Studies on the Host Genetic Resistance and Susceptibility to Influenza A Virus

Thesis

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1. Abstract

Studies on Host Genetic Resistance and Susceptibilities to Influenza A virus

Dai-Lun Shin

Influenza infections represent a serious threat to human health and are causing respiratory disease in human and animals. Many of the biological mechanisms of the host-pathogen interactions after influenza A virus (IAV) infection are still unclear. Both intrinsic and extrinsic factors determine the severity of influenza disease.

To study the importance of host and virus virulence factors, I investigated in my thesis how different genetic backgrounds of the host influenced the function of orthomyxoviridae resistance gene 1 (Mx1). It was shown previously that congenic B6.A2G-Mx1<sup>+/+</sup> (B6-Mx1<sup>+/+</sup>) mice carrying a functional Mx1 allele are highly resistant against IAV. In particular, the type I interferon induced protein MX1 has been shown to represent a strong effector of innate immunity and to act by blocking nuclear import of viral RNA and inhibits viral replication. To assess the influence of genetic background on Mx1 function, I compared the host response to IAV infection in congenic mice B6-Mx1<sup>+/+</sup> to congenic D2(B6).A2G-Mx1<sup>+/+</sup> (D2-Mx1<sup>+/+</sup>) mice. Most surprisingly, congenic D2-Mx1<sup>+/+</sup> mice harboring a functional Mx1 wild type allele were highly susceptible to H1N1 virus infection. I observed that the survival proportion was related to both the genetic background and the copy numbers of functional Mx1 alleles. Furthermore, the genetic background influenced Mx1 antiviral activity by regulating virus titer in the lungs after infection. Infection of D2-Mx1<sup>+/+</sup> mice led to a 100-fold higher viral load lungs compared to infection of B6-Mx1<sup>+/+</sup>. In contrast, congenic B6-Mx1<sup>+/+</sup> mice started to reduce virus earlier and did not develop severe disease. Additionally, D2-Mx1<sup>+/+</sup> mice which were pretreated with interferon α were fully protected from lethal infections. These observations suggest that B6 mice carry genetic factors which initiate Mx1 function and which are absent in D2 mice. Furthermore, in a D2 background, IAV replicates very rapidly at early time points and Mx1 protective functions are activated too late to prevent the severe outcomes.

In the second part of my thesis I investigated in the importance of fibrinolysis and the influence of vascular permeability in the context of IAV infection. Recent studies have shown that IAV alters the plasminogen conversion pathway and hemostasis after infection. Here, I studied infections with two IAV strains, PR8 and WSN. The WSN
strain has a unique neuraminidase which can convert plasminogen into plasmin and allows cleavage of the hemagglutinin via activated plasmin in the absence of other host serine proteases. In mice, WSN can disseminate to extra-pulmonary organs while PR8 is restricted to the respiratory tract. In my studies, I investigated if dissemination of WSN was related to hyperfibrinolysis from plasminogen conversion. For this, I studied the host response and virus replication in a mouse knockout mutant of the Serpine1 gene. I showed that Serpine1 mutants were more susceptible to H1N1 infections compared to wild type mice. The increase in susceptibility was caused by an enhancement of fibrinolysis, resulting in leaking of red blood cells into the alveolar space and dissemination of virus into the kidneys. Also, WSN-infected wild type mice showed higher levels of protein leakage into the bronchoalveolar space fluid compared to PR8-infected mice. In conclusion, my results strongly suggest that dysregulation of the plasminogen activation pathway contributes to the enhanced severity in IAV-infected mouse lungs. Hyperfibrinolysis increases lung hemorrhage and enhances vascular permeability which may lead to virus dissemination.

Furthermore, I investigated the kinetics of cellular changes in the peripheral blood after IAV infection of B6 and D2 mice. We observed that the ratio of granulocytes to lymphocytes in the peripheral blood and lung correlated well with disease severity. By fluorometric analysis, a massive infiltration of Ly6G+CD11b+ cells in the lung was detected which was strongly associated with the lethal infection in B6 mice, whereas D2 mice showed an increase in proinflammatory cells in the lungs after IAV infections. These immune cell infiltrates of the lung reflected quantitative and qualitative differences in the periphery.

In addition, I investigated genetic variants of the NK cell gene Klrd1. It was reported that Klrd1 is deleted in DBA/2J mice. After performing fluorometric analyses of NK cell from D2 mice, I could further show that the sub-strain DBA/2Rj stock from the Janvier Breeding Centre in France expresses the CD94 protein. On the other hand, I identified a spontaneous deletion spanning the last coding exon of the Klrd1 gene in DBA/2J mice from the Jackson laboratory by high throughput sequencing. An additional deletion in the intronic region between exons 2 and 3 was also identified in both D2 sub-strains. These results revealed the presence of different Klrd1 alleles in these two D2 sub-strains.
2. Zusammenfassung

Untersuchungen zur genetischen Wirtsresistenz und der Empfänglichkeit für Influenza A Viren

Dai-Lun Shin


Um Virulenzfaktoren auf der Virus- und Wirtsseite zu untersuchen, analysierte ich in meiner These, wie unterschiedliche genetische Hintergründe des Wirtes die Funktion des Orthomyxoviridae-Resistenzgens 1 (Mx1) beeinflussen. Es ist früher gezeigt worden, dass kongene Mäuse, B6.A2G-Mx1<sup>fr</sup> (B6-Mx1<sup>fr</sup>), die ein funktionelles Mx1-Allel tragen, höchst resistent sind gegenüber IAV. Insbesondere für das vom Typ I Interferon induzierte Protein Mx1 ist gezeigt worden, dass es einen starken Effektor der angeborenen Immunantwort darstellt und dass es den Kerntransport der Virus-RNA blockiert und die virale Replikation hemmt. Um den Einfluss des genetischen Hintergrundes auf die Mx1-Funktion zu bestimmen, verglich ich die Wirtsantwort auf eine IAV-Infektion in kongenen B6-Mx1<sup>fr</sup>-Mäusen mit der in kongenen D2(B6).A2G-Mx1<sup>fr</sup> (D2-Mx1<sup>fr</sup>)-Mäusen. Äußerst überraschend waren kongene D2-Mx1<sup>fr</sup>-Mäuse, die ein funktionelles Mx1-Wildtyp-Allel exprimierten, äußerst empfänglich für eine H1N1-Virusinfektion. Ich beobachtete, dass die Zahl der überlebenden Tiere sowohl vom genetischen Hintergrund als auch von der Kopienzahl funktioneller Mx1-Allele abhing. Außerdem beeinflusste der genetische Hintergrund die antivirale Aktivität von Mx1, indem es nach einer Infektion den Virustiter in der Lunge regulierte. Die Infektion von D2-Mx1-Mäusen führte zu einer 100-fach höherer Viruslast in den Lungen im Vergleich zur Infektion von B6-Mx1<sup>fr</sup>. Im Gegensatz dazu reduzierten die kongenen B6-Mx1<sup>fr</sup>-Mäuse das Virus früher und entwickelten keine schwere Krankheit. Weiterhin waren D2-Mx1<sup>fr</sup>-Mäuse, die mit Interferon α vorbehandelt wurden, vollkommen geschützt vor tödlichen Infektionen. Diese Beobachtungen legen nahe, dass B6-Mäuse genetische Faktoren tragen, die die Mx1-Funktion initiieren und die bei D2-Mäusen fehlen. Weiterhin repliziert das Virus bei einem D2-Hintergrund sehr schnell zu frühen Zeitpunkten und protektive Mx1-Funktionen werden zu spät aktiviert, um einen schweren Infektionsverlauf zu verhindern.

Im zweiten Teil meiner Dissertation habe ich die Bedeutung der Fibrinolyse und der vaskulären Permeabilität im Kontext einer IAV Infektion untersucht. Aktuelle Studien haben gezeigt dass eine IAV Infektion zu einer Veränderung des


3. Introduction

3.1. Biological characteristic of influenza A virus

500 million people are infected by the influenza A virus (IAV) worldwide each year which represents a serious health threat to humans (Fauci, 2006). Severe pandemics are caused by the emergence of new influenza subtypes (Fauci, 2006; Kilbourne, 2006; Klenk et al., 2011). In 2009, new variant of the H1N1 virus from a swine origin cause a world-wide pandemic (Fraser et al., 2009; Garten et al., 2009; Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009; Neumann et al., 2009; Wang and Palese, 2009). Moreover, seasonal influenza A viruses transmit from human-to-human naturally, while bird influenza virus may infect humans via direct contact. There are different variants circulating in birds which have the potential to infect humans and may cause severe disease (Gambotto et al., 2008; Gao et al., 2013; Krug, 2006; Morens et al., 2013; Watanabe et al., 2013).

Taxonomy

Influenza A, influenza B, influenza C viruses, thogotovirus and isavirus belong to the family of orthomyxoviridae (Horimoto and Kawaoka, 2005). The classification of influenza viruses in type A, B or C are based on antigenic differences in the nucleoprotein (NP) and matrix protein (M1) (Horimoto and Kawaoka, 2005). Influenza A viruses are further divided into different subtypes based on the antigenicity of their surface proteins hemagglutinin (HA) and neuraminidase (NA). Currently, 18 HA subtypes and 11 NA subtypes are known (Neumann et al., 2009; Tong et al., 2013). The official designation of an influenza A viral strain (e.g. A/California/7/2009 (H1N1)) represents viral isolates in the form of "A (virus type) / host / origin of geographic / number of the isolates / year of isolated (H- and N-subtype) " (CDC, 2010).
Influenza A consists of the following proteins: hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), non-structural proteins 1 and 2 (NS1 / 2), nucleoprotein (NP) and the polymerase complex consisting of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). (Figure adapted from Horimoto and Kawaoka, 2005)

**Virus structure**

Like all members of the family orthomyxoviridae, influenza A virus is an enveloped virus with a segmented, single-stranded ribonucleic acid (RNA) genome of negative polarity (Knipe et al., 2007; McCauley and Mahy, 1983). The spherical viral particles have a diameter of about 80 to 130 nm (Elton et al., 2006). The genome consists of eight RNA segments that contain approximately 15,000 nucleotides in total (Chen et al., 2001). Each gene segment carries highly conserved, non-coding regions at the 3' and 5' ends. The RNA segments form a panhandle-like shape by intramolecular base-pairing (Klumpp et al., 1997). These eight genomic segments encode for nine structural and two non-structural proteins.

As shown schematically in Figure 3.1, the viral particles carry a lipid envelope derived from the host cellular membrane as an outer shell. Two viral glycoproteins, the trimetric hemagglutinin (HA) and the tetrameric neuraminidase (NA), and matrix protein 2 (M2), which acts as a proton channel, can be found on the virus surface. The
HA is responsible for the binding of the virus to sialic acid residues on the host cell surface. After endocytosis of the virus particle, HA mediates membrane fusion with the infected cell. NA is important in releasing the newly synthesized virus particles. It cleaves sialic acid residues of glycoproteins of the host cell and prevents adhesion of the newly formed viral particles to the host cell surface. The matrix protein 1 (M1) is directly located below the lipid envelope. The viral RNA segments are surrounded by nucleoprotein (NP) and bound to viral polymerase complex (Horimoto and Kawaoka, 2005).

The polymerase complex contains three viral proteins: polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2). These proteins are positioned at the ends of the RNA segments. The whole complex is referred to as viral ribonucleoprotein complex (vRNP) (Brown, 2000; Webster et al., 1992).

The shortest segment encodes two proteins which are called non-structural protein 1 and 2 (NS1 and NS2). NS1 plays many roles in the viral replication cycle regulating vRNA synthesis and antagonizing host innate immune responses. NS1 represents an important virulence factor of influenza A virus influencing interferon production and interfering with other antiviral mechanism of the host (Hale et al., 2008). NS2, which is also called nuclear export protein (NEP), is necessary for exporting of newly synthesized vRNPs from the nucleus (O'Neill et al., 1998).

Additionally, the PB1-F2 has been found as the eleventh influenza A virus protein. PB1-F2 is encoded by an alternative open reading frame of the PB1 segment and contributes to viral pathogenicity by exerting a pro-apoptotic activity in the infected cell (Chen et al., 2001; Conenello and Palese, 2007; Zamarin et al., 2005).
Figure 3.2. Virus infection is initiated by binding of the virus to sialylated host cell-surface receptors, and entry is mediated by endocytosis. In the host cell, fusion of viral and endosomal membranes occurs at low pH which enables the release of the segmented viral genome into the cytoplasm. The viral genome is subsequently translocated to the nucleus where it is transcribed and replicated. Following synthesis in the cytoplasm, viral proteins are assembled into viral ribonucleoproteins (vRNPs) in the nucleus. Export of vRNPs to the cytoplasm is mediated by M1 and NS2. Virus particles are assembled at the cell membrane, and the newly generated progeny virus buds into extracellular fluid. (Figure adapted from Shi et al., 2014)

Virus replication cycle

Figure 2.2 illustrates the infection cycle of influenza A viruses. It begins with the binding of HA to sialic acid-bearing glycoproteins on the host cell surface (Weis et al., 1988). The virus particles are taken up by endocytosis vesicles which are mediated by clathrin-dependent or independent receptors (Sieczkarski and Whittaker, 2002). After transport of the viral particles into the late endosomes, the conformation of the HA will change because of low pH. The fusogenic peptide of the HA is exposed and the viral
lipid envelopes then fuse with the endosomal membrane of the host cell (Harrison, 2008).

The viral core becomes then acidified by the activation of the M2 ion channel due to lower pH in late endosomes. The vRNPs are released into the cytoplasm (Bui et al., 1996; Nayak et al., 2004). Followed by vRNP translocation into the cell nucleus, the viral RNA-dependent RNA polymerase transcription and replication begins. The viral RNA is recognized and transcribed by cellular polymerase II with the help of PB2 cap-snatching function (Shapiro and Krug, 1988). Viral glycoproteins (HA and NA) are glycosylated, oligomerized and transported via Golgi apparatus to the plasma membrane together with M2 proteins. Translated PA, PB1, PB2, and NP are transported back to the nucleus to participate in the synthesis of new vRNA. NS1 is synthesized in large quantities compared to other viral proteins, and secreted out of the cell to suppress interferon responses of the host (Garcia-Sastre et al., 1998b; Kochs et al., 2009).

The newly synthesized vRNPs interacts with the M1 protein and binds to NS2. The vRNP-M1-NEP complex is exported into the cytoplasm where it attaches to the cytoplasmic membrane (Resa-Infante et al., 2011). Here the budding process occurs and newly synthesized virus particles are released (Brown, 2000; Nayak et al., 2004).

**Virus strains**

The pathogenicity of influenza A viruses is related to HA cleavage. The cleavage potential of the hemagglutinin relates to differences of host proteases in different organs (Horimoto and Kawaoka, 1994; Kawaoka et al., 1984). Low pathogenic IAVs carry a single arginine in their HA cleavage site and therefore need specific trypsin-like enzymes that can activate HA (Kido et al., 1992; Sugiyama et al., 2001). In contrast, highly pathogenic IAVs possess a series of basic amino acids in their
cleavage site and can therefore be cleaved by ubiquitous proteases such as furin (Horimoto et al., 1994; Stieneke-Grober et al., 1992).

