HIGHLY PATHOGENIC AVIAN INFLUENZA A VIRUS: PATHOGENESIS, VACCINE AND ANTIVIRAL

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ZUSAMMENFASSUNG

Infektionen des Menschen mit hochpathogenen aviären Influenzaviren (HPAIV) weisen im Vergleich zu den saisonalen Influenza-Fällen einen besonders schweren Krankheitsverlauf auf. Die Sterberate humaner H5N1-Infektionen liegt derzeit bei etwa 60%. Im August 2010 sind nachweislich 504 Personen an vom H5N1-Virus verursachter aviären Influenza erkrankt und 299 daran gestorben. Der Krankheitsverlauf einer HPAIV-Infektion ist durch eine Reduktion der Lymphozyten (Lymphopenie), einer Hyperinduktion von Zytokinen und Chemokinen (Hyperzytokinämie), sowie ein plötzliches akutes Lungen- und Multiorganversagen gekennzeichnet. Die immunologischen und viralen Faktoren, die zu einem solch kritischen Verlauf der H5N1-Infektion führen, sind bislang nicht ausreichend bekannt. Ein weiteres Problem für die Behandlung der HPAIV-Infektion stellt die zunehmende Resistenz der humanpathogenen H5N1-Isolate gegenüber den gängigen antiviralen Medikamenten dar. Auch wenn eine Adaptation des Virus, die eine Weiterverbreitung von Mensch zu Mensch in großen Dimensionen ermöglichen würde, bisher nicht erfolgt ist, lässt sich diese für die Zukunft nicht ausschließen. Die begrenzten Möglichkeiten zur Bekämpfung von HPAIV-Infektionen verdeutlichen den dringenden Bedarf an neuen, effektiven Maßnahmen gegen diese Krankheit. Für die Entwicklung neuer antiviraler Medikamente und Impfungen gegen das Influenza A Virus müssen die Vorgänge, die den kritischen Verlauf der HPAIV-Infektion beeinflussen besser verstanden werden.

Das Ziel dieser Dissertation war es, einen besseren Einblick in genau diese Vorgänge zu bekommen und nach alternativen Möglichkeiten zur Behandlung der HPAIV-Infektion zu suchen. Im Rahmen der hier zusammengefassten wissenschaftlichen Publikationen wurde die Rolle des NF-κB-Signalweges bei der Bekämpfung viraler Infektionen untersucht. Es folgte eine Studie zur Beteiligung der Hyperzytokinämie an der H5N1-vermittelten Pathogenese. Des Weiteren konnte ein möglicher Mechanismus der die virusvermittelte Lymphopenie verursacht aufgezeigt werden. Weiterhin erfolgte die Charakterisierung immunologischer Mechanismen, die maßgeblich am Schutz gegen HPAIV-Infektionen nach Vakzinierung beteiligt sind. Schließlich, konnte die antivirale Wirkung von Polyphenolen als Alternative zu den gängigen antiviralen Medikamenten *in vitro*, sowie im Mausmodell gezeigt werden.

HIGHLY PATHOGENIC AVIAN INFLUENZA A VIRUS: PATHOGENESIS, VACCINE AND ANTIVIRAL

K. Droebner

Influenza virus leads to acute respiratory infection in humans with severity ranging from morbidity to mortality. Seasonal epidemic outbreaks of human influenza viruses occur annually during autumn and winter and cause estimated 250.000 – 500.000 deaths worldwide each year (World Health Organisation; WHO 2009a). In addition to the epidemic form of the disease, pandemic outbreaks occur regularly. Beside the current swine origin influenza virus (SOIV) H1N1v outbreak, three of these pandemics occurred in the last century, in 1918, 1957, and 1968 resulting in millions of deaths within the human population (Oxford 2000; Palese 2004; Hsieh *et al.* 2006).

Influenza A viruses are enveloped viruses with a single-stranded RNA genome of negative polarity that is divided into eight RNA segments. Influenza virions have two major surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). They are distinguished in several subtypes according to those surface proteins. Until today, 16 different HA and 9 different NA proteins are known. All combinations of HA and NA can be found in wild waterfowl, but only combinations of H1, H2, H3, and N1 and N2 circulate within the human population (Whittaker 2001; Julkunen *et al.* 2001). Some strains of avian influenza viruses lead to lethal infection in birds. Such outbreaks of highly pathogenic avian influenza are caused by H5 and H7 subtypes.

Although, avian viruses were previously thought to be incapable of infecting humans, in 1997 a direct avian to human transmission occurred. Highly pathogenic avian influenza viruses (HPAIV) of the subtype H5N1 were transmitted from domestic poultry to humans in Hong Kong (de Jong *et al.* 1997; Claas *et al.* 1998). Infections of humans with the HPAIV are much more fatal, in contrast to seasonal influenza infections and the current pandemic outbreak. The mortality rate of human HPAIV H5N1 infections is around 60%. In August 2010, 504 confirmed cases of avian H5N1 infections in humans have been reported, while 299 of them were lethal (WHO 2010a). HPAIV-mediated disease is characterized by lymphopenia, cytokine dysregulation, acute respiratory distress syndrome and multiorgan failure (Beigel *et al.* 2005).

Virus-host interactions are not only crucial for the defence against influenza virus infections, moreover these sometimes can negatively impact H5N1 mediated disease severity. However, both viral and immunological factors leading to H5N1 mediated severe influenza are only poorly understood. In addition, many of the human H5N1 virus isolates are already resistant

to the two main classes of anti-influenza drugs, namely inhibitors of the viral M2 ion channel protein or viral neuraminidase inhibitors (Hayden & Hay 1992; de Jong *et al.* 2005; McKimm-Breschkin *et al.* 2007). Therefore, options for control and treatment of H5N1 infections are limited, demonstrating the urgent need for new effective countermeasures against this important disease. Fortunately, the spread of the virus is limited by a rare human-to-human transmission. Although, HPAIV H5N1 has not evolved to a form that allows to spread easily between humans, it is still considered by WHO (WHO 2010b) as a potential pandemic strain. Moreover, the possibility of a reassortment between the pandemic SOIV H1N1v and H5N1 influenza virus strain is indeed a frightening but feasible association. A mixed strain capable of efficient human-to-human transmission may cause a serious pandemic with fatal mortality rates. A basic requirement for the development of new antiviral agents and vaccines against the influenza A virus is the understanding of how and why influenza viruses cause disease in humans and what influences disease severity.

The aim of this Ph.D thesis was to provide a better understanding of the immunological mechanisms that influence the critical outcome of HPAIV infection. The second goal of this work was to identify a new promising anti-influenza agent which can be used against infections with HPAIV. In the range of the scientific publications presented here I investigated the role of the NF-κB signalling pathway in viral infections. I determined the impact of two major hallmarks of HPAIV infection, hypercytokinemia and lymphopenia on the critical H5N1-mediated disease outcome. Furthermore, I characterized immune mechanisms that provide cross-protection against lethal influenza A H5N1 virus infection. Finally, as an alternative approach to the common anti-viral drugs I studied the antiviral activity of polyphenols against influenza virus.

Studies on the pathogenesis

Infections with H5N1 differ from the common influenza virus infections. While seasonal influenza epidemics affect the very young, the elderly and persons with an impaired immune system, infections with most avian influenza subtypes cause either mild infections or no disease at all, in humans (Beare & Webster 1991; Koopmans *et al.* 2004; Butt *et al.* 2005). In contrast, the HPAIV H5N1 is much more virulent than other avian influenza A viruses and targets mostly young and middle-aged people. Human infections with H5N1 result in severe disease leading to death. The role of virological and host-specific factors responsible for the establishment of such critical disease outcomes and for the altered host specificity still needs to be determined. The pathogenesis of fatal HPAIV infection involves a number of viral

factors that have been suggested to be involved in increased virulence of H5N1 viruses (Hatta *et al.* 2001; Salomon *et al.* 2006; Mansfield 2007; Neumann *et al.* 2007; Kortweg & Gu 2008; Gabriel *et al.* 2005 and 2009). The dysfunction of the immune system including a dysregulation of cytokines and chemokines, an up-regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and reduced cytotoxicity of $CD8^+$ T-lymphocytes after avian influenza virus infections may also be some of the key mechanisms in the H5N1 mediated pathogenesis (Hsieh & Chang 2006; Zhou *et al.* 2006).

In my Ph.D thesis I focused on two hallmarks of the H5N1 influenza virus infection that may be involved in the severe H5N1 mediated disease outcome. One difference between seasonal influenza virus infections and H5N1 cases are elevated levels of chemokines and cytokines that are present in the lungs of H5N1-infected humans and animals. This overreaction of the host immune system is called hypercytokinemia or cytokine storm and is a hallmark of H5N1 infection. Because of the fact that cytokine storm has the potential to do significant damage to body tissues and organs, it is assumed that increased levels of those immune modulators contribute to the serve pathology after H5N1 infection (Peiris *et al.* 2004; de Jong *et al.* 2006; Lee *et al.* 2007). In this case, therapeutic strategies targeting the local cytokine response may be adequate for the improvement of the severe H5N1 mediated disease outcome in human infections. However, the role of this hypercytokinemia in the pathogenesis of H5N1 remains controversial. Recent studies in H5N1 infected knockout mice, lacking a single cytokine response have given contradictory results. The absence of one cytokine in particular, interleukin 6 (IL-6) or tumour necrosis factor-α (TNF-α), did not reduce mortality or disease severity after H5N1 infection (Salomon *et al.* 2007; Szretter *et al.* 2007). Nevertheless, it should not be disregarded that hypercytokinemia is a multicytokine event (Hayden *et al.* 1998; Sladkova & Kostolansky 2006). Therefore, the effect of deleting only one cytokine might not be sufficient to influence the hypercytokinemia after H5N1 infection. An advantage for such studies would be the interference with the majority of cytokines and chemokines involved in the cytokine storm during H5N1 infection. In fact, the manipulation of the nuclear factor kappa B (NF-κB) pathway provides such an opportunity. In particular, the classical NF-κB pathway which operates through p50 and p65 subunits is involved in the regulation of gene expression of many immune modulators (Pahl 1999; Beinke & Ley 2004). Lethal H5N1 infection of nuclear factor kappa B (NF-κB) p50 deficient mice may represent a useful model to study the influence of hypercytokinemia in the pathogenesis after H5N1 infection. However, the importance of NF-κB in the defence of viral infections is still not completely understood. A manipulation of the NF-κB signalling pathway may not only reduce the

