⁻ mice. Influenza-specific CD4⁺ T cell immunity, B cell activation and antibody responses were not assessed. Given poor total B and CD4⁺ T cell numbers observed in influenza virus infected Adams5⁻/⁻ and Adams7⁻/⁻ mice, we speculate antigen-specific immune cell subsets would also be affected. Complicating things is
the availability and effectiveness of MHCII tetrameric complexes for enumerating influenza-specific CD4$^+$ T cells as they are difficult to make and hard to access (395). Future studies should endeavour to assess these responses in $Adams5^{-/-}$ and $Adams7^{-/-}$ mice.

**Concluding remarks**

The environment outside of the cell is an essential mediator of development and homeostasis for cell types and tissues. Studying members of the metalloproteinase family in the context of the ECM is an essential part of cell biology research. There are significant gaps in our knowledge describing the functional roles of ADAMTS5 and ADAMTS7 enzymes. This knowledge is essential to underpin and develop specific pharmacological targets. The interactions between ECM components and the impact on infectious disease is a new and emerging field of research that requires further study, with the potential for significant advances both clinically and therapeutically.
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