For my thesis work, three mouse-adapted influenza A viruses were used: A/Puerto Rico/8/34 H1N1 viruses, A/WSN/33 H1N1 virus, and A/Hong Kong/1/68 H3N2 virus. The PR8 Freiburg variant (PR8F) from Prof. Dr. Otto Haller in University Freiburg was adapted by several passages in a mouse strain without functional \( Mx1 \) gene. High virulent PR8 (hvPR8) was adapted to a congenic B6.A2G-\( Mx1^{r/r} \) mice with a functional \( Mx1 \) allele. The hvPR8 shows an unusually strong virulence with lethal dose of 100 ffu in B6.A2G-\( Mx1^{r/r} \) mice. The pathogenicity of PR8F virus corresponds approximately to that of other A/Puerto Rico/8/34 virus isolates (Haller, 1981a). In contrast, the PR8 Muenster variant (PR8M) which we received from Prof. Dr. Stefen Ludwig in University Muenster shows a relatively low virulence compared to PR8F or hvPR8. PR8M has a higher type I IFN inducing capacity and exhibits reduce pathogenicity compared to other PR8 variants (Liedmann et al., 2014). WSN virus exhibits a unique neuraminidase function which allows cleavage of the viral hemagglutinin with the help of plasminogen. The carboxy terminus of the WSN NA can bind to plasminogen and convert it into activated plasmin. The enzymatically active plasmin cleaves viral HA and initiates viral infection (Goto and Kawaoka, 1998, 2000; Zhirnov et al., 1982). This ability allows WSN virus to replicate more efficiently and thus increase pathogenicity in mice (Garcia-Sastre et al., 1998a; Goto et al., 2001). In addition, WSN can also replicate in the mouse brain because of the recruitment of host plasminogen (Goto and Kawaoka, 1998; Lazarowitz et al., 1973; Takahashi et al., 1995; Wolf et al., 1974). Additionally, the human influenza strain H3N2 (A/Hong-Kong/1/68; H3N2) was adapted to mice by passages in mouse lungs. This H3 subtype virus carries a mono-basic cleavage site in the HA (Haller and Lindenmann, 1975).
3.2. Host genetic influences the infection phenotype in the mouse model

Introduction to animal models

Animal models are wildly used nowadays to investigate host-pathogen interactions after IAV infection. The crucial virulence factors of IAV and the influence of host immunity need to be investigated to understand severe disease outcomes in humans. However, experiments under controlled conditions are very difficult to perform in humans because of obvious ethical restrictions and they are thus extremely rare. Additionally, the highly diversity of the genetic variantion in humans brings the difficulty to validate important factors. Therefore, various animals have been used as a model system to investigate disease progression and to evaluate genetic factors influencing pathogenicity and outcomes of severe influenza disease. To date, several animal models have been established for IAV research. In general, mice and ferrets are the most preferred ones. Others like cotton rats, pigs, and nonhuman primates are also used in some studies.

Ferrets model are valuable because they are susceptible to human influenza viruses and can develop similar disease symptoms as humans, including fever, nasal discharge, coughing, anorexia. (Belser et al., 2011). Also, most airborne transmission studies are done in the ferret model because they can be infected by aerosols and contact (Herlocher et al., 2001). However, the absence of immunological analysis reagents and diverse genetic backgrounds limit the possibility to study the host genetic factors in ferrets (van der Laan et al., 2008). Moreover, the main disadvantage of the ferret model is the cost for maintenance (Hers and Mulder, 1961).

One of the advantages to use mouse model is low costs, short reproduction times and easy handling. The biggest benefit for using mouse models is that researchers can rely on the fast knowledge of the biology and genetics as well as many phenotype studies that were performed on mouse mutants and inbred strains (Schughart et al., 2013; van der Laan et al., 2008). Inbred mouse strains are well genotyped and therefore represent an ideal condition to study on the influence of host factors after influenza A viruses infection (Wilk and Schughart, 2012a).
Many IAV strains can infect and replicate in mice without species adaptation. However, some viral strains still need prior adaptation with several passages in mouse lungs before one can study them. Unlike ferret model, mice do not show similar symptoms as humans. Body weight loss, stop of grooming, and restricted movement are the major clinical signs for IAV infection in mice (Belser et al., 2011).

Other animals like cotton rats exhibit similar innate and adaptive immune responses as humans. Viruses isolated from human do not require prior adaptation (Boukhvalova et al., 2009). However, cotton rats are more aggressive and no transgenic or gene-modified strains have been constructed. Recently, pigs have also been used as animal model to study reassortment and vaccines after the emergence of swine-origin H1N1 viruses (van der Laan et al., 2008). Non-human primates are also used as human disease models. Several therapeutic and prophylactic strategies are studied in rhesus macaques (Baas et al., 2006; Ibricevic et al., 2006). In all animal models, ethical issues have to be respected.

**Orthomyxovirus resistance gene (Mx) mediated resistance to IAV infection**

The most intensively studied interferon stimulated gene is *Mx1*. The orthomyxovirus resistance (MX) proteins have been discovered accidentally in 1962. Lindemann and coworkers infected inbred A2G mice with a mouse-adapted neurotrophic influenza A virus. A2G mice were resistant whereas other mouse strains were highly susceptible (Lindenmann, 1962), even at high infection doses (Lindenmann et al., 1963). The orthomyxovirus resistance gene (*Mx*) was further described as an autosomal dominant inherited gene (Lindenmann, 1964). Genetic analysis showed also the presence of the *Mx* wild type gene in wild mice. In contrast, most laboratory mouse strains have mutations or deletions in the *Mx* gene and lost their resistance to orthomyxoviruses infection (Staeheli et al., 1988). *Mx* does not only confer resistance to IAV but also to other viruses especially single strand RNA viruses, for example measles virus, thogoto viruses, vesicular stomatitis virus and bunyaviruses. Furthermore, other RNA viruses are also restricted by *Mx* genes, such as semliki
forest virus and coxsackie B viruses (Chieux et al., 2001; Frese et al., 1996; Frese et al., 1995; Landis et al., 1998; Pavlovic et al., 1995; Pavlovic et al., 1990; Schwemmle et al., 1995a; Schwemmle et al., 1995b).

Further studies showed that the $Mx$ gene encodes for a 72 kDa large protein localized in the nucleus of murine cells (Dreiding et al., 1985). The antiviral activity of $Mx$ in primary mouse embryo fibroblast cultures from A2G mice can be triggered by type I IFN (Staeheli et al., 1986). MX proteins belong to the family of GTPases. Other proteins such as Dynamine and the IFN-regulated guanylate binding proteins also belong to this family. The mouse has two $Mx$ genes: $Mx1$ and $Mx2$, both genes exhibit antiviral activity (Figure 3.4). During influenza A virus infection, MX1 inhibits the
interaction between PB2 and NP, blocks the vRNP formation and decreases the viral polymerase activity (Figure 3.3) (Verhelst et al., 2012).

*Mx* genes have been found in various vertebrate species including fish and birds. Humans also have two *Mx* genes (Aebi et al., 1989), called *MxA* and *MxB* which are located on chromosome 21. Unlike mouse *Mx1* which is located in the nucleus, human MXA proteins are found in the cytoplasm and have been shown to exhibit antiviral activity against influenza A virus, influenza B virus, bunyaviruses, measles virus and hepatitis B virus (Haller and Kochs, 2002).

![Figure 3.4. Mx-mediated inhibition steps of the life cycles of influenza A virus.](image)

*Figure 3.4. Mx-mediated inhibition steps of the life cycles of influenza A virus.*

The nucleocapsids (vRNPs, for viral ribonucleoprotein complexes) of FLUAV and THOV consist of genomic RNA segments associated with viral nucleoprotein and RNA polymerase. Human MxA blocks nuclear translocation of incoming vRNPs and inhibits secondary transcription and replication of FLUAV genomes by interfering with synthesis and/or nuclear import of newly synthesized viral components. Mouse Mx1 acts in the nucleus and inhibits primary transcription by viral RNA polymerases. (Figure modified from Haller et al., 2015)
**Host genetic background differences**

Although $Mx$ genes are the most dominant factors for resistance to IAV infection, there are also other host genes that influence pathogenesis of influenza virus. Studies have shown that host genetic background plays an important role in susceptibility to influenza infection. Experiments performed on inbred mouse strains that carried a $Mx1$ mutant allele have confirmed that highly susceptible mouse strains developed an elevated inflammatory response, severe pathological changes in the lung, and high virus titers after infection with various IAV subtypes and variants (Blazejewska et al., 2011; Trammell et al., 2012a). The susceptibility of seven inbred mouse strains to PR8 virus were examined in our group. We showed that C57BL/6J (B6) represents a more resistant inbred strain while DBA/2J (D2) exhibited highly susceptibility (Srivastava et al., 2009). F1 offspring from these two inbred strains showed the resistant phenotype, although more weight loss than the parental B6 strain was observed. Mapping studies in recombinant inbred strains suggest that the susceptibility is a polygenic trait (Alberts et al., 2010; Nedelko et al., 2012). Other studies demonstrated that pathogenicity is co-determined by host and pathogen factors.

**Fibrinolysis causes lung injury after IAV infection**

Plasmin is a serine protease involved in fibrinolysis, a process that dissolves fibrin polymers into soluble fragments (Figure 3.5A). Plasmin was activated through cleavage of plasminogen mainly through the urokinase or tissue plasminogen activator pathway. Both pathways can be inhibited by $Serpine1$ (serpin peptidase inhibitor, clade E, member 1). Plasminogen/ plasmin plays an important role in fibrinolysis-mediated inflammation (van Hinsbergh, 2012) and downstream fibrinolysis activation during IAV infections (Keller et al., 2006). Lacking the plasminogen protein results in weakening of the inflammatory response and impairs recovery (Moons et al., 1998; O'Connell et al., 2010; Ploplis et al., 1998). On one hand, extensive
inflammation after IAV infection might contribute to severe pathogenicity of IAV infections in humans. On the other hand, absence of plasminogen may reduce a hyper-inflammatory response in the lung (Figure 3.5B) (Berri et al., 2013; de Jong et al., 2006). Additionally, other studies showed that fibrin deposition balances hemostasis after IAV infection (Gralinski et al., 2013; Keller et al., 2006). Also, studies with severe acute respiratory syndrome (SARS) virus infections have shown that dysregulation of plasminogen activation pathway results in diffused hemorrhages in the lung of infected mice (Gralinski et al., 2013). Although it is clear that plasminogen-driven lung inflammation is happening through the fibrinolysis reaction, the interactions between fibrinolysis and IAV infection are still unclear.

**Figure 3.5.** Schematic overview of the proposed model for plasminogen-mediated lung injury. (A) Representation of the unperturbed plasminogen activation signaling pathway. Red T shaped lines indicate inhibition and blue arrows indicate activation. (B) During IAV infection, plasminogen is converted into plasmin and promotes IAV replication of some influenza strains. On the other hand, plasmin promotes inflammation via fibrinolysis and increases permeability. (Figure adapted from (A) Gralinski et al., 2013 and (B) Berri et al., 2013)
4. Objectives

Influenza A viruses (IAV) is the contagious agent of respiratory disease in human and animals. Many of the biological mechanisms of the host-pathogen interactions are not fully understood. The mouse infection model is one suitable tool to investigate the importance of viral and host factors that determine disease severity and outcome. In humans, there is circumstantial evidence that genetic factors may play an important role for susceptibility or resistance to IAV. In mice, it has been clearly shown that both the genetic background and the mutations in specific genes greatly influence the severity of disease after IAV infections.

The overall objective of my thesis was to investigate host-pathogen interactions in mouse models to determine the influence of host genetic background, the function of specific genes and IAV virulence factors.

In the main part of my thesis work, I wanted to investigate if the wild type orthomyxovirus resistance gene 1 (\(Mx1\)) can provide antiviral activity against IAV in different genetic backgrounds. Most inbred laboratory mice carry a non-functional allele of \(Mx1\). Our previous studies showed that in the absence of \(Mx1\), DBA/2J (D2) mice were highly susceptible to IAV infection whereas C57BL/6J (B6) mice were more resistant. To further elucidate how these two genetic backgrounds influence \(Mx1\) antiviral function, two congeneric mouse strains should be compared: B6.A2G-\(Mx1^{+/+}\) (B6-\(Mx1^{+/+}\)) and D2(B6).A2G-\(Mx1^{+/+}\) (D2-\(Mx1^{+/+}\)). A/Puerto Rico/8/34 (PR8) H1N1 should be used to challenge the mice, and phenotypic comparison between B6 or D2 genetic backgrounds in the presence of \(Mx1\) mutant or wild type allele should be investigated.

In the second major part of my thesis, I wanted to investigate both viral and host factors involved in the plasminogen activation pathway. WSN expresses a unique neuraminidase protein which is able to convert plasminogen into plasmin. It thus allows plasmin to cleave viral HA in the absence of other host serine proteases. Also, recent studies showed that IAV may trigger hyperfibrinolysis after infection. Moreover, WSN can disseminate from the lung to other organs. The link between WSN virulence factors, host genes and systemic dissemination are not well understood. Therefore I investigated the possible involvement of the plasminogen conversion pathway for the
susceptibility of the host to IAV infections, by comparing pathogenesis in mice after infection with PR8 or WSN H1N1 virus. In addition, I wanted to define the involvement of the host protease inhibitor Serpine1 using Serpine1 knock-out mice which have a general of coagulation defect and reduce fibrosis. Furthermore, I wanted to investigate the pathogenicity of PR8 virus using histopathological analyses to identify the parameters that correlate lung damage with the cellular composition of the peripheral blood. Also, we showed in previous studies that natural killer cell (NK) were one of the important innate immune cells to prevent IAV infection. NK cell function is regulated by CD94 (Klr d1) gene. Therefore, I wanted to study genomic differences of Klr d1 alleles in different mouse sub-strains.
5. Results

5.1 Manuscript I: The protective function of the *Mx1* influenza resistance gene strongly depends on genetic background

5.2 Manuscript II: Mutation of *Serpine1* in mice results in increased susceptibility to influenza A virus infection due to increased vascular leakage and virus dissemination

5.3 Manuscript III: Cellular changes in blood indicate severe respiratory disease during influenza infections in mice

5.4 Manuscript IV: Segregation of a spontaneous *Klrd1* (CD94) mutation in DBA/2 mouse substrains
5.1 Manuscript I: The protective function of the \textit{Mx1} influenza resistance gene strongly depends on genetic background

\textbf{Shin DL, Hatesuer B, Bergmann S, Nedelko T, and Schughart K.}

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\textbf{Title}

The protective function of the \textit{Mx1} influenza resistance gene strongly depends on genetic background