cytokine storm, but also have opposing effects on the host's immune response. Therefore, as a part of my Ph.D thesis I wanted to investigated the role of the NF-κB signalling pathway in viral infections. Since the immune response to influenza A viruses is multifaceted and highly complex, I decided to use the Lymphocytic Choriomeningitis virus (LCMV) model in a pilot study rather than the model of influenza virus infection of mice. The LCMV infection of mice represents an excellent model for studying antiviral immune responses, because the immunological mechanisms that are required for the defence against the noncytopathic LCMV infection of mice are well characterized (Zinkernagel 2002; Oldstone 2002). The role of NF-κB in viral infection was determined after LCMV infection of mice deficient in NF-κB subunits from either the classical ($p50^{-/-}$ mice) or the alternative NF-κB pathway ($p52^{-/-}$ mice) (Publication #1: Droebner *et al.* 2010). LCMV infection of NF-κB $p52^{-/-}$ mice by three different routes resulted in the disability of those mice to control the infection, while NF-κB $p50^{-/-}$ mice efficiently eliminated the virus (Publication #1: Fig. 1). After LCMV infection a robust $CD8^+$ T-cell response is responsible for the elimination of infected cells (Kagi *et al.* 1996). This control mechanism was significantly reduced in NF-κB $p52^{-/-}$ mice. I was able to demonstrate that the dysfunction of the cellular immune response after LCMV infection in NF-κB $p52^{-/-}$ mice was not due to a direct failure of the $CD8^+$ T-lymphocytes. Since NF-κB $p52^{-/-}$ lymphocytes were responsive after transfer to wild type recipients an impaired priming might be responsible for the inadequate cellular immune response in NF-κB $p52^{-/-}$ mice (Publication #1; Fig. 7). Mice lacking NF-κB family members have severe alterations in the microarchitecture of secondary lymphoid organs, where, the priming of naive B- and T-cells occurs (Karrer *et al*. 1997; Bonizzi & Karin 2004). The T-cell chemoattractant CCL21 is associated with the modulation of immune responses by positioning of T-cells and dendritic cells (DCs) within T-cell zones of secondary lymphoid organs (Marsland *et al.* 2005; Veerman *et al.* 2007). In fact, in this project I have demonstrated the absence of CCL21 in the spleens of NF-κB $p52^{-/-}$ mice (Publication #1; Fig. 8).The lack of the NF-κB p52 subunit had negative effects on the maturation and priming processes of the CTL response against LCMV. This work clearly demonstrates the importance of the alternative, but not the classical NF-κB pathway in the defence against LCMV infection. The NF-κB p52 subunit has a potential role in the maturation and priming processes of the CTL response against LCMV. The understanding of the effects caused by modulation and manipulation of multiple adaptive immune responses through NF-κB impairment may provide new opportunities for the development of antiviral strategies, prevention of autoimmune diseases and also might be beneficial for tumour vaccine strategies.

Moreover, the knowledge that the depletion of the subunit from the classical NF-κB signalling pathway had no impact on an appropriate cellular immune response against the virus enabled me to use NF-κB p50 deficient mice for defining the role of the hypercytokinemia in the H5N1 mediated pathogenesis (Publication #2: Droebner *et al.* 2008a). After H5N1 infection of wild type mice I was able to demonstrate an up regulation of many cytokines and chemokines in the lungs of those mice. In contrast, NF-κB p50 deficient mice revealed a strong reduction of most of these immune modulators after H5N1 infection, indicating a lack of hypercytokinemia (Publication #2: Fig. 1). Interestingly, the absence of the cytokine storm had no influence on the pathogenesis after H5N1 infection in these animals. The mouse lethal dose 50% (MLD_{50}) titers were similar in wild type and p50 deficient mice. No differences in the onset of disease, changes in body weight and survival rates were found (Publication #2; Fig. 2). The viral tropism as well as virus loads in NF-κB p50 mice was comparable with those of wild type mice (Publication #2; Table 1). These results indicate that hypercytokinemia does not contribute to the fatal H5N1 disease outcome which is in line with studies, where glucocorticoid treatment neither improved the outcome of infection in mice nor altered the course of fatal disease in humans (Arabi *et al.* 2007; Carter 2007; Salomon *et al*. 2007).

In studies with H5N1 infected mice a strong reduction of peripheral blood lymphocytes can be observed. I have seen this phenomenon also during the previous study (Publication #2; Fig. 3). This alteration in lymphocytes is called lymphopenia and is the second hallmark of the H5N1 infection (Gao *et al.* 1999; Lu *et al.* 1999; Tran *et al*. 2004; Maines *et al.* 2005). The depletion of lymphocytes in the presence of influenza virus is not only restricted to the blood, but was also detected in primary and secondary lymphoid organs (own studies and observations; Tumpey *et al*. 2000). Lymphopenia is associated with the highly pathogenic nature of the H5N1 viruses, particularly because infections with H5N1 influenza viruses often lead to an impairment of the adaptive immune response. It has been hypothesized that this impairment may result from viral infection of immune cells or virus-mediated reduction of effector function of $CD8^+$ T-cells by an insufficient perforin expression (Hsieh & Chang 2006; Kortweg & Gu 2008). During our dissections of H5N1 infected mice a size reduction of the thymus has been noticed (Publication #3: Vogel *et al.* 2010). This atrophy of the primary lymphoid organ was also observed after infection with other HPAIV, but was absent when mice were infected with LPAIV and the pandemic H1N1v strain (Publication #3; Fig. 1A-F). The massive destruction of the thymus could be the result of an interaction of HPAIV with this organ and could be the basic cause for the lymphocyte depletion observed during H5N1

infections of mice. In fact, the HPAIV infection caused not only a size reduction, but also led to functional alterations of this primary lymphoid organ. We found reduced numbers of leucocytes in the thymus. The expression of chemokines and cell adhesion molecules involved in T-cell development was also altered after HPAIV infection. Furthermore, functional influenza virus specific cytotoxic $CD8^+$ T-cells and infectious virus were present within this organ (Publication #3; Fig. 1-5). This virus-induced thymic damage leading to a reduced outflow of naive T-lymphocytes could be an explanation for H5N1-induced lymphopenia, most notably because all the described features were only found after HPAIV infections, but not after infections with LPAIV. Nevertheless, it was still not clear, how the virus reached the thymus. It seemed implausible that the virus can pass the barrier between periphery and thymus by itself without causing a systemic infection. It was more likely that HPAIV spread into the thymus through the infection of migrating cells. A recent study revealed that circulating DCs in addition to mature peripheral T-lymphocytes can migrate into the thymus after the uptake of antigenic material in the periphery (Bonasio *et al.* 2006). Furthermore, it was demonstrated that DCs, murine T-cells and macrophages are a target for influenza virus infection (Hao *et al.* 2008; Gabriel *et al.* 2009). Using flow cytometry analysis we were able to demonstrate that in our study HPAIV mainly infected the mucosal DC population (Publication #3; Fig. 6). Moreover, DCs isolated from HPAIV infected C57BL/6-GFP^+ mice and transferred into the lungs of GFP^- mice were detectable in lung and most interesting in the thymus of recipient wild type mice (Publication #3; Fig. 7A). This analysis provided evidence that respiratory DCs served as carrier cells after infection with HPAIV (Publication #3; Fig. 7B). DCs that continuously migrate in to the thymus (Li *et al.* 2009) enable the virus to spread into secondary and primary lymphoid organs. The interference of the virus with the thymus can lead to a dysregulation of T-lymphocytes development. The presentation of viral antigens as pseudo self peptides by infected DCs in the thymus may cause an additional clonal deletion of influenza specific T-lymphocytes. This feature might be an explanation for the reduced or even missing adaptive immune responses to HPAIV especially in the younger population when the thymus is still highly active. This mechanism might not only be found in influenza virus infection but also might be the reason of the increased immune evasion of some new emerging pathogens.

Vaccination study

The best way to protect humans against H5N1 influenza virus infection is the vaccination. Traditional influenza vaccines focus on the humoral immune response to the surface

glycoproteins HA and NA of the vaccination virus and are only protective against this particular virus strain (Clements *et al.* 1986; Gerhard 2001). Because H5N1 influenza viruses contain antigenic variations of those surface proteins the common vaccines are not effective against them. The development of a broadly protective vaccine against seasonal, avian and in addition potentially pandemic strains would be the ultimate goal. One idea is to develop a vaccine that instead of inducing antibodies stimulates cross-reactive $CD8^+$ T-cell responses (LaGruta *et al*. 2009; Brown & Kelso 2009; Mueller *et al.* 2010). However, when HPAIV negatively affects the adaptive immune response (Vogel *et al.* 2010), vaccine approaches that activate the production of cytotoxic T-lymphocytes may not be adequate for the protection against H5N1 influenza virus. Therefore, further characterization of the immunological mechanisms involved in protection against HPAIV is still needed to demonstrate the adequate immunogenicity of such vaccine approaches. In refining our understanding of the immunological mechanisms involved in cross-protection against H5N1 infection I carried out a vaccination study with immunodeficient mice (Publication # 4: Droebner et al. 2008b). For this study a H5N2 LPAIV was used as a vaccine strain. Wild type as well as antibody deficient (μMT), $CD4^+$ and $CD8^+$ T-cell deficient mice showed no clinical symptoms or weight loss after the H5N2 infection, while infection with HPAIV H5N1 lead to severe disease resulting in death of wild type and immunodeficient mice (Publication # 4; Fig. 2 + Table 1-3). Due to the fact that the H5N2 infection caused no symptoms and the virus efficiently replicated in lungs of infected mice, I decided to use this LPAIV as a live-attenuated vaccine strain. In my study, H5N2 infected mice, 80 days post-infection (p.i.) were challenged with hundred-fold MLD_{50} of the H5N1 isolate. The immunization with H5N2 protected wild type mice against the lethal H5N1 infection. However, no protection was observed in antibody-deficient mice ($\mu MT^{-/-}$). All $\mu MT^{-/-}$ mice succumbed to the H5N1 infection, independently of their vaccination status. When $CD8^+$ T-cell deficient mice were used for challenge infection vaccinated mice showed a mild reduction of their body weight. Nevertheless, all vaccinated $CD8^+$ T-cell deficient mice survived the lethal H5N1 challenge infection. Vaccination of $CD4^+$ T-cell deficient mice resulted in a partially protection, only 50% of the immunized mice survived the lethal challenge infection (Publication # 4; Fig. 3-4). Challenged wild type, $CD8^+$ and $CD4^+$ T-cell deficient mice had comparable hemagglutinin antibody titers in their sera. These antibodies were reactive against both isolates, revealing the induction of cross-protective antibodies after vaccination with H5N2 (Publication # 4; Fig. 3 + 5). Taken together, in this work I was able to demonstrate that $CD8^+$ T-cells can not promote protection against H5N1 influenza virus infection. Even though, T-lymphocytes are

essential for viral clearance during acute influenza infections (Doherty *et al.* 2006), they play probably only a minor if any role in cross-protective primary immunity to HPAIV after vaccination. Furthermore, this study underlines the mandatory role of an effective antibody-mediated immune response in protection against H5N1 influenza virus infection and is in line with other studies indicating the importance of the humoral immunity for influenza vaccination (Cox *et al.* 2004; Lee *et al.* 2005; Song *et al*. 2010). Our results contribute to the characterization of immune responses that correlate with vaccine protection and may help improve the development of new vaccines against HPAIV.

Antiviral properties of polyphenol against influenza virus

The growing number of influenza virus strains that are resistant to the most commonly used drugs underscores an urgent need for the development of new effective antiviral drugs. One idea for future therapeutic strategies also recommended by the WHO (WHO 2009b) is the investigation of the antiviral activity of natural products. Proanthocyanidins are important natural products. They belong to the group of polyphenols, a large family of natural compounds widely distributed in the plant kingdom and characterized by the presence of more than one phenol group per molecule (Harborne 1980). Proanthocyanidins exhibit a wide range of biological effects, including antiviral and antibacterial activity (Shahat *et al.* 2002; Cos *et al.* 2004; Taguri *et al.* 2006). CYSTUS052 is a plant extract that is very rich in highly polymeric polyphenols, in particular flavan-3-ols and proanthocyanidins (Petereit *et al.*1991; Danne *et al.*1993). The extract is a preparation of a selected variety of the biochemical polymorphic species *Cistus incanus*. In traditional folk medicine *Cistus* species are used for treatment of various skin diseases and against infectious agents without having any apparent side effects (Petereit *et al.* 1991; Danne *et al.*1993).

Given that many reports describe the anti-influenza virus activity of proanthocyanidins (Nakayama *et al.* 1993; Serkedjieva & Hay 1998; Palamara *et al.* 2005; Song *et al.* 2005; Jariwalla *et al.* 2007) and that CYSTUS052 is very rich in proanthocyanidins, I decided to investigate the antiviral properties of CYSTUS052 against common and highly pathogenic influenza viruses. First, we investigated the antiviral activity of CYSTUS052 in cell culture experiments (Publication #5: Ehrhardt *et al.* 2007). CYSTUS052 was very effective in reducing the replication of several human and avian influenza virus strains (H1N1, H7N7 and H5N1) at non-cytotoxic concentrations revealing broad antiviral properties (Publication #5; Fig.1). The extract exhibited antiviral activity without adversely affecting several parameters of cell viability and function such as the proliferative and metabolic capacity, gene

transcription and translation and cellular ligand/receptor system in cells (Publication #5; Fig. 2). Evidently, CYSTUS052 inhibited a very early stage in the replication cycle of the influenza virus, since titer reduction could only be observed if cells were either pre-incubated with CYSTUS052 or if the extract was added simultaneously with infection. CYSTUS052 blocked the hemagglutinating activity of pre-treated influenza A virus particles, possibly by binding to the viral surface protein HA and preventing entry into the cells (Publication #5; Fig. 3 + 4). In addition, continued passage of HPAIV in the presence of the plant extract did not result in the emergence of resistant variants, in contrast the M2 ion channel blocker amantadine which was tested in parallel and which rapidly generated resistant virus variants (Publication #5; Fig. 5). After those promising *in vitro* studies, we asked whether CYSTUS052 was also effective against influenza virus infections in a mouse model. The problem for *in vivo* studies is the poor bioavailability of polymeric polyphenols, since only few polymeric polyphenols are metabolized (Manach *et al.* 2004). However, the antiviral compound must be present within the lungs for effective anti-influenza therapy. Therefore, I have used an aerosol formulation for the local lung administration of CYSTUS052 (Publication #6; Droebner *et al.* 2007). The aerosol formulation of CYSTUS052 was first investigated in an *in vitro* experiment, where virus titer reduction was observed in MDCK cells treated with CYSTUS052 aerosol prior to infection. Next, I determined the effect of CYSTUS052 extract on the health and immune status of uninfected mice using an aerosol chamber. Inhalative application of CYSTUS052 three times a day for five consecutive days induced no apparent side effects in treated animals (Publication #6; Figure 1-2). For all these reasons CYSTUS052 seemed to be a suitable anti-influenza A agent. In fact, the inhalative application of CYSTUS052 was effective to prevent disease and death in HPAIV infected mice, whereas a normal oral administration of CYSTUS052 extract gave no protection at all due to the limited bioavailability of the high molecular weight polyphenols. Mice treated with the aerosolized plant extract neither showed changes in their body temperature nor changes in their gross motoric activity, while drastic differences were detectable in mice from the control groups. This observation was supported by reduced amounts of virus detected in the lungs of CYSTUS052 treated animals. The aerosol application of the polyphenol extract was also well-tolerated, since bronchial epithelial cells from treated mice showed normal histology, indicating that such a preparation is safe and could be beneficial in human influenza (Publication #6; Fig. 3-5). Furthermore, in this work I could approve the physical mode of antiviral action of CYSTUS052, where the polymeric polyphenols bind to the virus prior to influenza A virus infection by two facts. First, in my *in vitro* studies with CYSTUS052

aerosol the extract showed no antiviral properties, when given after or during the infection. Second, pre-treatment of virus with the plant extract prior to infection prevented the development of disease and dead of infected mice. In the range of those publications (Droebner *et al.* 2007; Ehrhard *et al.* 2007) I provided evidence that the polyphenol extract CYSTUS052 exhibits antiviral activity against several influenza A viruses *in vitro* and against a highly pathogenic influenza virus in a mouse model. In addition to my studies, Kalus and co-workers (Kalus *et al.* 2009) demonstrated in a randomized, placebo controlled clinical study that CYSTUS052 reduced cold symptoms by 2.5 days compared to the placebo group in a randomized, placebo controlled clinical study. The antiviral activity of CYSTUS052 is presumably due to binding of the polymeric polyphenol components to the virus particle surface, in particular the viral surface protein HA. The extract inhibits the binding of the HA to cellular receptors. An unspecific interaction with the viral HA has also been reported for the polyphenolic compound epigallocatechin-gallate (Nakayama *et al.* 1993). The rather unspecific action of CYSTUS052 is a preference in its use as anti-influenza drug. The action of CYSTUS052 directly on the pathogen may not result in the emergence of virus drug resistance and may also be active against opportunistic bacterial infections that represent a major complication in severe influenza virus infections. Moreover, the unspecific activity of CYSTUS052 might be directed against different subtypes of influenza A viruses, including those already resistant to common drugs. Recently, I was also able to demonstrate that CYSTUS052 is also highly effective against natural H5N1 isolates. In the first 20 hours after cell culture infection CYSTUS052 was up to 100 fold more effective against those viruses than oseltamivir. Moreover, the plant extract exhibited antiviral activity against H5N1 isolates that were unresponsive to oseltamivir treatment (Droebner unpublished data). Not only because of its high degree of efficacy and low toxicity demonstrated in my studies, the plant extract might be considered as a new candidate drug against influenza A viruses in particular for a save prophylactic and therapeutic use. In addition, beneath the direct antiviral activity of CYSTUS052, it may also weaken the severity of the influenza disease caused by bacterial co-infections, because extracts from *Cistus* plants possess antibacterial and antifungal activities (Bouamama *et al.* 1999).