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Abstract

Influenza infections represent a serious threat to human health. Both extrinsic and intrinsic factors determine the severity of influenza disease. The MX dynamin-like GTPase 1 (Mx1) gene has been shown to confer strong resistance to influenza A virus infections in mice. Most laboratory mouse strains, including C57BL/6J, carry nonsense or deletion mutations in Mx1 and thus a non-functional allele whereas wild-derived mouse strains carry a wild type Mx1 allele. Congenic C57BL/6J (B6-Mx1<sup>r/r</sup>) mice expressing a wild type allele from the A2G mouse strain are highly resistant to influenza A infections, to both mono- and poly-basic subtypes. Furthermore, in genetic mapping studies, Mx1 was identified as the major resistance locus to influenza infections. Here, we investigated if Mx1 protective function may be influenced by genetic background. We generated a congenic mouse strain carrying the A2G wild type Mx1 resistance allele on the DBA/2J background (D2-Mx1<sup>r/r</sup>). Most remarkably, congenic D2-Mx1<sup>r/r</sup> mice expressing a functional Mx1 wild type allele are still highly susceptible to H1N1 virus. Pre-treatment of D2-Mx1<sup>r/r</sup> mice with interferon α protected them from lethal infections. Our results showed, for the first time, that the presence of a Mx1 wild type allele from A2G as such does not protect mice from lethal influenza A virus infections. These observations are also highly relevant for susceptibility to influenza infections in humans.
**Introduction**

Influenza A virus represents a major health threat to humans. Seasonal influenza epidemics cause high economic loss, morbidity and deaths every year (Fauci, 2006). Each year, about 500 million people are infected by the influenza A virus worldwide, of which about 500,000 die (Fauci, 2006). In recent history, the emergence of new influenza subtypes has caused severe pandemics (Kilbourne, 2006; Klenk et al., 2011; Russell and Webster, 2005), the most severe Spanish Flu pandemics in 1918 resulted in 30–50 million deaths worldwide (Johnson and Mueller, 2002). And a new variant of the H1N1 virus, pH1N1, caused a world-wide pandemic in 2009 (Fraser et al., 2009; Garten et al., 2009; Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009; Neumann et al., 2009; Wang and Palese, 2009). Seasonal influenza A viruses are transmitted from human-to-human, but bird influenza A viruses may also directly infect humans who have been in close contact with infected birds. There are presently three virus subtypes, H5N1, H9N2 and H7N9 that are circulating in birds and which have the potential to infect humans. Infection with these subtypes may cause severe disease with lethal outcome (Gambotto et al., 2008; Gao et al., 2013; Krug, 2006; Morens et al., 2013; Watanabe et al., 2013). There is some evidence from animal models that H7N9 virus may be able to spread by contact and air transmission (Zhang et al., 2013; Zhu et al., 2013) making it a likely candidate for future pandemics in humans. Therefore, it is important to better understand the biological mechanisms that result in severe outcomes after influenza A infection.

The course and outcome of an influenza A virus infection is influenced by viral and host factors. Host risk factors, like obesity or pregnancy, became evident during the 2009 swine flu pandemics (Scriven et al., 2009; Yates et al., 2010). Furthermore, genetic factors in humans associated with a higher susceptibility to influenza infections and severe disease outcome have been suspected for the 1918 pandemics, as well as H5N1 infections in patients (Albright et al., 2008; Gottfredsson et al., 2008; Horby et al., 2010). However, evidence for genetic predisposition in humans is circumstantial (Albright et al., 2008; Gottfredsson et al., 2008; Horby et al., 2010) and the details of the biological mechanisms for health and genetic factors predisposing to severe influenza in humans remain largely unknown (Albright et al., 2008;
Gottfredsson et al., 2008; Horby et al., 2010; Karlsson et al., 2012; Mancuso, 2012; O'Brien et al., 2011). Recently, the importance of \textit{IFITM3} as a crucial factor for host susceptibility has been demonstrated in mice and humans (Everitt et al., 2012).

The mouse is one of the most important mammalian model systems for studying host responses to influenza A virus and for assessing, for example, virus virulence, disease severity, genetic predisposition, immune responses, and vaccine efficacy [(Wilk and Schughart, 2012b) and references therein]. The importance of host factors to host susceptibility and resistance has been demonstrated clearly in animal models. We and others have shown in mouse models that susceptibility of the host to influenza A infection strongly depends on the genetic background (Boon et al., 2009; Boon et al., 2010; Boon et al., 2011; Ding et al., 2008; Otte et al., 2011; Pica et al., 2011; Srivastava et al., 2009; Trammell et al., 2012b; Trammell and Toth, 2008).

Also in mice, the MX dynamin-like GTPase 1 (\textit{Mx1}) gene has been identified as one of the most important influenza resistance gene (reviewed in (Haller, 1981b; Haller et al., 2007; Haller et al., 2009]). \textit{Mx1} acts as a cell-autonomous restriction factor against many viral pathogens. Expression of \textit{Mx1} is induced by type I or type III interferons (Holzinger et al., 2007). Structure analysis of \textit{Mx1} proteins revealed globular G domain connected to a stalk region (Gao et al., 2011). The stalk is able to mediate self-assembly into a ring-like oligomer that is thought to interact directly with viral RNP particles and thereby block replication (Gao et al., 2011). The amino acid sequence in the L4 loop of the stalk determines specificity against different virus pathogens (Patzina et al., 2014). It has been further suggested that additional cellular host factors may be involved in the anti-viral activity of \textit{Mx1} (Wisskirchen et al., 2011).

The protective activity of \textit{Mx1} against myxoviruses has been originally discovered in A2G mice that carry a wild type allele (Lindenmann et al., 1963). However, most laboratory mice are deficient for \textit{Mx1} because of deletions or nonsense mutation (Ferris et al., 2013; Staeheli et al., 1988) whereas many wild-derived strains carry a functional \textit{Mx1} allele (Ferris et al., 2013; Jin et al., 1998). The A2G allele of \textit{Mx1} has subsequently been demonstrated to be highly protective from lethal influenza infections in various mouse models (Cilloniz et al., 2012; Grimm et al., 2007; Hodgson
et al., 2011; Moritoh et al., 2009; Tumpey et al., 2007). Congenic C57BL/6J.A2G-\textit{Mx}^{1/r} (B6-\textit{Mx}^{1/r}) survive infections with mouse-adapted H1N1 and are also resistant to lethal infections with highly virulent poly-basic H5N1 virus (Tumpey et al., 2007). Furthermore, SPRET/Ei mice which carry another \textit{Mx}1 wild type allele are strongly protected against influenza infections (Vanlaere et al., 2008). A genetic mapping study in a backcross of (C57BL/6 x SPRET/Ei)F1 x C57BL/6 identified \textit{Mx}1 as the major resistance locus (Vanlaere et al., 2008). Furthermore, the founder strains of the Collaborative Cross recombinant inbred population (Collaborative Cross Consortium, 2012) carry five different haplotypes in the \textit{Mx}1 genomic region, two of which (PWK/PhJ, NZO/HILtJ) were highly protective against influenza infections (Ferris et al., 2013). A/J, C57BL/6J, 129S1/SvImJ and NOD/ShiLtJ carry a deletion or stop codon in the \textit{Mx}1 gene and were highly susceptible (Ferris et al., 2013). A third wild-derived allele was found in CAST/EiJ mice exhibiting one amino acid difference to the presumed ancestral PWK/PhJ allele. It was expressed after influenza A virus infection but did not protect CAST/EiJ mice from a lethal infection (Ferris et al., 2013). It is yet unclear whether genetic background or the specific \textit{Mx}1 allele in CAST/EiJ mice is responsible for the susceptible phenotype. In a mapping study using pre-Collaborative Cross mice, \textit{Mx}1 was found as the strongest resistance Quantitative Trait Locus (QTL) explaining 42% of the variation in body weight loss in this population (Ferris et al., 2013).

We showed previously that in the absence of \textit{Mx}1, C57BL/6J (B6-\textit{Mx}^{1/-}) mice survive infections with a less virulent strain of a mouse-adapted H1N1 (PR8M) virus whereas DBA/2J (D2-\textit{Mx}^{1/-}) mice were highly susceptible (Srivastava et al., 2009). On the other hand, \textit{Mx}1-deficient (B6-\textit{Mx}^{1/-}) mice were highly susceptible to the more virulent mouse-adapted H1N1 (PR8F) virus (Blazejewska et al., 2011; Grimm et al., 2007). However, in the presence of the \textit{Mx}1 allele from A2G mice, congenic B6-\textit{Mx}^{1/r} mice were strongly protected against infections with this virus (Grimm et al., 2007). To further investigate the role of \textit{Mx}1 in different genetic backgrounds, we generated a congenic D2(B6).A2G-\textit{Mx}^{1/r} (D2-\textit{Mx}^{1/r}) mouse line carrying the wild type \textit{Mx}1 allele from A2G and challenged these mice with PR8F virus. Most surprisingly, we found that D2-\textit{Mx}^{1/r} mice were highly susceptible to PR8F infections even in the presence of the wild type A2G \textit{Mx}1 allele.
Methods

Ethics statement
All experiments in mice were approved by an external committee according to the national guidelines of the animal welfare law in Germany (BGBl. I S. 1206, 1313 and BGBl. I S. 1934). The protocol used in these experiments has been reviewed by an ethics committee and approved by the ‘Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany’ (Permit Number: 3392 42502-04-13/1234).

Virus
Original stocks of viruses were obtained from Prof. Dr. Peter Stäheli, University of Freiburg (PR8F, A/PuertoRico/8/34 H1N1, Freiburg variant), from Prof. Dr. Stefan Ludwig, University of Münster (PR8M, A/PuertoRico/8/34 H1N1, Münster variant). Both viruses and their pathogenicity in C57BL/6J and DBA/2J mice were described previously (Blazejewska et al., 2011; Liedmann et al., 2014). Mouse-adapted H3N2 virus (A/Hong Kong/1/68 H3N2) was obtained from Prof. Dr. med. Otto Haller, University of Freiburg. All viruses were propagated in the chorio-allantoic cavity of 10-day-old pathogen-free embryonated chicken eggs, aliquoted and stored at -80°C.

Mice
Laboratory C57BL/6J (B6-\textit{Mx1}\textsuperscript{r/r}) and DBA/2J (D2-\textit{Mx1}\textsuperscript{r/r}) mice carrying mutant \textit{Mx1} alleles were purchased from Janvier, France. Congenic B6.A2G-\textit{Mx1}\textsuperscript{r/r} (B6-\textit{Mx1}\textsuperscript{r/r}) mice carrying a functional A2G \textit{Mx1} allele were provided by Prof. Dr. Peter Staeheli, University of Freiburg, Germany. Congenic D2(B6).A2G-\textit{Mx1}\textsuperscript{r/r} (D2-\textit{Mx1}\textsuperscript{r/r} mice) carrying a wild type \textit{Mx1} allele were generated in our laboratory by backcrossing D2-\textit{Mx1}\textsuperscript{r/r} mice for ten generation onto B6-\textit{Mx1}\textsuperscript{r/r}. In each generation, the presence of the \textit{Mx1} wild type containing region on chromosome 16 was confirmed by PCR genotyping.
Genotyping of mice
For genotyping, genomic DNA was extracted from mouse tails with DNeasy Blood & Tissue Kit according to the manufacturer instructions (Qiagen). DNA concentration was quantified with spectrophotometer (NanoDrop 1000, Thermo Scientific). A total of 100 ng DNA and 10 pmol primer oligonucleotides were used for PCR with LightCycler ® 480 Probes Master (Roche) according to the manufacturer instructions. For the PCR genotyping, polymerase was activated at 95 °C for 10 minutes, followed by 40 cycles of denature step at 94 °C for 1 minute, primer annealing at 61°C for 1 minute, and elongation reaction at 72 °C for 1 minute. A three-primer PCR strategy was used for Mx1 allele genotyping (Peter Stäheli, personal communication). Primers were designed for sequences flanking the Mx1 locus (exon8 forward, e8fn 5'-GGA GCT CAC CTC CCA CAT CT-3'; exon8 reverse, e8r: 5'- AGC ATG GCT GTG TCA CAA GCA-3'; exon12 reverse, e12r: 5'-CGA AGG CAG TTT GGA CCA TCT-3'). Mice carrying a wild type Mx1 gene yielded a 950bp product whereas mutant Mx1 alleles were detected by the presence of a 1255 bp product (Supplementary Fig. 1B).

Infection of mice
Female mice at the age of 8-12 weeks were anesthetized by intra-peritoneal injection of Ketamin-Xylazine solution in sterile NaCl (100 mg/ml Ketamine, WDT, Garbsen, Germany; 20 mg/ml Xylavet®, CP-Pharma, Burgdorf, Germany) with a dose adjusted to the individual body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS). Subsequently survival and body weight loss were monitored until day 14 p.i. In addition to mice that were found dead, mice with a weight loss of more than 30% of the starting body weight were euthanized and recorded as dead.

RT-PCR for Mx1 transcript analysis
RT-PCR was performed to confirm wild type Mx1 expression in D2.A2G-Mx1fr- mice. Mice were anesthetized and infected intranasal with 2x10^3 FFU PR8F in 20 µl PBS. Lungs were prepared, washed in PBS and stored in 2 ml RNA Later (Qiagen). Subsequently, lungs were homogenized using PolyTron 2100 homogenizer. Total RNA was prepared using Trizol® chloroform according to the manufacturer
instructions (Invitrogen). 1 µg of total RNA was reverse transcribed into cDNA using the SuperScript® III reverse transcriptase (Invitrogen TM, USA) according to the manufacturer instructions. 5 µl of cDNA product were amplified with specific primers (e8fn and e12r) to determine expression of the Mx1 wild type allele. Only D2-Mx1+/r but not B6-Mx1−/− mice yielded a product of 467bp that is generated from expression of the Mx1 wild type allele (Supplementary Fig. 1).

Determining of infectious viral particles
For determining viral load in lungs, lungs were prepared and put into 2 ml PBS containing 0.1 % BSA. Lung tissue was subsequently homogenized using the Poly Tron 2100 homogenizer. Debris was removed by centrifugation, and aliquots stored at -70 °C. Virus titers were determined on MDCK II (Madin–Darby Canine Kidney II) cells as focus forming units (FFU) as described previously (Blazejewska et al., 2011). Briefly, MDCK II cells were seeded in 96-well plates and serial 10-fold dilutions of homogenized lung samples in DMEM containing 5 µg/ml NAT (N-Acetylated Trypsin, Sigma) were added. After incubation for 24 hours at 37 °C, cells were washed, fixed with 4 % formalin and permeabilized with quencher buffer (0.5 % Triton X-100 with 20 mM glycine in PBS), followed by incubation with a primary anti-influenza polyclonal antibody (Virostat) and a secondary HRP antibody (KPL). Subsequently, substrate (True Blue, KPL) was used for immunological staining. Foci were counted and calculated as FFU per lung homogenate. The detection limit of the assay was 80 infectious particles/ lung. Thus, for samples where no foci were detected, data points were set to 80 FFU/ lung.