CONCLUSIONS

The existence of a massive reservoir of influenza A viruses in avian species and the ability of these pathogens to cross species barriers means that they cannot be easily eradicated and might always pose a threat to the health of humans. New and better antiviral therapies are

needed to deal with pandemic strains and to reduce the toll of disease and death produced every year by influenza virus infections. The understanding of cellular but also viral mechanisms involved in the pathogenesis of influenza viruses is crucial for the defeat of influenza in its pandemic, zoonotic and seasonal epidemic forms. Currently available antiviral therapies were developed to reduce the impact of most common forms of seasonal disease, but many of them are no longer sufficient to protect against the disease. Moreover, their effectiveness against the highly virulent H5N1 virus is questionable (Beigel & Bray 2008; Maltezou & Tsiodras 2009). Their significant limitations point out the need for additional modalities. Herbal and natural products are not only promising alternatives to current interventions, but their investigation is also recommended by the WHO (Bing *et al.* 2009; Kalus *et al.* 2009; Pleschka *et al.* 2009; WHO 2009b). My studies on the antiviral activity of CYSTUS052 demonstrated the great potential of this plant extract against newly emerged HPAIV (Droebner *et al*. 2007; Ehrhardt *et al.* 2007). New insights into the immunological mechanisms involved in host-defence after viral infections can help develop novel therapeutic options that minimize immunopathology without impairing beneficial host defences (Ludwig & Planz 2008; Burke & Fish 2009). The determination of the role of the NF-κB signalling pathway in viral infections (Droebner *et al*. 2010) offers new cellular targets for such therapies. Furthermore, a better understanding of the underlying factors in viral pathogenesis, such as hypercytokinemia and lymphopenia may help to prevent fatal disease outcomes (Droebner *et al.* 2008a; Vogel *et al.* 2010). Knowledge of immune responses involved in protection after H5N1 influenza virus infections can improve vaccine approaches (Droebner *et al.* 2008b). Although, many efforts had been taken in the vaccination field (Chen & Subbarao 2009; Ellebedy & Webby 2009; Keitel & Atmar 2009) still more research is needed to develop effective influenza vaccines.

Taken together, a robust scientific knowledge is mandatory for decreasing the risk and impact of influenza infections. The studies presented in my publications have contributed to the clarification of some aspects of H5N1 mediated pathogenesis. They provide a basis for further scientific research concerning the mechanisms of pathogenicity and the contest between the virus and the immune system.

AUTHORS CONTRIBUTIONS

PUBLICATION #1: Reprinted from Viral Immunology, Volume: 23, Droebner K, Klein B, Paxian S, Schmid R, Stitz L and Planz O.: The Alternative NF-κB signalling pathway is a prerequisite for an appropriate immune response against Lymphocytic Choriomeningitis Virus infection. Pages: 295-308. Copyright 2010, with permission from Mary Ann Liebert, Inc.; available online at: http://www.liebertonline.com.

K. Droebner performed animal experiments, flow cytometry, CCL21 ELISA, immunohistology analysis (Fig. 1-2, 3A, 5, 6 and 8) and prepared the manuscript layout
B. Klein supported animal experiments and flow cytometry and performed LCMV-ELISA (Figure 1, 4 and 6)
Paxian M. and Schmidt R. generated and provided NF-κB2 deficient mice
L. Stitz and Planz O. performed adoptive transfer experiment (Figure 3B-C, Figure 7 and Table)
K. Droebner and O. Planz designed the experiments and wrote the manuscript
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PUBLICATION #2: Reprinted from Journal of Virology, Volume: 82(22), Droebner K, Reiling SJ, Planz O.: Role of hypercytokinemia in NF-kappaB p50-deficient mice after H5N1 influenza A virus infection. Pages 11461-11666. DOI: 10.1128/JVI.01071-08. Copyright 2008, with permission from American Society for Microbiology.

K. Droebner performed all animal experiments, flow cytometry, RNA isolation, western blot analysis (Figure 2, 3 B and C, Table 1) and prepared the manuscript layout.
S.J. Reiling carried out qRT-PCR analysis (Fig. 1 and 3A)
K. Droebner and O. Planz designed the experiments
K. Droebner, SJ. Reiling and O. Planz wrote the manuscript
The studies were supported by Grants provided by O. Planz

PUBLICATION #3: Reprinted from Journal of Immunology, Volume:185(8); Vogel A., Haasbach E., Reiling S.J., Droebner K., Klingel K. and Planz O.: Highly pathogenic avian influenza virus infection of dendritic cells modulates the adaptive immune response. Pages 4824-4834. Copyright 2010, The American Association of Immunologists,Inc.

A.B.Vogel performed the animal experiments, flow cytometry, cell isolation, immunhistology analysis, partly qRT-PCR and prepared the manuscript layout (Fig. 1 and Fig. 4-8)
E. Haasbach carried out qRT-PCR analysis and supported the animal experiments (Fig. 2 and 5a)
S.J. Reiling analyzed isolated cells for their immunofluorescence (Fig. 7)
K. Droebner performed infectious assays, supported the animal experiments and flow cytometry (Table and Fig. 1 and 6)
K. Klingel accomplished the *in situ* experiments (Fig. 5B)
O. Planz performed the *in vitro* cytotoxicity assay (Fig. 3)
A.B.Vogel and O. Planz designed the experiments and wrote the manuscript
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PUBLICATION #4: Reprinted from Vaccine, Volume:26(52), Droebner K, Haasbach E, Fuchs C, Weinzierl AO, Stevanovic S, Büttner M, Planz O.: Antibodies and $CD4^+$ T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain. Pages: 6965-6974;

K. Droebner performed all animal experiments, flow cytometry, hemagglutination inhibition assays, *in vitro* growth kinetics (Figure 1-5, Table 1-3) and prepared the manuscript layout
E. Haasbach supported the animal experiments (Fig. 2 and 4) and further propagated the H5N1 virus strain in embryonated chicken eggs
C. Fuchs and M. Büttner isolated and provided both avian strains
A.O. Weinzierl and S. Stevanovic constructed and provided MHC class I tetramers predicted for both avian strains containing $NP^{366–374}$ peptide
K. Droebner and O. Planz designed the experiments and wrote the manuscript
The studies were supported by Grants provided by O. Planz

PUBLICATION #5: Reprinted from Antiviral Research, Volume:26(52), Ehrhardt C, Hrincius ER, Korte V, Mazur I, Droebner K, Poetter A, Dreschers S, Schmolke M, Planz O, Ludwig S.: A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance. Pages 38-47.

C. Ehrhardt, ER. Hrincius, V. Korte, I. Mazur, S. Dreschers and M. Schmolke performed all experiments (Fig. 1-5)
C. Potter took part in the development of CYSTUS052 extract
K. Droebner was involved in the experimental setup, discussions and corrections of the manuscript
O. Planz was involved in discussions, experimental design and corrections of the manuscript
C. Ehrhardt and S. Ludwig designed the experiments and wrote the manuscript
The studies were supported by Grants provided by S. Ludwig and O. Planz

PUBLICATION #6: Reprinted from Antiviral Research, Volume 76(1), Droebner K, Ehrhardt C, Poetter A, Ludwig S, Planz O.: CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice. Pages 1-10.

K. Droebner performed all animal experiments, hemagglutination assay and immunohistology analyses (Fig. 1-5 and Table 1-2) and prepared the manuscript layout
C. Ehrhardt was involved in the experimental setup, discussions and corrections of the manuscript
C. Potter took part in the development of CYSTUS052 extract
S. Ludwig was involved in discussions and experimental design
K. Droebner and O. Planz designed the experiments and wrote the manuscript
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The Alternative NF-κB signalling pathway is a prerequisite for an appropriate immune response against Lymphocytic Choriomeningitis Virus infection

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Abstract

Two major NF-κB signalling pathways are involved in the regulation of the immune response. While the classical NF-κB pathway is responsible for regulation of genes encoding components of the innate immune response, the alternative NF-κB signalling mediates processes of the adaptive immune system. To evaluate the role of the NF-κB signalling pathways in the control of viral infection, we have used the Lymphocytic Choriomeningitis Virus infection of mice that is known as an excellent model for studying antiviral immune responses. By the use of mice that were deficient in NF-κB subunits from either the classical ($p50^{-/-}$ mice) or the alternative NF-κB pathway ($p52^{-/-}$ mice), we were able to demonstrate, that the alternative NF-κB pathway is required for the T-cell mediated immune response against LCMV. Mice that were deficient in the alternative NF-κB pathway subunit p52 showed an impaired T-cell response against LCMV infection. Furthermore, those mice showed also an impaired T-cell dependent humoral immune response against VSV infection. Adoptive transfer experiments revealed that impaired priming but not the T-cell response itself was responsible for the defective cellular immune response against LCMV infection. Our data demonstrate that a functional alternative NF-κB signalling pathway is required to assure adequate immune response after viral infection.

Introduction

The NF-κB/Rel family of transcription factors plays a crucial role in the gene regulation that is responsible for cellular proliferation and apoptosis (18) NF-κB is also involved in viral infections and viral clearance (64, 70). Mammalian cells express five NF-κB proteins: NF-κB1 (the DNA binding subunit p50 and its precursor p105), NF-κB2 (the DNA-binding subunit p52 and its precursor p100), RelA (p65), RelB and c-Rel (19). In non-stimulated cells homo- and heterodimers composed of NF-κB/Rel proteins are retained in the cytoplasm by a family of inhibitory kappaB proteins (IκBs) (64). Proinflammatory cytokines and pathogen-associated molecular patterns (PAMPs) invoke the NF-κB signalling pathway (58). The stimulus leads to the activation of the IκB kinase (IKK) complex, containing the IκB kinase α and β (IKKα/IKKγ) and a regulatory subunit IKKγ. This kinase complex is responsible for the phosphorylation and subsequent degradation of IκBs. Consequently, the released NF-κB dimers translocate to the nucleus, bind DNA and activate gene transcription (3). Two major signalling pathways can be distinguished within this regulation. Signalling through the classical NF-κB pathway leads in most cases to the nuclear translocation of RelA/p50 dimers. This pathway is responsible for the regulation of the innate immune response (61); the

adaptive immunity is controlled via the alternative NF-κB signalling pathway. The alternative signalling pathway involves the subunits RelB/p52 for gene regulation (6, 7, 12, 35, 40).

The immune response after infection with the non-cytopathic Lymphocytic Choriomeningitis virus (LCMV) is well characterized (54, 72). The course of LCMV infection in mice depends on virus dose, infection route and the age of the infected mouse. In immunocompetent mice an acute LCMV infection is efficiently controlled by cytotoxic effector mechanisms, namely natural killer cells (NK-cells) and cytotoxic T-cells (CTL) (20, 27, 33, 53, 60, 73). Virus elimination during acute phase infection mainly depends on the massive induction of virus specific $CD8^+$ T-cells. The maximum CTL activity is found by day 8 after LCMV infection (10, 21, 22). Transplacental or neonatal infection of mice or infection of immunodeficient mice leads to a lifelong, persistent infection (9, 31, 53, 55, 56). Intracerebral inoculation of LCMV generally results in fatal T-cell mediated meningitis (15, 24, 47, 48). LCMV infection of mice serves as an excellent model to study the immunological mechanisms that are required for the defence against viral infections. Although our knowledge on mechanisms of the cellular immune response against viral infection has increased in the past, tremendously, the influence of NF-κB in this process is not very well understood. The knowledge of how NF-κB is involved in the immune defence against viral pathogens however could help to improve therapeutic strategies against viral infections. Thus, the present study was focussed on better understanding the role of NF-κB for the immunity against viral infection. We demonstrate that the alternative NF-κB pathway is a prerequisite for the T-cell mediated immune response against LCMV. Mice that were deficient in the NF-κB subunit p52 showed an impaired T-cell response against this virus. Interestingly, T-cells from NF-κB $p52^{-/-}$ mice were responsive when transferred into wild type mice, indicating the importance of the alternative NF-κB pathway for priming rather than for signalling processes in the T-cell itself.

Materials and Methods

Virus

LCMV, WE-strain was obtained from R.M. Zinkernagel (Institute of Experimental Immunology, Zürich, Switzerland) and further propagated on L929 cells at a low multiplicity of infection (MOI) at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tübingen, Germany. VSV Indiana was also obtained from R.M. Zinkernagel (Institute of Experimental Immunology, Zürich, Switzerland).

Animals and infections

C57BL/6 mice were obtained from the animal breeding facilities 10 at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tübingen, Germany. NF- κ B2 ($p52^{-/-}$) mice were originally obtained from Schmidt and Paxian from the Department of Internal Medicine II, Technical University of Munich. The generation of NF-κB$2^{-/-}$ mice was reported elsewhere (59). NF-κB$2^{-/-}$ mice were backcrossed into C57Bl/6 strain at least 10 times at the animal breeding facilities of the Friedrich-Loeffler-Institute. NF- κ B1 ($p50^{-/-}$) mice were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA) and were backcrossed onto a C57BL/6 background as previously described (8, 16).

Female mice at the age of 6-8 weeks were used throughout all the experiments. For LCMV infection, mice were either infected with 1 x 10^2 pfu or 1 x 10^6 pfu intravenously into the tail vein or were infected intracerebrally with 1 x 10^3 pfu. For VSV infection, animals were infected with 2 x 10^6 pfu VSV-IND intravenously into the tail vein.

Cells

The murine fibroblast line MC57 obtained from C57Bl/6 mice (H-^{2b2}) was used for *in vitro* infections with LCMV-WE. The cells were cultured with MEM medium supplemented with 5% FCS and 2 mM L-glutamine and 100 U/ml gentamycin.

Infectivity assay

To assess the number of infectious particles (plaque titers) in blood and organs of LCMV infectedwild type, NF-κB $p50^{-/-}$ and $p52^{-/-}$ 8 mice standard focus forming assay on MC57 cells (5) was performed in 24-well plates. Virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the LCMV nucleoprotein (VL4) followed by a 30 min incubation with an anti-rat biotin-labelled antibody (Dianova) and and by 30 min incubation with streptavidin-peroxidase-conjugate (Dianova). The reaction was visualized with *ortho* phenylendiamine substrate (Sigma). Titers are expressed as pfu (plaque forming unit) per ml of 10% organ homogenates or per ml of blood. The detection limit of this focus forming assay is 1.7 log10 pfu/ml.

Foot pad swelling reaction

Delayed-type hypersensitivity (DTH) response was induced by injecting 1.5 x 10^3 pfu LCMV WE into the left hind foot pad of either four C57Bl/6, NF-κB $p50^{-/-}$ or NF-κB $p52^{-/-}$ mice. Foot pad thickness was measured with a spring-loaded caliper (Kroepelin). Increased foot pad

thickness was expressed as the percent swelling relative to the thickness of the uninfected right hind foot pad.

T-cell depletion

For the depletion of T-cells, C57Bl/6 and NF-κB $p52^{-/-}$ mice were inoculated intra peritoneal (i.p.) with monoclonal antibodies against CD4 (YTS 191.1, rat IgG2b) or CD8 (YTS 169.4, rat IgG2b) two weeks prior to the experiment. Antibodies were produced as ascitic fluids and purified by affinity chromatography. For depletion of CD4 cells mice were treated with the anti-CD4 specific antibody YTS 191.1 diluted 1:500 in balanced salt solution (BSS). CD8 cells were depleted by injection of the anti CD8 specific antibody YTS 169.4 diluted 1:200 in BSS in four day intervals. The depletion of T-lymphocytes was assessed using FACS-analysis with monoclonal antibodies to CD8a (clone 53-6.7), CD4 (clone H129.19) and CD3e (clone145-2c11, all from BD Bioscience). Only mice with complete depletion of T-cells were used for further experiments.

Cytotoxic T-cell response

Spleens from C57Bl/6 and NF-κB $p52^{-/-}$ mice were harvested on day 8 post i.v. LCMV infection with 1.5 x 10^2 pfu. For the assay of CTL activity, MC57 target cells were either infected with LCMV-WE at a multiplicity of infection (MOI) of 0.1 or were left uninfected. Two days later, the cells were labelled with $Na_2\ ^{51}CrO_4$ and used as targets in a 6-hr 5116 Cr-release assay (57). Single cell suspensions of spleen cells were used as effector cells at the indicated effector to target ratios. Cytotoxicity assays were performed in MEM (minimal essential medium, GibcoBRL) supplemented with 2% FCS (PAA Laboratories), 2 mM L-glutamine and 100 U/ml gentamycin. At the end of the 6-hr incubation period, plates were centrifuged and 25µl of supernatants from each well were harvested and counted in a micro-beta counter (Wallac). For determining spontaneous and maximum release, target cells were only incubated with medium without effector cells or 1 N HCl, respectively. The percentage of specific lysis was calculated as (experimental release – spontaneous release)/(total release – spontaneous release) x 100. Spontaneous release was always < 20%.

Measurement of VSV-specific antibody responses

To determine total Ig or IgG serum titers against VSV mice were infected intravenously into the tail vein with 2 x 10^6 pfu VSV-IND. On day 0, 4, 8 and 12 post-infection animals were bled and serum samples were collected. Polystyrene microtiter plates were coated at 4°C

overnight with 0.5 µg of purified VSV per 100 µl of carbonate-bicarbonate buffer (pH 9.6) per well. The plates were washed five times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween-20, and then 100µl of serial dilutions of sera in phosphate-buffered saline-0.05% Tween 20 was added to each well and incubated for 2h at room temperature. Plates were washed again, and 100 µl of goat anti-mouse total immunoglobulin (Ig) or goat anti-mouse immunoglobulin G (IgG) labeled with horseradish peroxidase diluted optimally in phosphate buffered saline-0.05% Tween 20 was added and then incubated for 2 h at room temperature. The plates were washed again, and 200 µ1 of substrate containing 2 mg of 2,2'-azino-di-3-ethyl benzthiazoline sulfonate in 20 ml of 0.1 M NaH2PO4 (pH 4.0) with 15 µl of 30% H_2O_2 was added to each well. After 30 min of incubation at room temperature, all samples and appropriate controls were measured at 405 nm with a micro-ELISA reader.

Detection of anti-LCMV antibodies

For detection of the different LCMV-specific Ig subtypes (IgM, IgG) and the IgG subclasses an enzyme-linked immunosorbent assay (ELISA) was performed. Serum samples from high dose (10^6 pfu) LCMV-WE infected mice were used. ELISA plates were coated overnight at 4°C with baculovirus-derived LCMV-NP (1 µg/ml) in carbonate buffer (pH 9.6). Afterwards the plates were blocked with 1% BSA-PBS/0.05% Tween-20, 40-fold–prediluted sera in 1% BSA PBS/0.05% Tween-20, titrated 1:2 over 8 dilution steps were added. After washing, the plates were incubated with the HRP-conjugated secondary antibodies anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3 (Biozol/Southern Biotech). Incubations were performed at RT for 60 min; PBS/0.05% Tween-20 was used for all washing steps. Color reactions were performed at RT for 20 min. with ABTS (2.2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonate]; Boehringer Mannheim) substrate and H_2O_2 (Fluka). OD was measured at 405 nm in an ELISA reader, and antibody titers were determined as the highest dilution of serum yielding an optical density (OD) of twice background levels (naive mouse serum) or higher.

Immunohistochemistry

Mice were infected i.v. with 1 x 10^6 pfu LCMV-WE. At the indicated time points freshly removed organs were immersed in OCT (tissue tek; Torrance, CA) and snap frozen in liquid nitrogen. Seven-µm-thick tissue sections were cut in a cryostat, placed on SuperFrost Plus slides, air dried, fixed with acetone for 10 min, and stored at -80°C. For blocking of endogenous peroxidase activity rehydrated sections were incubated for 10 min with with 0.3% H_2O_2 in methanol. After blockage of unspecific binding with 5% goat serum for 30 min,

section were incubated with the primary antibody to CD4 (BD Pharmingen) or CD8a (BD Pharmingen) diluted (1:50 or 1:10, respectively) in PBS/5% goat serum overnight at 4°C. Subsequently, after washing of the sections with PBS (three times for 5 min) they were incubated with the secondary biotinylated goat anti rat IgG (1:1000, Dianova) antibody for 30 min at room temperature. Afterwards the incubation with a streptavidin-avidin-biotin peroxidase complex (30 min RT; ABC Peroxidase Elite, Vector Labs) was followed by visualization of the peroxidase activity by DAB. The counterstaining was performed with haematoxylin.