Cytokine and chemokine analysis in BAL fluid
Female B6-Mx1+/r and D2-Mx1+/r mice (five in each group and time point) at the age of ten to twelve weeks were infected with 2x10^3 FFU PR8F. Control mice were mock-infected with PBS. After 3 and 5 days p.i., mice were euthanized by isoflurane. A sterile 22G catheter was inserted into the exposed trachea lumen. By instillation of PBS a volume of 0.5 ml broncho alveolar lavage fluid (BAL) per mouse was collected. BAL was stored at -70 °C until measurement. Chemokine and cytokine levels of G-CSF, GM-CSF, IFNg, IL-1a, IL-6, IL-10, IL-17, IP-10, KC, MCP-1, MIP-1a, RANTES,
TNFa and VEGF were analyzed using the Mouse Cytokine/Chemokine Magnetic Bead Panel MCY TOMAG-70K from Millipore following the instruction manual of the manufacturer. Plates were read in the Luminex 100™ apparatus.

**Interferon pre-treatment**

One day prior to influenza infection, mice were anesthetized and treated with 1 µg recombinant human interferon α B/D (type I interferon, IFN-I, provided by Prof. Dr. Peter Stäheli, University of Freiburg) in 20 µl of sterile phosphate-buffered saline by intranasal application. The control group received 20 µl of sterile phosphate-buffered saline.

**Statistical analysis**

Data and statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software, California). Heatmaps were generated using the R software package (R_Core_Team, 2013). Results were presented as means ± SEM for body weight change and virus titers. Statistical significance between groups was determined using the Mann-Whitney U test for body weight and virus titers. The log-rank test was used to determine significant differences between survival curves.
Results

**D2-Mx1<sup>fr</sup> mice are not resistant to lethal H1N1 influenza A infections**

We wanted to investigate if the wild type *Mx1* allele was able to protect DBA/2J mice from lethal infection or whether the genetic background of the highly susceptible DBA/2J strain may modify the function of the wild type *Mx1* allele. For this, we generated a congenic DBA/2J(B6).A2G-*Mx1<sup>fr</sup>* (D2-*Mx1<sup>fr</sup>*) mouse strain by backcrossing DBA/2J mice for ten generations with a congenic C57BL/6J.A2G-*Mx1<sup>fr</sup>* (B6-*Mx1<sup>fr</sup>*) mice (received from Peter Stäheli, Freiburg) that carried the A2G *Mx1* wild type allele (Supplementary Fig. 1A). By SNP-genotyping (data not shown), we confirmed that the congenic D2-*Mx1<sup>fr</sup>* stain carried a 32.73 Mb region from the B6-*Mx1<sup>fr</sup>* on chromosome 16 which includes 1.5 Mb of the original A2G region. Furthermore, presence of the wild type allele was confirmed by diagnostic PCR (Supplementary Fig. 1B). Also, congenic D2-*Mx1<sup>fr</sup>* mice expressed the *Mx1* wild-type allele after infection with H1N1 (PR8F) by RT-PCR (Supplementary Fig. 1C).

We then infected D2-*Mx1<sup>fr</sup>* and B6-*Mx1<sup>fr</sup>* mice as well as D2-*Mx1<sup>/</sup>* and B6-*Mx1<sup>/</sup>* mice with a highly virulent PR8F virus (Blazejewska et al., 2011). As described before, B6-*Mx1<sup>/</sup>* and D2-*Mx1<sup>/</sup>* were highly susceptible to these infections. They rapidly lost body weight and died between days 4 and 8 p.i. (Fig. 1). On the other hand, B6-*Mx1<sup>fr</sup>* mice exhibited less body weight loss and survived the infection confirming previous observations (Fig. 1). Most surprisingly, infected D2-*Mx1<sup>fr</sup>* mice were not protected from lethal infections. They showed severe clinical symptoms, lost body weight similar to *Mx1* deficient DBA/2J mice, and all infected D2-*Mx1<sup>fr</sup>* mice were dead at day 9 p.i. (Fig. 1). Furthermore, D2-*Mx1<sup>fr</sup>* mice produced high levels of chemokines and cytokines in their lungs (Fig 2 and Supplementary Fig. 2) indicating strong inflammatory response that associated with high levels of virus replication and severe course of infection.

To confirm that congenic D2-*Mx1<sup>fr</sup>* mice carried a functional *Mx1* allele, we outcrossed them to B6-*Mx1<sup>/</sup>* and compared the phenotype of the resulting F1 mice with the phenotype of F1 mice deriving from an outcross of B6-*Mx1<sup>fr</sup>* to D2-*Mx1<sup>/</sup>*. Thus, in the first case, the *Mx1* wild type allele is inherited from the congenic D2-*Mx1<sup>fr</sup>* mouse whereas in the second case the wild type allele is derived from the original B6-*Mx1<sup>fr</sup>* congenic strain. After infection with PR8F, F1 mice from both crosses
exhibited similar body weight loss but increased survival compared to D2-\textit{Mx1}−/− mice (Supplementary Fig. 3). These observations further demonstrated that the A2G \textit{Mx1} allele in D2-\textit{Mx1}−/− mice is fully functional.

**The protective effect of \textit{Mx1} on survival and virus replication is influenced by copy number and genetic background**

We then compared systematically the effect of \textit{Mx1}−/− copy number and combinations of DBA/2J and C57BL/6J background on survival after PR8F infections (Table 1, Fig. 3). The presence of one instead of two copies of \textit{Mx1} increased mortality in C57BL/6J mice rates to 21.5% and mice died between day 10 and 12 (B6-\textit{Mx1}−/− versus B6-\textit{Mx1}−/−). The increase in mortality was also observed for mice with a hybrid B6xD2 genetic background (F1(B6xD2).A2G-\textit{Mx1}−/− versus F1(B6xD2).A2G-\textit{Mx1}−/−). Mice that were homozygous for the mutant \textit{Mx1} allele were most susceptible and succumbed to the infection, independent of their genetic background (D2-\textit{Mx1}−/− and B6-\textit{Mx1}−/−). Furthermore, hybrid B6xD2 genetic background decreased survival in the presence of either one or two wild type \textit{Mx1} alleles compared to a pure C57BL/6J background (F1(B6xD2).A2G-\textit{Mx1}−/− versus B6-\textit{Mx1}−/− and (F1(B6xD2).A2G-\textit{Mx1}−/− versus B6-\textit{Mx1}−/−). Mice with a pure DBA/2J background did not survive infection in the presence or absence of \textit{Mx1} (D2-\textit{Mx1}−/− and D2-\textit{Mx1}−/−).

Next, we compared virus replication in the lung of D2-\textit{Mx1}−/−, B6-\textit{Mx1}−/−, F1 mice expressing one copy of the wild type \textit{Mx1} allele, and D2-\textit{Mx1}−/− and B6-\textit{Mx1}−/− carrying a mutant allele. After infection with PR8F virus, DBA/2J mice (with or without a functional \textit{Mx1} allele) exhibited very high levels of viral load in infected lungs at day 1 p.i. (Fig. 4B, D) whereas infected B6-\textit{Mx1}−/− mice rapidly reduced viral titers in lungs at day 3 p.i. (Fig. 4A). Most interestingly, F1(B6xD2).A2G-\textit{Mx1}−/− mice initially exhibited high viral loads in infected lungs but reduced viral titers in their lungs at day 3 p.i. which further decreased until day 5 p.i. (Fig. 4E). Thus, the \textit{Mx1} restrictive function on viral replication requires a hybrid or pure C57BL/6J background.

**D2-\textit{Mx1}−/− mice are partially resistant to low virulent H1N1 and H3N2 influenza A virus**

We then studied D2-\textit{Mx1}−/− mice after infection with the less virulent influenza A virus PR8M. All D2-\textit{Mx1}−/− mice infected with PR8M survived whereas all infected D2-\textit{Mx1}−/− mice died (Fig. 5A). Also, D2-\textit{Mx1}−/− mice were partially protected against infections
with the H3N2 virus subtype (Fig. 5B). B6-\textit{Mx1}^{-/-} mice did not survive infections with H3N2 but all infected B6-\textit{Mx1}^{+/+} survived (Fig. 5B). Of note, F1(B6xD2).A2G-\textit{Mx1}^{-/-} mice with a hybrid C57BL/6J genetic background were fully protected against mortality from H3N2 infections (Fig. 5B). These observations again confirmed that the \textit{Mx1} allele in D2-\textit{Mx1}^{+/+} is functional and is able to protect D2-\textit{Mx1}^{+/+} mice from an infection that is lethal in the absence of \textit{Mx1} in D2-\textit{Mx1}^{-/-}.

\textbf{D2-\textit{Mx1}^{+/+} mice are resistant to H1N1 influenza A virus after interferon pre-treatment}

Finally, we investigated the pathology in D2-\textit{Mx1}^{+/+} mice after interferon α pre-treatment since it had been shown that such pre-treatment protects from severe pathology after IAV infection (Grimm et al., 2007). All infected D2-\textit{Mx1}^{+/+} mice survived an infection with PR8F virus when pre-treated with IFN-I whereas all PBS mock-treated mice lost body weight and died (Fig. 5A). Furthermore, D2-\textit{Mx1}^{+/+} mice pre-treated with IFN-I exhibited lower viral loads compared to mock-treated animals at day 1 p.i. (Fig. 6B). In immunohistochemical staining, we did not observe viral antigen at the day 3 p.i. time point in IFN-I pretreated mice compared to a wide spread of virus in D2-\textit{Mx1}^{+/+} mice that were pre-treated with PBS (data not shown).
Discussion

Most laboratory mouse strains, including C57BL/6J, are deficient in $Mx1$ and susceptible to H1N1 (mouse-adapted PR8) infections. However, the presence of a wild type $Mx1$ allele makes B6-$Mx1^{+/r}$ resistant to H1N1 infections (Grimm et al., 2007). Furthermore, many studies have shown that $Mx1$ is a strong genetic resistance factor controlling influenza virus replication and protecting the host from severe pathology and mortality (Cilloniz et al., 2012; Grimm et al., 2007; Hodgson et al., 2011; Moritoh et al., 2009; Tumpey et al., 2007). These studies combined suggested that the $Mx1$ allele from A2G mice is able to protect from lethal infections independent of genetic background.

Most surprisingly, we found that congenic D2-$Mx1^{r/r}$ that carry the $Mx1$ wild type allele, still exhibited a highly susceptible phenotype as D2-$Mx1^{+/r}$ mice after infection with H1N1 virus (PR8F). All infected D2-$Mx1^{r/r}$ mice rapidly lost body weight and died. We confirmed in D2-$Mx1^{r/r}$ infected mice that the wild type allele in D2-$Mx1^{r/r}$ was expressed after infection.

Furthermore, ((B6 x D2(B6).A2G-$Mx1^{r/c}$)F1 which received the $Mx1$ allele from the congenic D2-$Mx1^{r/c}$ strain were as resistant to PR8F infections as F1 mice that were generated by crossing B6-$Mx1^{r/c}$ to D2-$Mx1^{+/r}$ mice. These experiments confirmed that D2-$Mx1^{r/c}$ mice carried a fully functional protective $Mx1$ allele. We also showed that, in agreement with previously published results (Grimm et al., 2007), B6-$Mx1^{r/c}$ were resistant to mouse-adapted PR8F (H1N1) virus infections and that $Mx1$-deficient B6-$Mx1^{+/r}$ mice succumbed to the infection.

Thus, our results show for the first time that the presence of the A2G $Mx1$ allele which is able to rescue A2G and congenic C57BL/6J mice from lethal influenza A virus infections does not exert its protective function in a DBA/2J genetic background. These observations suggest that additional genetic factors are required for the protective $Mx1$ functions or that the DBA/2J background is highly permissive to infections and that expression of $Mx1$ comes too late.

Recently, the wild derived mouse strain CAST/EiJ mice was found to be highly susceptible to H1N1 infections although these mice express a full length $Mx1$ allele with only one amino acid difference to the ancestral PWK/PhJ allele (Ferris et al., 2013). However, it is yet unclear if the high susceptibility in CAST/EiJ mice is caused
by the genetic background or the polymorphism in the \( Mx1 \) allele. Furthermore, we tested systematically different background combinations and \( Mx1 \) allele combinations to determine how resistance and susceptibility were influenced by wild type \( Mx1 \) and combinations of C57BL/6J and DBA/2J backgrounds. We observed that changing the genetic background from a pure DBA/2J to a hybrid B6xD2 and then to a pure C57BL/6J background incrementally increased survival. Thus, for protective non-lethal outcome of an H1N1 infection, at least one C57BL/6J genome had to be present. In the presence of a C57BL/6J genome, an increase from one to two copies of the wild type \( Mx1 \) allele also increased survival. These observations suggest that the \( Mx1 \) gene and presumed resistance factors from C57BL/6J act in an additive fashion. Furthermore, we found that the presence of a DBA/2J background in pure DBA/2J or in hybrid B6xD2 mice resulted in high viral loads in the lung at day 1 p.i., regardless whether the wild type \( Mx1 \) allele was present or not. However, in mice with a C57BL/6J or hybrid B6xD2 background, the presence of the \( Mx1 \) wild type allele always resulted in a reduction of virus lung titers on day 3 p.i. Absence of wild type \( Mx1 \) allele resulted in very high titers in a DBA/2J background which was not reduced at day 3 and lower initial virus titers in mice with a C57BL/6J background increased until day 3 p.i.

These observations suggest that a pure DBA/2J background renders the host highly permissive to an early rapid viral replication whereas the presence of at least one copy of the wild type \( Mx1 \) gene results in reduction of virus replication on day 3 p.i. The most likely explanation for the high susceptibility of D2-\( Mx1^{r/r} \) mice is therefore that \( Mx1 \) is induced too late after infection with H1N1 (PR8F) to exert its protective functions.

It has been shown previously that pre-treatment of B6-\( Mx1^{r/r} \) mice with interferon type I (IFN-I) can rescue mice from an otherwise lethal infection with a very highly virulent H1N1 virus (hvH1N1) (Grimm et al., 2007). We therefore investigated whether IFN-I pre-treatment may have a beneficial effect in D2-\( Mx1^{r/r} \) mice. Indeed, pre-treated D2-\( Mx1^{r/r} \) mice survived lethal infections with PR8F virus. Thus, our results showed that wild type \( Mx1 \) is able to protect DBA/2J mice when it is already present at the time of infection.
These results further support the hypothesis that in non-treated DBA/2J mice viral replication during the first two days is very rapid and expression of *Mx1* is too late to restrict the massive viral replication and prevent severe tissue damage and subsequent death.

*D2-Mx1<sup>+/−</sup>* mice showed a lower mortality rate when infected with 2x10<sup>3</sup>FFU H3N2 virus. In this case, we speculate that H3N2 virus may exhibit a slower replication rate very early after infection or that H3N2 does not suppress induction of *Mx1* as efficiently as PR8F and induction of Mx1 protein is early or strong enough to partially rescue infected D2-*Mx1<sup>+/−</sup>* mice.