Flow Cytometry

Mice were infected intravenously into the tail vein with a high dose of LCMV-WE (1 x 10^6 pfu/100 µl) at the indicated time points the animals were bleed, spleens were removed and mechanically disrupted by passage through a 70 µm cell strainer (BD Falcon). A single cell suspension was stained in FACS-buffer (PBS, 2% FCS, EDTA, pH 7.5) with 1:100 dilution of required fluorochrome conjugated monoclonal antibodies to CD8a (clone 53-6.7), CD4 (clone H129.19) and CD3e (clone145-2c11, all from BD Bioscience) for 30 min at 4°C. Before FACS analysis, cells were washed, erythrocytes were lysed and cells were fixed with FACS lysing solution (BD Bioscience). Flow cytometry was performed on a dual laser FACSCalibur and analysed with Cell Quest software (BD Biosciences).

Adoptive transfer of spleen cells

Donor NF-κB $p52^{-/-}$ and wild type mice were infected i.v. with 10^2 pfu LCMV-WE. Eight or 60 days post-infection single cell suspensions of spleens were prepared by passage through a 70 µm cell strainer (BD Falcon). Isolated spleen cells were washed twice, counted and 5 x 10^7 donor cells were injected intravenously in a volume of 500 µl BSS into the tail vein of either NF-κB $p52^{-/-}$ or wild type thymectomized recipients**.** Recipient mice were thymectomized at the age of four weeks and additionaly treated with monoclonal antibodies against CD4 (YTS 191.1, rat IgG2b) or CD8 (YTS 169.4, rat IgG2b) two weeks prior to the experiment to remove residual T-cells. Successful depletion of T-lymphocytes was assessed using FACS-analysis with monoclonal antibodies to CD8a (clone 53-6.7), CD4 (clone H129.19) and CD3e (clone145-2c11, all from BD Bioscience) as described above.

Determination of mouse CCL21

The amount of CCL21 in the spleens of mice was investigating using the DuoSet® Mouse CCL21 (6 Ckine) ELISA-Kit (R&D Systems). Animals were infected i.v. with LCMV-WE (10^6 pfu) at the indicated time points spleens from sacrificed mice were homogenized to a ten percent homogenate in saline buffer. The supernatants of those 10% spleen homogenates were used in the CCL21 ELISA according to manufacturer's protocol.

Statistical analysis

Error bars are given as the standard error of the mean. For the investigation of significant differences (p-value < 0.05), *t-test* was performed.

Results

Impaired anti-viral immune response in NF-κB p52 but not in NF-κB p50 deficient mice

To investigate the ability of NF-κB p52- and NF-κB p50-deficient mice to control a LCMV infection, these mice and C57Bl/6 wild type animals were infected intravenously with 10^6 pfu LCMV-WE. Virus titer was determined at different time points after LCMV infection in blood (Fig. 1A) and spleens (Fig. 1B) of infected animals. Whereas LCMV was already detectable 2 days p.i. in the blood of $p50^{-/-}$ mice (Fig. 1A, grey circles), the virus was first found in the blood of NF-κB $p52^{-/-}$ (Fig. 1A, open triangles) and wild type (Fig. 1A, black squares) mice on day 4 after infection. At this time-point LCMV reached maximum titers in wild type (3.08 ± 0.33 $\log_{10}$ pfu/ml) and $p50^{-/-}$ mice (2.72 ± 0.02 $\log_{10}$ pfu/ml). Thereafter, the amount of infectious virus in the blood of those animals slowly decreased. Already 10 days after infection no LCMV was detected in the blood of wild type and p50 deficient mice. In sharp contrast, $p52^{-/-}$ mice were unable to eliminate the virus and high viral loads (4.77 ± 0.65 $\log_{10}$ pfu/ml) persisted in the blood for as long as 130 days, when the experiment was terminated.

A similar situation was observed in the spleens of LCMV-infected mice. As shown in Figure 1B, already 2 days p.i. LCMV replicated to high titers in wild type (5.69 ± 0.06 $\log_{10}$ pfu/ml), NF-κB $p50^{-/-}$ (3.89 ± 0.52 log10 pfu/ml) and $p52^{-/-}$ mice (4.96 ± 0.37 $\log_{10}$ pfu/ml). Again wild type (Fig. 1B, black squares) and NF-κB p50 deficient mice (Fig. 1B, grey circles) were able to eliminate the virus from the spleen within 15 days post infection. Again, no virus clearance was observed in p52 deficient mice (Fig. 1B, open triangle).

Intracerebral (i.c.) LCMV-infection with 1 x 10^3 pfu of wild type (Fig. 1C, black squares) as well as NF-κB $p50^{-/-}$ mice (Fig. 1C, grey circles) resulted in massive weight loss in

association with death of these animals between day 6 and 9 after LCMV inoculation to the brain. Surprisingly, NF-κB $p52^{-/-}$ mice survived the i.c. infection (Fig. 1C, open triangles). Those mice lost less than 20% of their body-weight throughout the experiment, gained the full body mass after 30 days and completely recovered from clinical symptoms (data not shown).
LCMV inoculation into the foot pad induces a local delayed-type hypersensitivity (DTH) reaction that results in a foot pad swelling sequentially mediated by class I-restricted cytotoxic $CD8^+$ T-cells and class II-restricted $CD4^+$ T-cells, cytokines and inflammatory cells (32, 50). To investigate LCMV-mediated foot pad swelling in wild type, NF-κB $p50^{-/-}$ and $p52^{-/-}$ mice, LCMV (1 x 10^4 pfu) was administered subcutaneously into the left hind foot. As seen in Figure 1D foot pad swelling in wild type mice shows a maximum between day 8 and 9 (Fig. 1D, black squares) and a subsequent decrease in foot pad thickness after day 9 p.i.. Foot pad swelling in $p50^{-/-}$ mice (Fig. 1D, grey circles) was similar to that of wild type mice, although the maximum was reached 10 days p.i.. In NF-κB p52 deficient mice (Fig. 1D, open triangles) a maximum of foot pad swelling was also found at day 9, but the intensity was reduced three fold as compared to wild type animals. Taken together, all experiments using different routes of infection demonstrated no differences in the ability of NF-κB $p50^{-/-}$ mice to control LCMV-infection in comparison to wild type mice, whereas NF-κB p52 deficient mice were unable to control LCMV-infection. To investigate the underlying mechanism of the impaired immune response of NF-κB $p52^{-/-}$ mice in more detail, the following experiments were performed with wild type and NF-κB $p52^{-/-}$ mice only.

Depletion of $CD4^+$ T-lymphocytes prevent DTH reaction in NF-κB *p52 deficient mice*

To analyse the impact of T-cell populations in the cellular immune response against LCMV in NF-κB p52 deficient mice in more detail, experiments were performed in which wild type and NF-κB $p52^{-/-}$ mice were treated with antibodies to deplete either $CD4^+$ or $CD8^+$ T-lymphocytes or both immune cell populations. Immunocompetent untreated wild type mice showed the classical swelling reaction with the highest peak at 9 days p.i. (Fig. 2A, black squares), whereas the foot pad swelling after LCMV-infection of NF-κB $p52^{-/-}$ mice was 4-fold reduced (Fig. 2A, open triangles). Depletion of $CD8^+$ T-cells alone results in delayed and decreased swelling of the foot pad in wild type mice (Fig. 2B, black squares) as described earlier (50-51). In contrast, hardly any foot pad swelling was observed in $CD8^+$ T-cell depleted NF-κB $p52^{-/-}$ mice (Fig. 2B, open triangles). The depletion of $CD4^+$ T-cells in wild type mice resulted in a diminished but not delayed swelling reaction (Fig. 2C, black squares). The maximum was reached 9 days p.i.. Similar to wild type mice, $CD4^+$ T-cell depletion in

NF-κB p52$^{-/-}$ mice resulted in a drastic attenuated foot pad swelling (Fig. 2C, open triangles). The maximum swelling was achieved 11 days after virus inoculation, similar to immunocompetent NF-κB p52$^{-/-}$ mice. Depletion of CD4^{+} plus CD8^{+} T-cells in NF-κB deficient mice resulted in complete suppression of foot pad swelling (Fig. 2D, open squares), whereas a minimised swelling reaction was detectable in wild type mice 12 days p.i..

Reduced cellular and humoral immune response in p52$^{-/-}$ mice after virus infection

The suppressed foot pad swelling after LCMV infection in T-lymphocytes depleted NF-κB p52$^{-/-}$ mice suggested that the function of both CD4^{+} and CD8^{+} T-cells was impaired. To verify this finding, the cellular immune response mediated by cytotoxic antigen-specific CD8^{+} T-cells in the spleens of wild type and NF-κB p52$^{-/-}$ mice was investigated. By the use of a classical ^{51}Cr release-assay as described in materials and methods, a ten-fold reduced CTL activity was found in the spleen of NF-κB p52$^{-/-}$ mice (Fig. 3A, open triangles) 8 days after LCMV-infection as compared to the CTL response in wild type mice (Fig. 3A, black squares)

To investigate the CD4^{+} T-cell help in NF-κB p52$^{-/-}$ mice, we chose the Vesicular stomatitis virus (VSV) infection as a model for CD4^{+} T-cell function analysis. VSV-infection of immunocompetent mice induces a rapid T-independent IgM response followed by a long-lived T-cell dependent IgG response (34). Mice were infected with VSV-IND and bled at days 0, 4, 8 and 12 post-infection. VSV-specific antibodies were found as early as at 4 days p.i. (9.7 ± 0.7 –log$_2$ x 40) in wild type mice (Fig. 3B, black squares). The titer slightly increased at 8 (11.0 ± 0.6 –log2 x 40) and 12 days p.i. (11.3 ± 0.3 –log2 x 40). NF-κB p52$^{-/-}$ mice showed a similar titer of VSV specific antibodies compared to wild type mice (Fig. 3C, open triangles). Here, VSV-specific immunoglobulins were also detectable at 4 days p.i. (7.0 ± 0.6 –log$_2$ x 40), day 8 p.i. (8.7 ± 1.2 –10 log$_2$ x 40) and day 12 p.i. (8.7 ± 2.5 –log$_2$ x 40). In contrast, the overall amount of IgG-specific VSV antibodies was altered in NF-κB p52$^{-/-}$ compared to wild type mice. Antibodies were first found at day 8 (9.7 ± 0.7 – log$_2$ x 40) and the amount increased at day 12 p.i. (10.7 ± 0.7 –log$_2$ x 40) in wild type mice (Fig. 3B, black squares). In NF-κB p52 deficient mice VSV-specific IgG antibodies were also found at day 8 and 12 p.i. but at low titers (0.6 ± 0.3 – log$_2$ x 40 at day 8 p.i. and 2.6 ± 1.5 – log$_2$ x 40 at day 20 p.i.). These data indicate that the humoral immune response against VSV in p52$^{-/-}$ mice is mediated by IgM, while the T-cell dependent IgG response against VSV is impaired.

Since the LCMV specific non-neutralizing humoral immune response is also T-cell dependent, we evaluated the humoral immune response against the LCMV nucleoprotein (NP) by ELISA assay. Mice were infected with high dose LCMV (10^{6} pfu) and were bled at

days 0, 4, 8 and 20 post-infection. The first IgM titers in wild type mice appeared at day 4 p.i. (3.3 ± 0.3 $-\log_2$; Fig.4A, black squares) and increased at day 8 p.i. to 5.7 ± 0.3 $-\log_2$. At day 20 post-infection, LCMV specific IgM titer dropped to 4.3 ± 0.3 $-\log_2$. In NF-κB $p52^{-/-}$ mice the LCMV specific IgM titer was almost 3 $\log_2$ higher at day 4 p.i. compared to the titer in wild type mice (Fig. 4A, open triangles). The titer slightly decreased at day 8 p.i. (5.7 ± 0.3 – $\log_2$), and increased again at day 20 p.i. (6.5 ± 0.4 $-\log_2$). LCMV infection led to higher IgG titers in $p52^{-/-}$ mice (7.7 ± 0.6 – $\log_2$ Fig. 4B, open triangles) at 4 days p.i. compared wild type mice (6.7 ± 0.3 $-\log_2$; Fig. 4B, black squares). The IgG titer in wild type mice increased at day 8 (10.0 ± 0.9 $-\log_2$) and 20 p.i. (10.7 ± 0.7 $-\log_2$). In $p52^{-/-}$ animals a slight increase of the titer at day 8 p.i. (8.0 ± 0.1 $-\log_2$ x 40) was found, whereas IgG titer decreased at day 20 post-infection (6.0 ± 0.3 $-\log_2$; Fig. 4B, open triangles).

The determination of IgG subclasses in the sera of LCMV-infected mice demonstrated almost no IgG1 response after LCMV infection in both mice strains (Fig. 4C) while the titers of IgG2b (Fig. 4E) were almost equal in $p52^{-/-}$ animals compared to wild type mice. In contrast, differences in the amounts of IgG2a and IgG3 between wild type and NF-κB $p52^{-/-}$ mice were found (Fig. 4D +F). Even though $p52^{-/-}$ mice had higher IgG2a and IgG3 titers (2.0 ± 0.1 – $\log_2$ x 40 and 1.5 ± 0.6 $-\log_2$ x 40, respectively) 4 days after LCMV-infection compared to wild type mice (no LCMV specific antibodies detectable), a reduction of the two IgG subclasses was observed in NF-κB p52 deficient mice at day 8 and 20 p.i. (IgG2a: 4.0 ± 0.8 – $\log_2$ and 2.0 ± 0.8 $-\log_2$; IgG3: 1.0 ± 0.6 – $\log_2$ x 40 and no antibodies) compared to wild type animals (IgG2a: 6.0 ± 0.6 $-\log_2$ and 5.7 ± 0.3 $-\log_2$; IgG3: 4.3 ± 0.7 $-\log_2$ and 4.3 ± 0.7 – $\log_2$).

Reduced numbers of $CD8^+$ T-cells in the spleen of NF-κB p52 deficient mice after LCMV infection

To investigate the distribution of $CD4^+$ and $CD8^+$ T-cells in wild type and $p52^{-/-}$ mice after LCMV infection immunohistochemistry of spleens was performed. $CD4^+$ T-cells of wild type mice were located within the T-cell zones of the splenic white pulp (Fig. 4A, white arrows). Four days after infection $CD4^+$ T-cells migrated out of the white pulp towards the red pulp (Fig. 4A, red arrows). In the course of infection CD4+ T-cells were detected within the white and red pulp in the spleens of wild type mice. Twenty days post infection distinct germinal centres within the white pulp of LCMV infected wild type mice were visible (Fig. 5B, black arrow). In the spleens of uninfected NF-κB $p52^{-/-}$ mice the $CD4^+$ T-cells were distributed within the white pulp, due to the lack of a B- and T-cell zone separation in the splenic

microarchitecture (Fig. 5B, white arrows). The localization of $CD4^+$ T-cells in the spleen of NF-κB $p52^{-/-}$ mice did not change until day 8 post LCMV infection. Migration of $CD4^+$ T-cells out of the white pulp into the red pulp in the spleens of $p52^{-/-}$ mice was observed 8 days after LCMV infection (Fig. 5B, red arrows). Furthermore, no germinal centre formation was detected in the spleens of LCMV infected NF-κB $p52^{-/-}$ mice.

The dissemination and amount of $CD8^+$ T-cells in the white pulp within the spleens of uninfected wild type and NF-κB $p52^{-/-}$ mice revealed no differences (Fig. 5C + D, white arrows). Four days p.i. hardly any CD8+ T-cells were present in the spleens of wild type or NF-κB $p52^{-/-}$ mice (Fig. 5C + D, white arrows). Migration of $CD8^+$ T-cells out of the red into the white pulp was observed 8 days p.i. in wild type mice (Fig. 5C, red and white arrows). In contrast, at the same time point only very few $CD8^+$ T-cells were detected in the spleens of p52 deficient mice (Fig. 5D, red and white arrows). The same development was observed in spleens of infected mice 20 days p.i.. Both, wild type and NF-κB $p52^{-/-}$ mice showed accumulations of $CD8^+$ T-cells within the white and red pulp 20 days p.i.; however the overall amount of $CD8^+$ T-cells in spleens of NF-κB $p52^{-/-}$ animals was significantly reduced as compared to wild type mice.

FACS analysis confirms reduced $CD8^+$ T-cell numbers in NF-κB $p52^{-/-}$ mice

To further support the immunohistochemical observation, a quantitative analysis of $CD4^+$ and $CD8^+$ T-lymphocytes in the spleens of LCMV infected wild type and $p52^{-/-}$ mice was performed by FACS analysis. In wild type mice a 14.5% reduction of $CD4^+$ T-cells in the spleen was found four days after LCMV infection compared to uninfected animals (Figure 6A, black bars). A similar reduction of $CD4^+$ T-cells (10%) was observed in the spleens of NF-κB $p52^{-/-}$ mice (Figure 5A, white bars). Eight days p.i. $CD4^+$ T-cells were still reduced in wild type mice, while a slight increase of these cells (15%) was found in NF-κB $p52^{-/-}$ mice (Figure 6A, white bars). The amount of $CD4^+$ T-cells increased 20 days p.i. in wild type mice and reached almost the level of uninfected controls. NF-κB $p52^{-/-}$ mice had comparable numbers of $CD4^+$ T-cells as compared to day 8 p.i. Significant differences in the amount of $CD4^+$ T-lymphocytes in the spleens of wild type and NF-κB $p52^{-/-}$ mice were present ($p = 0.0069$) at day 8 p.i.

The amount of $CD8^+$ T-cells after LCMV infection in the spleen of NF κB $p52^{-/-}$ mice was also altered compared to wild type animals. Four days after LCMV infection in wild type mice there were no difference in the amount of $CD8^+$ T-cells in the spleen compared to uninfected animals (Figure 6B, black bars). NF-κB $p52^{-/-}$ mice showed a reduction (30%) of

the $CD8^+$ T-cell population at this time point (Fig. 6B, white bars). Clear differences ($p = 0.0051$) were observed days p.i. Wild type mice showed a 160% increase of CD8 positive lymphocytes at this time point compared to uninfected controls (Figure 6B, black bars). Although the amount of $CD8^+$ T-cells in NF-κB $p52^{-/-}$ mice increased by 40% 8 days after infection compared to uninfected controls, the number of $CD8^+$ T-lymphocytes was drastically reduced (120%) when compared to wild type mice (Fig. 6B, white bars). The number of $CD8^+$ T-cells in wild type mice (Figure 6B, black bars) decreased again at 20 days p.i. but was still elevated at about 60% compared to uninfected animals. A decrease of the $CD8^+$ T-cell population was also observed in the spleens of NF-κB $p52^{-/-}$ mice (15%) 20 days post LCMV infection. These results confirmed the data obtained from the immunohistochemistry, revealing differences in the amount and distribution of $CD8^+$ T-cell population in the spleen of NF-κB $p52^{-/-}$ mice after LCMV infection.

Lymphocytes from $p52^{-/-}$ mice are able to respond against viral pathogen when transferred into wild type recipient

It is well known that the splenic microarchitecture is impaired in mice lacking NF-κB family members. In NF-κB $p50^{-/-}$ mice only the marginal zone B-cell population is impaired, while in $p52^{-/-}$ mice major defects in germinal centres, FDC networks and in the architecture of the marginal zone were found (7). We therefore questioned whether the observed inability of $p52^{-/-}$ mice to control LCMV infection is due to defects in the effector immune cell population or due to defects in priming as a consequence of disrupted splenic microarchitecture.