In contrast to infections with PR8F and H3N2 virus, D2-*Mx1<sup>+/−</sup>* mice survived an infection with the low virulent mouse-adapted H1N1 (PR8M) virus. Influenza A viruses are known to suppress the host interferon induction by a variety of mechanisms, and it was shown recently, that PR8M is less efficient in suppressing the host interferon responses compared to PR8F (Liedmann et al., 2014). Also, *Ifnb1* expression was already detectable in PR8M infected mice but undetectable in PR8F infected mice at day 1 p.i. (Liedmann et al., 2014). Thus, a faster and stronger increase in *Mx1* due to the earlier activation of interferons in PR8M infected mice may well explain why D2-*Mx1<sup>+/−</sup>* mice survived infections with PR8M but not with PR8F.

Humans, as a species, carry functional orthologous genes of *Mx1*, named *MX1* and *MX2* (Haller et al., 2007). Thus, the differences in susceptibility and resistance to influenza A virus in humans that are attributed to genetic factors (Horby et al., 2012; Horby et al., 2010) are most likely not caused by presence or absence of *MX1*. Therefore, our observations which demonstrate that genetic background may render an individual highly susceptible, even in the presence of a functional *MX1* resistance gene, are also highly relevant for understanding genetic susceptibility to influenza infections in humans.

In summary, our results show that, in contrast to studies that were performed previously, the wild type influenza resistance *Mx1* gene (derived from A2G) does not necessarily result in high resistance against lethal influenza A virus infections. Rather, the protective effect of *Mx1* depends strongly on the genetic background, virulence of virus and kinetics of *Mx1* induction.
Acknowledgements

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Author contributions
DLS, BH, TN, and SB performed the experiments and analyzed the data. DLS and KS perceived the experiments. KS wrote the manuscript.

Additional information

Supplementary information accompanies this paper

Competing financial interest. The authors declare no conflict of interest.
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## Figures and Table

Table 1: Survival after H1N1 infections is influenced by wild type *Mx1* copy number and genetic background

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>Genetic background</th>
<th><em>Mx1</em> allele</th>
<th>Killed/infected mice</th>
<th>Survival proportions</th>
</tr>
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<tr>
<td>B6-Mx1&lt;sup&gt;cr&lt;/sup&gt;</td>
<td>B6</td>
<td>r/r</td>
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<tr>
<td>B6-Mx1&lt;sup&gt;1/-&lt;/sup&gt;</td>
<td>B6</td>
<td>-/-</td>
<td>14/14</td>
<td>0%</td>
</tr>
<tr>
<td>D2-Mx1&lt;sup&gt;cr&lt;/sup&gt;</td>
<td>D2</td>
<td>r/r</td>
<td>10/10</td>
<td>0%</td>
</tr>
<tr>
<td>D2 -Mx1&lt;sup&gt;tr&lt;/sup&gt;</td>
<td>D2</td>
<td>r/-</td>
<td>7/7</td>
<td>0%</td>
</tr>
<tr>
<td>D2-Mx1&lt;sup&gt;1/-&lt;/sup&gt;</td>
<td>D2</td>
<td>-/-</td>
<td>11/11</td>
<td>0%</td>
</tr>
<tr>
<td>F1(B6xD2).A2G-Mx1&lt;sup&gt;cr&lt;/sup&gt;</td>
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<td>r/r</td>
<td>1/14</td>
<td>92.83%</td>
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<tr>
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<td>r/-</td>
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<td>68.75%</td>
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<td>B6 x F1(B6xD2).A2G-Mx1&lt;sup&gt;tr&lt;/sup&gt;</td>
<td>B6 x F1(B6xD2)&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>D2 x F1(B6xD2).A2G-Mx1&lt;sup&gt;tr&lt;/sup&gt;</td>
<td>D2 x F1(B6xD2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>r/-</td>
<td>5/8</td>
<td>37.50%</td>
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</table>

* Secondary outcross performed by outcrossing F1(B6xD2).A2G-Mx1<sup>tr</sup> with B6-Mx1<sup>1/-</sup> or D2-Mx1<sup>1/-</sup>
Figure 1. D2-Mx1/r mice were highly susceptible to H1N1 (PR8F) infections whereas B6-Mx1/r mice were resistant.

Eight to twelve weeks old female mice (D2-Mx1/r, B6-Mx1/r, D2-Mx1/-, B6-Mx1/-) were infected intra-nasally with 2x10^3 FFU PR8F (H1N1) influenza A virus. Body weight loss (A) and survival rates (B) were monitored over a period of 14 days. Mice that reached 30% of the starting body weight were sacrificed and recorded as dead. Data represent mean percent of body weight change (+/- SEM) compared to starting body weight (100%). Differences in body weight loss were significant between D2-Mx1/r and B6-Mx1/r mice after day 3 p.i. (p<0.0001, non-parametric Mann Whitney test). Survival rates were significantly different between D2-Mx1/r and B6-Mx1/r mice (p<0.0001, Log rank Mantel-Cox test). n= number of mice per group.
Figure 2. D2-Mx1<sup>−/−</sup> mice showed high levels of chemokine and cytokines in BAL fluid.

Female D2-Mx1<sup>−/−</sup> (D2Mx1) and B6-Mx1<sup>−/−</sup> (B6Mx1) mice at 10-12 weeks of age were infected intranasally with 2x10<sup>3</sup> FFU PR8F. Bronchio-alveolar lavage (BAL) was collected from mock-infected control mice at day 3 post treatment and from infected mice at day 3 and day 5 p.i. The concentration of chemokines and cytokines was determined by using the Mouse Cytokine/Chemokine Magnetic Bead Panel MCYTOMAG-70K from Millipore. At each time point, five biological replicates were analyzed. The heatmap shows log2-transformed mean protein concentrations of the indicated chemokines and cytokines (representing rows) and colors indicates z-scores after scaling by rows from -2 (dark green) to 2 (red). Columns represent groups of mice as indicated. mo: mock-infected mice at day 3 post treatment, d3, d5: days 3 and 5 p.i.
Figure 3. Resistance to lethal H1N1 infections is controlled by Mx1 copy number and genetic background.

F1 mice of different Mx1 allele combinations and different C57BL/6J and DBA/2J background combinations were tested for susceptibility to PR8F H1N1 virus. Groups of eight to twelve weeks old female mice (F1(B6xD2).A2GMX1<sup>r/r</sup>, F1(B6xD2).A2GMX1<sup>r/-</sup>, B6.A2G-MX1<sup>r/-</sup>, and D2.A2G-MX1<sup>r/-</sup>) were infected intra-nasally with 2x10<sup>3</sup> FFU PR8F virus and survival was monitored until day 14 p.i. Mice that reached 30% of the starting body weight were sacrificed and recorded as dead. For the F1(B6xD2).A2G-MX1<sup>r/-</sup> group, data from reciprocal crosses ((B6xD2(B6).A2G-MX1<sup>r/-</sup>)F1 (n=6) and (D2xB6.A2G-MX1<sup>r/-</sup>)F1 (n=10)) were combined. Two copies of the wild type Mx1 locus increased resistance compared to one copy. Introduction of DBA/2J background in hybrid F1(B6xD2) Mx1<sup>r/r</sup> mice increased susceptibility and pure DBA/2J background in Mx1<sup>r/r</sup> mice increased susceptibility further. The results also confirmed that D2-Mx1<sup>r/r</sup> mice carried a functional protective Mx1 allele, since F1 mice from an outcross of D2-Mx1<sup>r/r</sup> to B6-Mx1<sup>r/-</sup> exhibited the same phenotype as F1 mice that carried the Mx1 allele from the original B6-Mx1<sup>r/r</sup> strain outcrossed to D2-Mx1<sup>r/-</sup>. 
Eight to twelve weeks old female B6-\(Mx1^{+/+}\) (A), D2-\(Mx1^{+/+}\) (B), B6-\(Mx1^{+/}\) (C), D2-\(Mx1^{+/}\) (D) and F1(B6xD2).A2G-\(Mx1^{+/}\) (E) mice were infected intra-nasally with \(2\times10^3\) FFU PR8F virus. Infectious virus particles in lung homogenates were determined by foci forming assay at days 1, 3, 5 p.i. Viral load on day 1 p.i. was significantly different between infected mice that carried a DBA/2J genetic versus C57BL/6J background (B6-\(Mx1^{+/+}\) compared to D2-\(Mx1^{+/+}\) using Mann-Whitney test, \(p=0.0022\)) and only mice carrying both a functional \(Mx1\) allele and C57BL/6J genetic background reduced viral loads from day 1 to day 3 p.i. D2-\(Mx1^{++}\), B6-\(Mx1^{++}\), and B6-\(Mx1^{++}\): \(n = 6\) mice per time point; D2-\(Mx1^{--}\): \(n = 5\) per time point, F1(B6xD2).A2G-\(Mx1^{+/}\): \(n = 3\) per time point.
Figure 5. D2-\textit{Mx}^{1\textit{ir}} mice were protected against infections with low virulent H1N1 (PR8M) virus and partially protected against infections with H3N2 virus.

Eight to twelve weeks old female mice (B6-\textit{Mx}^{1\textit{ir}}, D2-\textit{Mx}^{1\textit{ir}}, F1(B6xD2).A2G-\textit{Mx}^{1\textit{ir}}), B6-\textit{Mx}^{1\text{c}} and D2-\textit{Mx}^{1\text{c}}) were infected intra-nasally with 2x10\(^3\) FFU low virulent PR8M H1N1 virus (A) or with 2x10\(^3\) FFU H3N2 virus (B). Survival rates were monitored over a period of 14 days p.i. Mice that reached 30% of the starting body weight were sacrificed and recorded as dead. All infected D2-\textit{Mx}^{1\textit{ir}} mice survived infection with PR8M (H1N1) virus. D2-\textit{Mx}^{1\textit{ir}} mice were partially protected against H3N2 infections compared to B6-\textit{Mx}^{1\text{c}} (Log-rank Mantel-Cox test, p=0.0065). Also, all F1(B6xD2).A2G-\textit{Mx}^{1\text{c}} mice with a hybrid C57BL/6J genetic background survived the H3N2 infections. Please note that in A, survival curves of D2-\textit{Mx}^{1\textit{ir}} and B6-\textit{Mx}^{1\text{c}} mice overlap.
Eight to eleven weeks old female D2-\textit{Mx}^{1cr} \textit{mice} were pretreated with 1\(\mu\)g recombinant human interferon alpha B/D (IFN-\(\alpha\) ) intra-nasally 1 day prior to infection, PBS was given as mock control. All mice were subsequently infected intra-nasally with \(2\times10^3\) FFU PR8F virus. \textbf{(A)} Survival rate was monitored for 14 days p.i. Mice that reached 30\% of the starting body weight were sacrificed and recorded as dead. IFN-I pretreated mice showed higher survival rates compared to PBS-treated controls (Log-rank survival \(p=0.0019\), \(n=5\) mice per group). \textbf{(B)} Virus particles in lung homogenates from IFN-I pretreated and PBS-treated mice were determined in foci forming assay on day 1 p.i. Virus titers were significantly different between IFN-I- and PBS-pretreated groups (\(p=0.0079\), using Mann Whitney U test); \(n=5\) mice per group.
**Figure S1. Genomic organization of Mx1 locus in B6-Mx1<sup>r/r</sup> and B6-Mx1<sup>-/-</sup> mice, Mx1 genotyping and Mx1 expression analysis in infected mice**

(A) C57BL6J mouse strains carry a deletion in the Mx1 gene from intron 8 to intron 11 resulting in a truncated non-functional Mx1 protein. Congenic B6-Mx1<sup>r/r</sup> mice carry a wild type Mx1 allele. Primers used for genotype analysis are indicated by arrows (details see M&M). (B) PCR analysis with indicated primers showed two distinct fragment sizes for the Mx1<sup>r/r</sup> (950 bp) and the Mx1<sup>-/-</sup> (1255 bp) alleles. Heterozygous mice showed both products. (C) RT-PCR products confirmed expression of a full length Mx1 transcript (using primer pair e8Fn and e12R) in congenic D2-Mx1<sup>r/r</sup> mice after infection with 2x10<sup>3</sup> FFU PR8F virus at 12 and 24 h p.i.
Figure S2. Chemokine and cytokine levels in BAL fluid of D2-\(Mx^{1+}\) show stronger inflammatory response than B6-\(Mx^{1-}\) mice (continue on next page)
Figure S2. Chemokine and cytokine levels in BAL fluid of D2-\textit{Mx1}^{r/r} show stronger inflammatory response than B6-\textit{Mx1}^{r/r} mice

Female D2-\textit{Mx1}^{r/r} (circles) and B6-\textit{Mx1}^{r/r} mice (squares) were infected with 2x10^{3} FFU PR8F intranasally. Bronchio-alveolar lavage (BAL) was collected from mock-infected control mice at day 3 post treatment and from infected mice at day 3 and day 5 p.i. The concentration of chemokines and cytokines was determined by using the Mouse Cytokine/Chemokine Magnetic Bead Panel MCYTOMAG-70K from Millipore. At each time point, five biological replicates were analyzed.
Figure S3. Confirmation of functional wild type Mx1 in D2-Mx1r mice by outcrossing to B6-Mx1r mice

Congenic D2-Mx1r mice were outcrossed to B6-Mx1r mice and the phenotype of the resulting F1 mice [(B6 x D2(B6).A2G-Mx1-Mx1r)F1 or reciprocal crosses] were compared to the phenotype of F1 mice derived from an outcross of B6-Mx1r to D2-Mx1r [(D2 x B6.A2G-Mx1-Mx1r)F1 or reciprocal cross]. F1 mice from both crosses did not show significant differences in body weight loss or survival (Mann Whitney U test for body weight change analysis, Log-rank Mantel-Cox test for survival curves). n= number of mice per group.
5.2 Manuscript II: Mutation of *Serpine1* in mice results in increased susceptibility to influenza A virus infection due to increased vascular leakage and virus dissemination

Shin DL, Hatesuer B, Stricker RLO, and Schughart K

(Manuscript in preparation)

Title

Mutation of *Serpine1* in mice results in increased susceptibility to influenza A virus infection due to increased vascular leakage and virus dissemination

Authors

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Abstract

Influenza A viruses (IAV) cause a contagious respiratory disease in human and animals. Besides intensive studies on the virus and its virulence factors, the biological mechanisms of the host-pathogen interactions are still only partially understood. Recent studies showed that IAV alter the plasminogen conversion pathway after infection, which induces hyperfibrinolysis and results in lung injury. Serpine1 (PAI-1) is a member of the serine protease inhibitor (serpine) family that inhibits tPA (tissue type plasminogen activator) and urokinase (uPA). Both proteins activate the plasminogen pathway and lead to increased fibrosis in the lung. Deficiency of SERPINE1 can lead to bleeding due to unstable hemostatic plug formation. Edema in the lung is a characteristic pathology of severe influenza infection. Here, we studied virus dissemination and host susceptibility to IAV infection in Serpine1−/− mutant mice. Our results showed that Serpine1−/− mutant mice were highly susceptible to A/Puerto Rico/8/34 (PR8, H1N1) infections compared to wild type mice. Knock-out animals exhibited increased body weight loss, enhanced lethality and higher viral loads on day 5 post infection (p.i.). We also infected mice with another H1N1 IAV, A/WSN/33 (WSN) carrying a unique neuraminidase gene that binds and converts plasminogen into plasmin. Activated plasmin can then cleave viral hemagglutinin (HA) in the absence of serine proteases resulting in lung higher titers in infected mice. After infection with WSN virus, knock-out mice lost more body weight and showed higher mortality compared to wild type mice although virus titers were similar. Furthermore, red blood cells leaked into the alveolar space in Serpine1−/− mutant mice after infection with both PR8 and WSN which was associated with increased vascular permeability. Finally, we detected viral RNA in kidneys of infected Serpine1−/− mice indicating viral dissemination from the lung into the blood stream. In summary, our results suggest that due to the loss of SERPINE1, PR8 can replicate to higher titers and cause a more severe outcome. WSN by itself can convert plasminogen into plasmin which results in an even more severe phenotype than observed by PR8.
**Introduction**

Influenza A viruses (IAV) cause a contagious respiratory disease in human and animals (Fauci, 2006; Kilbourne, 2006). Most of the biological mechanisms of the host-pathogen interactions during the progression of influenza virus infection still remain to be understood. Recent studies in mice and ferrets illustrated the importance of hyperfibrinolysis in acute lung injury after IAV infection. In the mouse model, IAV is damaging the vascular capillaries and impairs fibrin deposition that provides an environment for immune cell recruitment and cytokine induction (Berri et al., 2013; Khoufache et al., 2013). In the ferret model, high pathogenic avian influenza (HPAI) H5N1 virus can deposit clotting fibrin in intra-capillary tissue and alter hemostasis after infection (Goeijenbier et al., 2014).

Hyperfibrinolysis is induced from activated plasmin, a product which is converted from plasminogen precursor protein. This process is controlled by the urokinase pathway or tissue plasminogen activator pathway, and both pathways are negatively regulated by SERPINE1 (Ghosh and Vaughan, 2012). The SERPINE1 protein is known to be an anticlotting agent that can be used to prevent stroke patients from enhanced breakdown of fibrin clots and optimizes hemostasis during infection progression (Hatcher and Starr, 2011). Recent study has also shown that SERPINE1 protein may inhibit the host serine protease function, thereby reducing IAV glycoprotein cleavage (Dittmann et al., 2015).

To date, only few studies addressed the role of plasminogen activation or fibrinolysis pathway during IAV infection. Some in vivo studies investigated the role of anti-thrombin III, endogenetic protein C and thrombin focusing on the direct protease activation of virus hemagglutinin (HA) cleavage (Dittmann et al., 2015; Schouten et al., 2011; Smee et al., 2014). Other reports showed that infected mice could maintain stable hemostasis after IAV infection by producing more thrombin or by indirect inhibition of plasminogen conversion (Keller et al., 2006). Some studies have also shown that plasminogen activation via annexin-II pathway may be considered as a potential enhancer for virus replication by altering HA cleavage (Khoufache et al., 2013; LeBouder et al., 2008).

Two commonly used mouse-adapted IAV strains of the H1N1 subtype, A/Puerto Rico/8/34 (PR8) and A/WSN/33 (WSN), were used in this study. Compared to PR8,
WSN virus carries a unique neuraminidase (NA) function allowing HA cleavage of the viral hemagglutinin (HA) by plasmin. The carboxyl terminus of the WSN NA can bind to plasminogen and convert it into activated plasmin. The enzymatically active plasmin cleaves viral HA and allows viral infection (Goto and Kawaoka, 1998, 2000; Zhirnov et al., 1982). This NA function allows WSN virus to replicate more efficiently resulting in increased viral loads (García-Sastre et al., 1998a; Goto et al., 2001). In addition, WSN can also replicate in the mouse brain due to recruitment of host plasminogen (Goto and Kawaoka, 1998; Lazarowitz et al., 1973; Takahashi et al., 1995; Wolf et al., 1974). PR8 virus is mainly restricted to the pulmonary tract while the WSN virus disseminates to other organs (García-Sastre et al., 1998a). The replication of WSN virus in lungs induces the protease activator receptor 2 signaling pathway which disturbs tight junction formation and increases vascular permeability (Wang et al., 2010).

Here we investigated the effect of PR8 and WSN IAV on a mouse knock-out mutant with a deletion in the Serpine1 gene, and we found that knock-out mice are more susceptible to infection with IAV. This leads to more lung damage and that increased vascular permeability, which in the end caused virus dissemination.
Material and methods

Ethics statement
All experiments in mice were approved by an external committee according to the national guidelines of the animal welfare law in Germany (BGBl. I S. 1206, 1313 and BGBl. I S. 1934). The protocol used in these experiments has been reviewed by an ethics committee and approved by the ‘Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany’ (Permit Number: 3392 42502-04-13/1234). Laboratory C57BL/6J purchased from Janvier, France. Serpine1-/- knock-out mice carrying mutant Serpine1 alleles were purchased from Jackson Lab, USA. Mice were maintained at specific pathogen free conditions at the animal facilities of the HZI. Embryonated chicken eggs were purchased from Charles River Laboratories, Germany.

Virus and mice
The serine peptidase inhibitor clade E (Serpine1) targeted null mutation (B6.129S2-Serpine1tm1Mlg/J) was made as described earlier (Carmeliet et al., 1993). Shortly, a PGK-neomycin resistance cassette replaced all of the Serpine1 coding sequence and part of the promoter region including the transcription initiation site. The targeting vector was inserted in D3 ES cells and mice were subsequently backcrossed to C57BL/6J background. Homozygous mutant mice (Serpine1-/-) were genotyped using a three 3-Primer PCR analysis (Serpine1-F: CTG GGC AGT AAC AGA GA; Serpine1-R: GTC GGT CGT CTA GAC CCT TG; Serpine1-KO: TGG ATG TGG AAT GTG TGC GAG). Influenza A virus A/PuertoRico/8/34 H1N1, Münster variant (PR8) and A/WSN/33 H1N1 virus (WSN) were obtained from Prof. Dr. Stefan Ludwig, University of Münster. All viruses were propagated in the chorio-allantoic cavity of 10-day-old pathogen-free embryonated chicken eggs, aliquoted and stored at -80°C.
Infection of mice

Female mice at the age of 9-12 weeks were anesthetized by intra-peritoneal injection of Ketamin-Xylazine solution in sterile NaCl (100 mg/ml Ketamine, WDT, Garbsen, Germany; 20 mg/ml Xylavet®, CP-Pharma, Burgdorf, Germany) with a dose adjusted to the individual body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS). Subsequently survival and body weight loss were monitored until day 14 p.i. In addition to mice that were found dead, mice with a weight loss of more than 30% of the starting body weight were euthanized and recorded as dead. For both, PR8 and WSN viruses, we challenge the mice with non-lethal dose for wild type mice in the following experiments, which were 2x10⁵ FFU for PR8 and 2x10³ FFU for WSN.

Determining of infectious viral particles

For determining viral loads, lungs were prepared into 2 ml PBS containing 0.1 % BSA. Tissue was homogenized using the Poly Tron 2100 homogenizer and aliquots were stored at -70 °C. Virus titers were determined on MDCK II (Madin–Darby Canine Kidney II) cells as focus forming units (FFU) as described previously (Blazejewska et al., 2011). Briefly, MDCK II cells were seeded in 96-well plates and serial 10-fold dilutions of homogenized lung samples in DMEM containing 5 µg/ml NAT (N-Acetylated Trypsin, Sigma) were added. After incubation for 24 hours at 37 °C, cells were washed, fixed with 4 % formalin and permeabilized with quencher buffer (0.5 % Triton X-100 with 20 mM glycine in PBS), followed by incubation with a primary anti-influenza polyclonal antibody (Virostat #1301) and a secondary HRP antibody (KPL). Subsequently, substrate (True Blue, KPL) was used for immunological staining. Foci were counted and calculated as FFU per lung homogenate. The detection limit of the assay was 80 infectious particles/ lung. Thus, for samples where no foci were detected, data points were set to 80 FFU/ lung, respectively.
**Histopathology**

For histopathology studies, groups of three mice were infected intra-nasally with 2x10^5 FFU PR8 or 2x10^3 FFU WSN. On day 8 p.i., mice were euthanized with CO₂, lungs were prepared and immersion-fixed in 4% formaldehyde solution for 24 hours, embedded in paraffin, sectioned at 0.5μm, and mounted on slides. Tissue specimens were stained with hematoxylin and eosin (H&E). All histopathological changes were examined microscopically by blinding identities of samples.

**In vivo permeability assay**

To determine protein leakage from alveolar septum into alveolar space, we performed a quantitative *in vivo* permeability assay using fluorescence conjugated bovine serum albumin (BSA). Groups of 3-6 mice were anesthetized and infected with 2x10^5 FFU PR8 or 2x10^3 FFU WSN intra-nasally. On day 5 and 7 p.i., mice were injected intravenously with FITC conjugated albumin (1.5mg/ mouse) one hour prior to sacrifice. Subsequently, mice were euthanized with CO₂, bronchoalveolar lavage fluid (BALF) was collected with two washes of 1ml PBS and serum were collected. Fluorescence in BALF and serum (tenfold dilution) was measured in a fluorometer at 492-nm absorbance and 520-nm emission wavelengths (Del Maschio et al., 1999; Maus et al., 2001). BSA leakage ratio was calculated as fluorescence A.U. (absolute fluorescence value of FITC-BSA in BALF / absolute fluorescence value of FITC-BSA in diluted serum *100).

**RT-PCR for virus dissemination assays**

Melting curve analysis with RT-PCR was performed to determine levels of virus RNA in different organs after infection. Mice were anesthetized and infected intranasally with 2x10^5 FFU PR8 or 2x10^3 FFU WSN in 20 μl PBS. On day 5 p.i., lungs, brains, spleens, livers, and kidneys were prepared, washed in PBS and stored in 2 ml RNA Later (Qiagen). Subsequently, tissues were homogenized using PolyTron 2100 homogenizer. Total RNA was prepared using Trizol® chloroform extraction method according to the manufacturer instructions (Invitrogen). One μg of total RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen TM, USA) according to the manufacturer instructions. Five μl of cDNA product were
amplified with specific primers (M52C and M253R) to amplify the Matrix gene of IAV as described previously (Fouchier et al., 2000). PCR and melting curve analyses were carried out with SsoFast™ Evagreen® supermix (Bio-Rad) by LightCycler 480 (Roche) according to manufacturer instructions. PCR products exhibiting melting peaks at 85±0.5 °C were counted as positive (Trevennec et al., 2012).

**Statistical analysis**

Data and statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software, California). Results were presented as means ± SEM for body weight change, virus titers, and BSA leakage levels. Statistical significance between groups was determined using the Mann-Whitney U test for body weight, virus titers and BSA leakage. The log-rank test was used to determine significant differences between survival curves.
Results

*Serpine1*−/− mice show enhanced lethality after infection with influenza A virus

To investigate the role of the plasminogen activation pathway during influenza A virus infection, we studied the host response in *Serpine1*−/− knock-out mice after infection with different influenza A virus H1N1 variants. *Serpine1*−/− mice exhibit increased pulmonary clot lysis and show a mild hyperfibrinolytic state, while hemostasis is normal compared to wild type mice ([http://jaxmice.jax.org/strain/002507.html](http://jaxmice.jax.org/strain/002507.html)). After intranasal infection with 2x10^5 FFU of PR8, both wild type and *Serpine1*−/− mutant mice lost body weight (Fig. 1A). However, 45.5% of infected *Serpine1*−/− mice died until day 8 p.i., whereas all wild type mice survived (Fig. 1B). Furthermore, when mice were infected with WSN at a dose of 2x10^3 FFU, *Serpine1*−/− mutant mice showed significantly more weight loss from day 3 p.i. compared to wild type mice (Fig. 1A). *Serpine1*−/− mice showed 100% mortality after WSN infection whereas all infected wild type mice survived (Fig. 1B). Foci forming assay from infected lungs on day 1, 3, 5, and 8 p.i. showed that viral load in PR8 infected *Serpine1*−/− mice was significantly higher at day 5 p.i., compared to wild type mice (Fig. 1C). Additionally, at day 8 p.i., most of the wild type mice had cleared the virus, whereas *Serpine1*−/− knock-out mice still carried infectious virus particles in their lungs (Fig. 1C). In contrast, WSN infected wild type and mutant mice group showed no differences in virus titration during the entire infection period (Fig. 1D).

Red blood cells leak into the alveolar space in *Serpine1*−/− mutant mice after influenza A virus infection

Although virus titers were similar in the lungs of wild type and *Serpine1*−/− mutant mice after WSN infection, *Serpine1*−/− mice were more susceptible to infection than wild type mice. Therefore, we performed the histopathological analysis of infected lungs to further evaluate the pathology in mutant mice. Polymorphonuclear leukocytes (PMN) infiltrated the interstitial space and scattered PMNs were apparent in the alveolar space of both wild type and mutant mouse strains (Fig. 2A-D). On day 8 p.i. with PR8 and WSN infection, *Serpine1*−/− mutant mice showed diffuse alveolar damage (Fig. 2B,
Most remarkably, red blood cells (RBC) leaked into the alveolar space in Serpine1−/− mutant mice on day 8 p.i. (arrow in Fig. 2B, D), but not in uninfected mice (data not shown), whereas in wild type mice RBC were only found in the alveolar septum (Fig. 2A, C).

WSN virus infection enhances vascular permeability in infected mouse lungs

To further assess the integrity of the endothelial-epithelial barrier, we performed in vivo permeability assays using fluorescence-labeled BSA. On day 5 and 7 after PR8 infection, Serpine1−/− knock-out mice had significantly more FITC-BSA in the bronchoalveolar lavage than wild type mice (Fig. 3). On the other hand, after WSN infection both wild type and knock-out mice showed increased FITC-BSA leakage into BALF on day 5 and 7 p.i. compared to mock infected mice (Fig. 3). At day 5 p.i. both levels were the same as in the knock-out mice after PR8 infection. Our results indicated that, Serpine1−/− mice exhibited a higher vascular permeability after PR8 infection compared to wild type mice, whereas WSN virus increased permeability in both mouse strains.

Dysregulation of the urokinase pathway results in virus dissemination

Finally, we investigated dissemination of virus since leakage of endothelial-epithelial barrier may allow virus to enter the blood stream. Using RT-PCR we could detect viral RNA in kidney, liver and spleen besides the lung on day 5 p.i. in mutant, but not in wild type mice, after infection with PR8. On the other hand, after infection with WSN virus, which is known to disseminate to extra-pulmonary organs, viral RNA was detected in brain, liver, and kidney in only one of four infected wild type mice, whereas all lung samples were positive. However, in Serpine1−/− knock-out mice infected with WSN viral RNA was found in one sample of liver and in all lungs and kidneys of all mutant mice (Table 1).
Discussion

In this study, we used a mouse knock-out model to investigate the role of SERPINE1 during influenza A infection. Our data suggest that hyperfibrinolysis contributes to higher severity in the lung, and allows virus to disseminate into other organs. Furthermore, our studies indicate that, Serpin1⁻/⁻ mice exhibited a higher vascular permeability after PR8 infection compared to wild type mice, whereas WSN virus increased permeability in both mouse strains.

Hyperfibrinolysis may lead to higher permeability of alveolar capillaries in the lung (Wygrecka et al., 2008). For the fibrinolysis pathway, urokinase (uPA) and tissue plasminogen activator (tPA) play important roles in the conversion of plasminogen, whereas activated plasmin can initiate the break down process of blood clots. SERPINE1 functions as the inhibitor of uPA and tPA. When Serpin1⁻/⁻ mice were treated with belomycin (an inducer of fibrosis), they induced fibrosis at a slower rate and generally reduced fibrin (Eitzman et al., 1996; Ghosh and Vaughan, 2012). Thus, Serpin1⁻/⁻ mice represent a well-suited mouse model for studying the interaction between fibrinolysis and viral infection.

To date, IAV studies related to fibrinolysis or fibrosis are very limited. Recent studies using plasminogen knock-out mice showed a strong resistance to PR8 virus infection, with less clot breakdown and less immune cell recruitment into the alveolar space. These responses were due to the block of plasminogen conversion and further reduction of hyperfibrinolysis (Berri et al., 2013; Khoufache et al., 2013; LeBouder et al., 2008). However, our data using Serpin1⁻/⁻ mice showed higher susceptibility to infections with different IAVs. Serpin1⁻/⁻ mutant mice exhibited higher viral load in their lungs only on day 5 p.i. compared to wild type mice after infection with PR8, while the differences between WSN infected mice were not significant. This is in agreement with Dittmann et al (2015) and Keller et al (2006), who detected a slightly increase of infectious viral particles on day 5 p.i. but not on day 4 p.i between wild type and Serpin1⁻/⁻ knock-out mice after infection with PR8. The mortality in WSN infected mutant mice reached 100% after day 10 p.i., while PR8 infected knock-out mice exhibited a mortality of 45%. These results indicate a difference in the pathological mechanism between both viruses.
Recent study showed that the size of infected lungs increased in Serpine1\(^{-/-}\) mutant mice at day 5 post IAV infection, which accompanied with bleeding into alveoli compared to wild type mice (Dittmann et al., 2015). Another study showed that after severe acute respiratory syndrome (SARS) virus infection lung lesions were restricted in C57BL/6 mice, while in infected Serpine1\(^{-/-}\) mice the phenotype was more severe and showed diffused hemorrhages in the lung. These studies suggest that the function of SERPINE1 was also relevant for pathogenicity after SARS infection (Gralinski et al., 2013). In our studies, we observed a mild hemorrhage phenotype with red blood cell leaking into the alveolar space in all infected Serpine1\(^{-/-}\) knock-out mice. These findings indicate that the alveolar membrane was damaged and became permeable for cells. Nevertheless, the leakage of RBCs into alveolar space is considered as a sign for dysregulation of the fibrinolytic response and lung injury in acute respiratory stress syndrome which is also associated with leakage of the endothelial-epithelial barrier (Idell et al., 1989).

In our study, we performed in vivo permeability assays to investigate if leakage only occurs in Serpine1\(^{-/-}\) mice. Surprisingly, we found that not only Serpine1\(^{-/-}\) mice but also WSN infected wild type mice showed high BSA leakage (Fig 3). Previous studies showed that WSN infected wild type mice exhibit protein leakage into the BALF. These observations suggest that WSN virus may enhance viral replication via plasminogen-NA binding and therefore result in higher virus titer and lung injury (Aeffner et al., 2014; Garcia et al., 2010; Wang et al., 2010). However, in our studies, both Serpine1\(^{-/-}\) and wild type mice showed the same viral load during the WSN infection period, which indicated that a high virus replication may not be the only factor for increased vascular permeability after WSN infection.

The neuraminidase of WSN virus is able to convert plasminogen which then facilitates HA cleavage (Goto and Kawaoka, 1998; Goto et al., 2001). Also, in vivo studies showed that through this conversion WSN can replicate more efficiently and thereby increase severity of infection (Garcia-Sastre et al., 1998a; Sun et al., 2010). Although plasminogen conversion was demonstrated for WSN virus, the downstream effects were still unknown. Here, we hypothesize that WSN virus may provide an alternative pathway for plasminogen conversion which enhances fibrinolysis (Fig 4). In wild type mice, SERPINE1 inhibits fibrinolysis and the absence of Serpine1 gene will result in
more severe lung injury and higher vascular permeability in alveolar membranes leading to hemorrhages. On the other hand, WSN virus alternatively triggers activation of plasmin, which results in fibrinolysis in wild type mice. This process will not occur in PR8 infected mice. Subsequently, the lung injury of WSN infected Serpine1−/− mice was more severe due to the lung leakage and resulted in death. Thus, this model of alternative plasminogen activator pathway may explain the observation which is described by Dittmann et al (2015). In previous description, the WSN spreading was more efficient in the human fibroblast cells containing natural Serpine1 deficiency than in cells contain wild type allele (Dittmann et al., 2015). Adapted these findings together with our model, the amount of activated plasmin may increase in the cells containing Serpine1 deficient allele, therefore the WSN virus may benefited by the dysregulation of plasmin in the environment. On the other hand, human fibroblast cell contain functional Serpine1 allele may restrict the WSN replication due to limited amount of converted plasmin. We suggested that the difference between the spreading of WSN in human fibroblast cells containing natural Serpine1 deficient or wild type alleles was not only due to inhibition of host TMPRSS2 or HAT (Dittmann et al., 2015) but also the alternative plasminogen conversion may enhanced the viral replication.

Increased vascular permeability might also enhance viral dissemination. In vivo studies with intra-nasal WSN inoculation in three to four weeks old BALB/c mice showed virus dissemination into the brain (Goto et al., 2001; Sun et al., 2010). Other study with protease activator receptor-2 antagonist showed that WSN virus replication can enhance cytokine-protease network in the lung, and thereby further result in the destruction of tight junctions in the brain-blood-barrier (Aeffner et al., 2014; Wang et al., 2010). In contrast to WSN, PR8 H1N1 virus was shown to be restricted to the respiratory tract whereas WSN could be detected in kidney, spleen, liver and brain (Garcia-Sastre et al., 1998a). Our results confirmed lung-restricted virus replication after PR8 infection in wild type mice and no dissemination to other organs. After infection of wild type mice with WSN virus, only limited dissemination into the kidney, liver and brain was observed. In contrast, viral RNA was found in kidney and liver in Serpine1−/− mutant mice after infection with both, PR8 and WSN virus. Interestingly, in all PR8 or WSN infected Serpine1−/− knock-out mouse viral RNA was found in the kidney.
Other studies have shown that SERPINE1 reduces fibrosis in aggressive renal disease and represents an important marker for chronic kidney disease (Eddy, 2002). This suggests that loss of SERPINE1 may play an important role in the observed dissemination of IAV to the kidney.

When the loss of SERPINE1 leads to increased pathogenicity after IAV infection, one could suggest that depletion of tPA or uPA, both plasmin cleaving proteins, has a protective function regarding infections. PLAT−/− mice, lacking the plasmin cleaving protein tPA, showed a mixed pathogenic phenotype depending on the dose of infection with SARS (Gralinski et al 2013). Similarly, depletion of uPA using PLAU−/− mice led to a slightly more severe phenotype after infection with PR8 (unpublished data). An explanation for this might be that tPA and uPA compensate for each other in the urokinase pathway.

Furthermore, other exogenous factors involved in plasminogen activation or fibrinolysis might also influence severity of IAV infections. For example, the streptokinase from _Streptococcus spp._ and staphylokinase from _Staphylococcus aureus_, which are widely used for myocardial infarct treatment (Kunadian and Gibson, 2012) might increase the fibrinolysis and alter the susceptibility in patients after IAV infection. Such conditions may arise during IAV and bacterial co-infections.

In conclusion, our results suggest that infections with influenza A viruses will become more severe if the plasminogen activation pathway is dysregulated. Fibrinolysis in the lung will enhance vascular permeability in the capillaries and facilitate virus dissemination and occurrence of hemorrhages. Taken together, our results show a link between WSN pathogenicity and fibrinolysis activity, and thus provide an extended model to explain lung injury, increased permeability and virus dissemination after IAV infection.
Author contributions
DLS, BH and RS performed the experiments and analyzed the data. DLS and KS perceived the experiments. DLS, RS and KS wrote the manuscript.

Competing financial interest.
The authors declare no conflict of interest.
References


Figure legends and table

Table 1. Virus dissemination to different organs on day 5 p.i. as determined by RT-PCR.

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<td>Kidney</td>
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Number indicates positive/test samples for the M gene using the high resolution melting RT-PCR method. Groups of mice were infected intra-nasally with 2x10^5 FFU PR8M or 2x10^3 FFU WSN. In all Serpine1−/− infected mice, viral RNA was found in lung and kidney.
Figure 1. Serpine1−/− mice show enhanced lethality after infection with influenza A virus

Nine to twelve weeks old female mice (Serpine1−/− and wild type) were infected intra-nasally with 2x10^5 FFU PR8M or 2x10^3 FFU WSN. Body weight loss (A) and survival (B) were monitored over a period of 14 days p.i.. Mice were sacrificed when they reached 70% of the starting body weight and recorded as dead. Data represent mean percent of body weight (+/− SEM) compared to initial body weight on day 0 (100%), n= number of mice per group. Groups of four Serpine1−/− and wild type mice were infected intra-nasally with 2x10^3 FFU PR8 (C) or 2x10^3 FFU WSN (D). Infectious viral particles in lung homogenates were determined by foci forming assay at days 1, 3, 5, and 8 p.i.. Each symbol represents one mouse. Body weight loss in (A) was significantly different between WSN infected Serpine1−/− and wild type group from day 3 to 7 p.i. (Mann Whitney U test, p=0.0186 for dpi 3; p=0.0062 for dpi 4; p=0.0016 for dpi 5,6, and 7). Survival rates were significantly different between wild type and
mutant mice after WSN infection (p=0.0004, Log rank Mantel-Cox test). Please note that in (B), survival curves of infected wild type mice overlap. Viral load on day 5 p.i. in the PR8 infected group was significantly different between Serpine1 mutant and wild type mice (Mann-Whitney test, p=0.0286, indicated with one asterisk in (C)).
Figure 2. *Serpine1*+/+ mice show red blood cell leakage into alveolar space after influenza A virus infection

Representative histological sections from lungs of wild type (A, C) and *Serpine1*−/− mice (B, D) at day 8 p.i.. Mice were infected intra-nasally with 2x10^5 FFU PR8 (A, B) or 2x10^3 FFU WSN (C, D). All wild type and *Serpine1*−/− infected lungs showed scattered polymorphonuclear neutrophil and mononuclear lymphocyte infiltration in the alveoli. However, leakage of red blood cells into the alveolar space (arrow) was only observed in *Serpine1*−/− mutant mice (B, D). Bar corresponds to 100µm.
Figure 3. Influenza infection results in increased vascular permeability

Mice were infected intra-nasally with $2 \times 10^5$ FFU PR8 or $2 \times 10^3$ FFU WSN. BSA-FITC was injected intra-venous one hour prior to sampling of BALF and serum. In vivo permeability assays were performed by calculating fluorescence A.U. (absolute BALF FITC-BSA value / FITC-BSA in diluted serum *100). Bars represent means ± SEM. BSA leakage was significant different between PR8 infected $\textit{Serpine1}^{+/+}$ and wild type mice on day 5 and 7 p.i. (p=0.0095 on day 5 p.i., p=0.0159 on day 7 p.i., non-parametric Mann Whitney test). In addition, wild type mice showed a significant difference after WSN infection compared to PR8 infection on day 7 p.i. (p=0.0286, non-parametric Mann Whitney test). PR8 infected $\textit{Serpine1}^{+/+}$ mice: day 5 p.i. n = 6, day 7 p.i. n = 5; mock control: 3 mice per group; all other groups: n = 4 mice. Asterisks * and ** represent significant differences at p<0.05 or p<0.01, respectively.
Figure 4. Model of alternative plasminogen activator pathway in the absence of Serpine1 or by WSN virus

In the absence of Serpine1 as the global inhibitor of plasminogen activator, plasminogen will be converted into active plasmin and induces fibrinolysis. On the other hand, WSN by itself is able to activate plasminogen, irrespective of the presence or absence of Serpine1. Serpine1−/− mutant mice or WSN virus infection will increase fibrinolysis which then results in increased vascular permeability, enhanced lung injury and viral dissemination. Blue arrows represent activation, red T symbols represent inhibition.
5.3 Manuscript III: Cellular changes in blood indicate severe respiratory disease during influenza infections in mice


Abstract

Influenza A infection is a serious threat to human and animal health. Many of the biological mechanisms of the host-pathogen-interactions are still not well understood and reliable biomarkers indicating the course of the disease are missing. The mouse is a valuable model system enabling us to study the local inflammatory host response and the influence on blood parameters under controlled circumstances. Here, we compared the lung and peripheral changes after PR8 (H1N1) influenza A virus infection in C57BL/6J and DBA/2J mice using virus variants of different pathogenicity resulting in non-lethal and lethal disease. We monitored hematological and immunological parameters revealing that the granulocyte to lymphocyte ratio in the blood represents an early indicator of severe disease progression already two days after influenza A infection in mice. These findings might be relevant to optimize early diagnostic options of severe influenza disease and to monitor successful therapeutic treatment in humans.
5.4 Manuscript IV: Segregation of a spontaneous *Klrd1* (CD94) mutation in DBA/2 mouse substrains


Abstract

Current model DBA/2J (D2J) mice lack CD94 expression due to a deletion spanning the last coding exon of the *Klrd1* gene that occurred in the mid- to late 1980s. In contrast, DBA/2JRj (D2Rj) mice, crosses derived from DBA/2J before 1984, and C57BL/6J (B6) mice lack the deletion and have normal CD94 expression. For example, BXD lines (BXD1-32) generated in the 1970s by crossing B6 and D2J do not segregate for the exonic deletion and have high expression, whereas BXD lines 33 and greater were generated after 1990 are segregating for the deletion and have highly variable *Klrd1* expression. We performed quantitative trait locus analysis of *Klrd1* expression by using BXD lines with different generation times and found that the expression difference in *Klrd1* in the later BXD set is driven by a strong cis-acting expression quantitative trait locus. Although the *Klrd1*/CD94 locus is essential for mousepox resistance, the genetic variation among D2 substrains and the later set of BXD strains is not associated with susceptibility to the Influenza A virus PR8 strain. Substrains with nearly identical genetic backgrounds that are segregating functional variants such as the *Klrd1* deletion are useful genetic tools to investigate biological function.
6. Contribution to Manuscripts

Manuscript I

I performed the majority of the experimental studies. I infected congenic B6-\textit{Mx}^{1/r} and D2-\textit{Mx}^{1/r} mice with PR8F, PR8M and H3N2 virus and monitored body weight loss and survival over the infection period. I also performed the analysis of histopathological changes and performed foci assay for virus titration. Furthermore, I analyzed the data and contributed to writing of the manuscript.

Manuscript II

I contributed in the experimental design of the studies. I performed most of the experiments described in this manuscript. I infected the wild type and knock-out mice and evaluated body weight loss, determined virus titer and performed the \textit{in vivo} permeability assay. I analyzed the histopathological change, generated the figures and contributed to writing of the manuscript.

Manuscript III

For this manuscript, I participated in the histopathological analysis. I performed the infection and analysis the histopathological changes after IAV infections. In addition, I contributed to writing of the manuscript.

Manuscript IV

Here, I was involved in the genotyping analysis. I performed the PCR studies, primer design, and analysis the sequencing results. I analyzed the data and contributed to writing of the manuscript.
7. Discussion

Our previous studies showed that different inbred mouse strains had diverse susceptibilities to IAV infections in the absence of functional $Mx1$ allele (Blazejewska et al., 2011; Pommerenke et al., 2012; Srivastava et al., 2009). Other studies have shown that $Mx1$ gene is a dominant resistance factor controlling viral replication and pathogenicity of IAV (Cilloniz et al., 2012; Grimm et al., 2007; Hodgson et al., 2011; Moritoh et al., 2009; Tumpey et al., 2007). These studies suggest that the $Mx1$ allele from A2G mice is protective and independent of genetic background. In humans, genetic variations have been proposed influence the host response to infections. Therefore, I wanted to study the influence of the host genetic to the resistance function of $Mx1$ after IAV infection using a highly susceptible and a resistant mouse strain. This knowledge could provide insights for further genetic predisposition studies and validated the interactions between $Mx1$ alleles and genetic backgrounds.

In the absence of functional $Mx1$ allele, B6 mice are more resistant and survived infection with IAV when infected with the low virulent PR8 Muenster variant. In contrast, D2 mice were highly susceptible and succumbed even at low infection doses of IAV (Blazejewska et al., 2011; Srivastava et al., 2009). In my thesis work, I found that congenic D2-$Mx1^{fr}$ mice exhibited a highly susceptible phenotype after PR8F infection even when they carry a functional $Mx1$ wild type allele. The offspring from an out-cross ((B6 x D2(B6).A2G-$Mx1^{fr}$)F1, which contain both $Mx1$ heterozygote allele and mix genetic background, had different susceptibility compare to the pure C57BL/6J background or presence of $Mx1$ in a homozygous state. As more D2 background was included in the genetic background, susceptibility increased. The systematically different background combinations and $Mx1$ alleles well determined how susceptibility was influenced by these factors. Our results demonstrated that the wild type A2G $Mx1$ allele did not exert a highly protective function in a pure DBA/2J genetic background. Additionally, an increase from one to two copies of functional $Mx1$ allele increased survival rates suggesting an additive function of the $Mx1$ gene in a C57BL/6J genetic background. Furthermore, in our previous studies we hypothesized that the susceptible phenotype DBA/2J after IAV infection were due to the extremely high viral load in the infected lungs. The LD50 in B6 mice was $10^4$ folds
higher after PR8M infection compared to D2 mice (Blazejewska et al., 2011; Srivastava et al., 2009). On the other hand, in the presence of functional \textit{Mx1} allele, we found out that a DBA/2J background in pure D2 or in hybrid B6xD2 mice results in high viral loads in the lung at day 1 p.i. compared to pure C57BL/6J background. However, a C57BL/6J or hybrid B6xD2 background combined with the presence of the \textit{Mx1} wild type allele could reduce viral replication at day 1 p.i. and showed lower viral load on day 3 p.i. Thus, at least one C57BL/6J genome had to be present to initiate the protective functions of MX1.

To get a deeper insight into why D2-\textit{Mx1}^{r/r} did not provide full antiviral activity of the wild type \textit{Mx1} allele, we investigated if prior activation of the interferon pathway may influence the response in D2-\textit{Mx1}^{r/r} mice. It had been shown previously that B6-\textit{Mx1}^{r/r} mice were highly resistant after exogenous interferon (IFN-I) pre-treatment to the very high virulent H1N1 virus (hvH1N1) (Grimm et al., 2007) or to the highly pathogenic H5N1 virus (Ferris et al., 2013). Our results showed that IFN pre-treatment protected D2-\textit{Mx1}^{r/r} mice from lethal infection. The wild type \textit{Mx1} allele was able to initiate the protective function and prevent death in DBA/2J mice after lethal infection. In addition, no virus antigen could be detected in the lungs on day 3 p.i. in the immunohistopathological studies in IFN-I pre-treated D2-\textit{Mx1}^{r/r} mice. Virus particles could only be detected in the bronchial epithelial cells of IAV infected B6-\textit{Mx1}^{r/r} mice. These results suggest that during the early stage of IAV infection in non IFN-I treated D2-\textit{Mx1}^{r/r} mice, viral replication was very fast and \textit{Mx1} was expressed too late to restrict the massive viral replication which then leads to severe tissue damage and subsequent death.

Moreover, I infected D2-\textit{Mx1}^{r/r} mice with other IAV subtype and variants. Surprisingly, when I infected D2-\textit{Mx1}^{r/r} mice with the low virulent mouse-adapted H1N1 (PR8M) virus, D2-\textit{Mx1}^{r/r} mice exhibited a high resistance and survived the infection with no dramatic body weight loss. PR8M was the virus we used to compare phenotypes of mice carrying non-functional \textit{Mx1} alleles. The best explanation for this outcome is that PR8M has a unique alternative way to induce IFN-I in infected cells. In PR8M infected mice, \textit{Ifnb1} expression was already detectable at day 1 p.i. (Liedmann et al.,
Thus, an early induction of IFN and *Mx1* protected D2-*Mx1r/r* mice from PR8M infection.

In the second main part of my thesis work, I established a mouse model to investigate the role of SERPINE1 during influenza A infection and the potential route for IAV dissemination. Recent studies showed that plasminogen associated fibrinolysis played a crucial role in lung injury after IAV infection. Plasminogen knock-out mice showed a strong resistance to PR8 infection, with less clot breakdown and recruitment of immune cell in the alveolar space. (Khouchache et al., 2013; LeBouder et al., 2008). For a better understanding of the relationship between fibrinolysis and IAV infection, I investigated a knock-out mouse model in which the *Serpine1* gene was deleted. In this study I used two H1N1 variants for phenotyping: the PR8 Muenster variant (PR8M) H1N1 virus and A/WSN/33 (WSN) H1N1 virus. My results showed that *Serpine1*−/− mice were more susceptible to infections with both IAVs. *Serpine1*−/− mutant mice exhibited 100% mortality after WSN infection and 45.5% mortality after PR8M infection whereas infections with the same dose did not cause mortality in wild type mice. Additionally, *Serpine1*−/− mutant mice exhibited higher viral loads in their lungs compared to wild type mice at late time points after PR8M infection. Interestingly, we observed a mild hemorrhage in the lung of all infected *Serpine1*−/− knock-out mice on day 8 p.i. in tissue sections. Red blood cells leaked into the alveolar space and this was accompanied with presence of a mild hyaline alveolar membrane. Such lesions are considered as dysregulation of the fibrinolytic response and lung injury associated with endothelial-epithelial barrier leakage (Idell et al., 1989). I evaluated the level of protein leakage in the lung of *Serpine1*−/− and wild type mice by *in vivo* permeability assays. My results showed that *Serpine1*−/− mice exhibited a higher vascular permeability after PR8M infection compared to wild type mice. Surprisingly, both wild type and mutant mice showed increased vascular permeability in lungs after WSN infection. In WSN infected wild type mice, neither RBC leakage in the alveolar space nor high virus titer could be detected. Next, I investigated the link between the increased vascular permeability and plasminogen conversion ability of WSN virus.

Previous studies suggested that WSN virus enhanced viral replication via plasminogen-NA binding and therefore resulted in higher virus titer in the lung.
(Aeffner et al., 2014; Garcia et al., 2010; Wang et al., 2010). However, my data showed that both Serpine1\textsuperscript{−/−} and wild type mice exhibited the same viral loads after WSN infection. These results indicated therefore suggest that other factors are influencing vascular permeability. However, the neuraminidase of WSN virus can convert plasminogen which facilitates HA cleavage (Garcia-Sastre et al., 1998a; Goto and Kawaoka, 1998; Goto et al., 2001; Sun et al., 2010), the downstream effects after plasminogen conversion are still unclear. We hypothesize that WSN virus provides an alternative pathway for plasminogen conversion and results in enhanced fibrinolysis.

In wild type mice, SERPINE1 inhibits fibrinolysis and thus the absence of Serpine1 gene would result in more lung injury and higher vascular permeability in alveolar membranes. On the other hand, WSN virus alternatively triggers activation of plasmin which results in fibrinolysis of infected wild type mice. This process would not occur in PR8M infected mice. Virus mediated vascular permeable was strongly related to lung injury and increases the chance for viremia (Aeffner et al., 2014; Tate et al., 2010; Wang et al., 2010). My results suggest that in wild type mice, PR8M was restricted to the lung without disseminated to other organs. After WSN infection in wild type mice, only limited dissemination into the kidney, liver and brain was observed. In contrast, viral RNA could be detected consistently in kidney, liver and spleen of Serpine1\textsuperscript{−/−} mutant mice after PR8M or WSN infection. All IAV infected Serpine1\textsuperscript{−/−} mutant mice revealed viral RNA in the kidney. Another study reported that SERPINE1 could reduce fibrosis in aggressive renal disease and may represent an important marker for chronic kidney disease (Eddy, 2002). Our findings suggest that Serpine1 may play an important role in nephrotropism and renal dissemination of IAV.

In addition to the influence of Mx1 alleles, I investigated the relationship between different genetic backgrounds in the absence of functional Mx1 (Dengler et al., 2014). For this part, I mainly worked on the comparison of pathological differences between lethal and non-lethal infection models in mice. Our results showed that C57BL/6J mice infected with lethal (PR8F) or non-lethal (PR8M) IAV variants could exhibit body weight loss, viral load, and histopathology changes to different degrees. The histological analyses revealed a massive infiltration of myeloid cell on day 2 p.i. in
PR8F infected group compares to PR8M infection. Diffuse damage to lung epithelial cells could be observed in PR8F infected lungs. A lower amount of myeloid cells on day 2 and 4 p.i. was seen in PR8M infected mouse lungs whereas the most abundant lymphocytic immune cells infiltrated on day 8 to 10 p.i. These findings underlined that during the lethal infection model (PR8F) of B6 mice, lung damage was much pronounced in the later stage accumulation of myeloid cells in the lungs. We further identified that Ly6G+CD11b+ cells in the lung were strongly associated to the lethal PR8F infections by flow cytometry analysis. Combined with our previous findings, we demonstrated that the severity of IAV infection was relied on the granulocytes in influenza pathology progress (Blazejewska et al., 2011; Pommerenke et al., 2012; Srivastava et al., 2009). On the other hand, flow cytometry analysis results showed that during PR8M infection in D2 mice, increasing proinflammatory cells in the lungs could be observed in lethal infections. These findings were published in PLoS ONE: Dengler et al., 2014 “Cellular changes in blood indicate severe respiratory disease during influenza infections in mice”.

During an infection, natural killer (NK) cells infiltrate the lungs. I therefore contributed to a study in which presence or absence of a natural killer cell receptor was investigated. It had been shown in previous studies, that the NK cell receptor NKp46 can directly bind to viral HA protein. This interaction was associated with early viral clearance via killing infected cells (Mandelboim et al., 2001). CD94 (Cluster of Differentiation 94) protein encoded by the killer cell lectin-like receptor subfamily D, member 1 (Klrd1) gene was one of the most important regulators for NK cell activation and inhibition. It was reported previously that DBA/2J mice carry a deletion in exon 5 and the 3’ end of Klrd1 gene (Vance et al., 2002). We found that in contrast to inbred DBA2/J (D2J) from the Jackson Laboratory, the DBA/2Rj (D2Rj) stock from Janvier Breeding Centre in France did express CD94 on NK cells. These differences of Klrd1 gene expression represent a valuable resource for further studies on NK cell-mediated resistance. These finding have been published in G3 (Bethesda) Shin et al., 2014: “Segregation of a spontaneous Klrd1 (CD94) mutation in DBA/2 mouse substrains”.
8. Conclusion & Outlook

In summary, my findings suggest that in the absence of functional \textit{Mx1} wild type alleles, IAV infection of C57BL/6J and DBA/2J mice result in a lethal infection which accompanied with huge amounts of myeloid cells infiltrating into the lung at early time points after infection. In a DBA/2J background, virus can replicate very rapidly compared to mice with a C57BL/6J background. On the other hand, if mice carry a functional \textit{Mx1} allele, the antiviral activity of \textit{Mx1} strongly depends on genetic factors, virulence factors of IAV and interferon induction. One on hand, a C57BL/6J background is essential for providing the genetic factors to activate \textit{Mx1} antiviral function and initiate early viral clearance. On the other hand, DBA/2J background combined with \textit{Mx1} wild type allele requires the early induction \textit{Mx1} expression by IFN-I.

My thesis work with \textit{Serpine1}^{-/-} mutant mice showed that lung injury was strongly related to the regulation of fibrinolysis after IAV infection. Deletion of the \textit{Serpine1} gene or activation of plasminogen by WSN may result in hyperfibrinolysis. The dysregulation of fibrinolysis results in an enhanced vascular permeability and increases the chance for viral dissemination.

Taken together, my thesis work could provide more highly valuable insights in host-pathogen interaction that are important for a lethal or non-lethal outcome after IAV infection.

To better understand the detailed mechanisms of \textit{Mx1} in different genetic backgrounds, more experiments will be required to determine the influence of other genes. Further transcriptome analysis between B6-\textit{Mx1}^{+/r} and D2-\textit{Mx1}^{+/r} would be highly informative. Additionally, QTL mapping analysis could be performed with outcrossing the congenic D2-\textit{Mx1}^{+/r} mice to BXD recombinant inbred mouse strains. A pair-wise outcross performed with B6-\textit{Mx1}^{+/r} and the selected BXDs could identify candidate genes which are associated with \textit{Mx1} antiviral activity. The susceptibility and resistance to influenza A virus in humans are most likely not caused by presence or absence of \textit{MX1} but attributed to genetic factors (Horby et al., 2012; Horby et al.,
Thus, relating our mouse to human studies might help to understand resistance and susceptibility to IAV infections in humans.

Furthermore, in the second major part of my thesis, I demonstrated the relationship between the dysregulation of fibrinolysis and lung injury by IAV infection. The dysfunction of plasminogen activator inhibitor 1 (Serpine1) resulted in lung damage and enhance vascular permeability. Here, it would be of great interest to look for alternative activators of plasminogen conversion that may enhance severity of disease and resulted in viral dissemination. The role of endogenous or exogenous plasminogen activators should be reconsidered in this context. Other activators involved in plasminogen activation or fibrinolysis might also influence severity of IAV infections. For example, the streptokinase from Streptococcus spp. or staphylokinase from Staphylococcus aureus might increase the fibrinolysis and alter the susceptibility in patients after IAV infection. Thus, the mechanisms of lung injury and bacterial co-infection should be deep investigated.
9. References


with a broad, but limited, range of influenza A and B viruses. Journal of virology 85(23), 12825-12829.


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