Wild type and NF-κB $p52^{-/-}$ recipient mice were thymectomized and treated with monoclonal antibodies to deplete the circulating $CD4^+$ and $CD8^+$ T-cells as described in material and methods. All recipient mice were controlled for successful depletion two weeks later by FACS analysis. Thereafter, wild type and NF-κB $p52^{-/-}$ recipient mice were reconstituted with naïve lymphocytes from wild type or NF-κB $p52^{-/-}$ sex matched donors (Fig. 7). Two days later, all mice were challenged i.c. with LCMV (10^3 pfu). Wild type mice, that either received lymphocytes from wild type or $p52^{-/-}$ donor mice, developed disease and immunopathology after virus inoculation into the brain. In contrast NF-κB $p52^{-/-}$ mice that either received lymphocytes from wild type or $p52^{-/-}$ donor mice did not develop disease and became virus carrier (Table 1). These data indicate that mice lacking NF-κB family members of the alternative pathway show an impaired cellular immune response against viral infection, due to the disrupted splenic microarchitecture but not due to impaired intracellular T-cell signalling.

T-cell chemoattractant CCL21 is almost absent in p52$^{-/-}$ mice

It is well known that the alternative NF-κB signaling pathway is responsible for the regulation of the T-cell chemoattractant CCL21. Therefore it was investigated, whether the level of CCL21 was altered in the spleens of wild type and p52$^{-/-}$ mice compared to CCL21 levels 4, 8 and 20 days after LCMV infection. In uninfected wild type mice 38 ±11 ng CCL21/g spleen were detected (Fig. 8A, black circles). LCMV infection led to a decrease of CCL21 levels in the spleens of wild type mice. At day 4 p.i. only 18 ± 3 ng CCL21/g spleen was found. Similar amounts were found at days 8 p.i. (18 ± 10 ng) and 20 days p.i. (17 ± 3 ng). In the spleens of NF-κB p52$^{-/-}$ mice only small amounts of CCL21 was detectable. In uninfected p52$^{-/-}$ mice 3 ± 0.6 ng/g spleen CCL21 was found (Fig. 8B, open circles). There were no changes in the CCL21 concentration after LCMV infection. Four days p.i. the spleens of p52$^{-/-}$ mice contained 3 ± 0.9 ng CCL21/g tissue, the amount of the cytokine did not alter at day 8 and 20 p.i. (4 ± 0.3 ng/g spleen). Taken together, these data indicate that down-regulation of the T cell chemoattractant CCL21 in p52$^{-/-}$ mice as a consequence of a disrupted splenic microarchitecture might be responsible for inadequate T-cell activity that is unable to control LCMV infection.

Discussion

The presented data demonstrate the importance of the alternative, but not the classical NF-κB pathway in the defence against LCMV infection. NF-κB p52$^{-/-}$ mice were unable to control LCMV infection when infected by three different routes, whereas NF-κB p50$^{-/-}$ efficiently eliminated the virus. Peripheral LCMV infection usually stimulates a robust CD8^{+} T-cell response that is responsible for eliminating infected cells and controlling LCMV infection (21). This CTL immune response was 10-fold reduced in NF-κB p52$^{-/-}$ mice. Those mice failed to clear LCMV and developed a persistent infection. Supporting this finding, the CTL-mediated immunopathology in the brain after i.c. LCMV infection was absent in NF-κB p52 deficient mice. The intensity of the LCMV mediated DTH reaction was also reduced. Depletion of participating T-cells revealed not only a reduced CD8^{+} T-cell activity, but also a defect in the CD4^{+} T-cell mediated immune response, since the elimination of those cells prevented any measurable swelling reaction. The failure of the T-helper response was also confirmed by investigating the humoral immune response after LCMV and in addition after VSV infection. NF-κB p52$^{-/-}$ mice showed only slightly reduced Ig levels but strongly impaired IgG responses after VSV infection compared to wild type mice. The analysis of the humoral immune response against LCMV nucleoprotein also demonstrated a reduced IgG

response in NF-κB p52$^{-/-}$ mice due to an abridged IgG2a and IgG3 response. These results give rise to speculate that the class switch from IgM to IgG was affected in those animals. This might be due to the disrupted splenic microarchitecture in NF-κB p52$^{-/-}$ mice. The T dependent B-cell response after antigen exposure infection is accompanied by formation of germinal centers (37, 66). Within these specialized microenvironments somatic mutation and affinity maturation take place (42). It was already described that NF-κB p52$^{-/-}$ mice lack germinal centers (62). Furthermore, the disorder of the humoral immune response to T-cell dependent antigens in those mice was demonstrated before (17, 62). In addition, NF-κB p52$^{-/-}$ mice were more susceptible than wild type mice when infected with *Toxoplasma gondii* (11, 13, 17). The clearance of this parasite is a CD4^{+} T-cell dependent process. The increased sensitivity to this parasite was explained by the insufficient activation of T-helper cells in NF-κB p52$^{-/-}$ mice. The data obtained in our study support these previous findings. However, the disability to control LCMV infection is primarily due to a dysfunction of the cytotoxic T-cell response. One might speculate that the CTL response is impaired because of the unavailable CD4^{+} T-cell help that often sustains the CTL response (14, 23, 45). In fact, mice deficient in CD4 are unable to control the LCMV infection by CTLs after high dose injection, but these mice mounted a normal CTL response after low dose inoculation (4). NF-κB p52$^{-/-}$ mice were unable to control a high dose as well as a low dose (data not shown) LCMV infection. The analysis of the cytotoxic activity of CTLs in the spleens of NF-κB p52$^{-/-}$ mice revealed a 10-fold reduction of the activity when compared to CTL activity in wild type mice. Additionally, we found substantial differences in the numbers of CD8^{+} T-cells in the spleen after LCMV infection. In NF-κB p52$^{-/-}$ mice the number of CD8^{+} T-cells was 4-fold reduced when compared to wild type mice. From these data one might argue that a functional defect of the CTL was responsible for these effects. However, the dysfunction of the cellular immune response after LCMV infection in NF-κB p52$^{-/-}$ mice was not due to a direct disorder of the CD8^{+} T-lymphocytes, since lymphocytes from NF-κB p52$^{-/-}$ mice achieved responsiveness when transferred into wild type recipient. This indicates that the disturbed splenic microarchitecture of NF-κB p52$^{-/-}$ mice was responsible for the inadequate cellular immune response after LCMV infection. In this regard the finding that after LCMV infection CCL21 is almost absent in NF-κB p52$^{-/-}$ mice supports the above hypothesis. CCL21 is a T-cell chemoattractant that is associated with modulation of responses. CCL21 is secreted by cells from the T-zone stromal cells and function as a ligand for CCR7 that is expressed on T-cells. CCL19 and CCL21 are crucial for the positioning of T-cells and dendritic cells (DC) within T-cell zones of secondary lymphoid organs (43-44, 67). Interestingly and in contrast to a

former publication using RAG deficient mice (52), the virus titer in the disrupted spleen of NF-κB deficient mice was not reduced (Fig. 1B) compared to wild type mice.

The splenic marginal zone is composed of metallophilic and marginal zone macrophages (28). Splenic marginal zone macrophages have been previously shown to be an essential cellular component in the clearance of LCMV (65). However, the spleens of NF-κB $p52^{-/-}$ mice contain marginal zone macrophages (62) and those cells are not relevant for the induction of cytotoxic T-cell responses (1). Certainly, within the highly organized microarchitecture of the spleen interactions between components of the innate and adaptive immune systems generate an efficient immune response. The priming of naive B- and T-cells occurs within the spleen (25). Dendritic cells (DCs) play a major role in the activation of the innate and adaptive immunity (2, 29-30). Those specialized antigen-presenting cells are able to induce antiviral cytotoxic T-lymphocytes responses (26, 38, 41). One of the factors determining the effectiveness of the T-cell response is the maturation status of the DCs. This maturation is highly dependent on the transcription factor NF-κB (68-69, 71). Moreover, two recent publications highlighted the essential involvement of the alternative NF-κB pathway in maturation and T-cell priming of DCs 20 (36, 49).

Conclusion

Taken together, we were able to demonstrate that the NF-κB p52 subunit is crucial for an adequate CTL response against LCMV. The underlying mechanism involves defects in maturation and priming rather than impaired intracellular signalling in the $CD8^{+}$ T-cells itself. The absence of NF-κB p52 leads not only to strong defects in lymphoid organs but it may also be essential for the defense against viral pathogens. The knowledge of the effects caused by modulation of multiple adaptive immune responses through NF-κB impairment may help developing new strategies for the prevention of autoimmune diseases. Manipulation of the immune system via the NF-κB pathway may also be beneficial for tumour vaccine strategies. Moreover, it was shown in the past that targeting cellular factors, like the classical NF-κB pathway, for an antiviral strategy is a very promising approach (39, 46). The present results further support this strategy since mice with a defect in the classical NF-κB signalling pathway ($p50^{-/-}$ mice) still mounted an appropriate cellular immune response against the virus.

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Disclosure Statement

No competing financial interests exist.

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Figure legends

FIGURE 1: Determination of immune responses after different routes of LCMV infections with wild type (black squares), NF-κB p50-/- (grey circles) and p52-/- (open triangles) mice. Kinetics of virus elimination in blood (A) and spleen (B): Mice were infected with 1 x 106 pfu LCMV-WE at day zero and spleen and blood samples were collected at the indicated time points. Infectivity was determined with diluted blood samples or diluted 10% spleen homogenates on MC57 cells after 48h cultivation. The specific staining of the infected cells was done with the LCMV-specific antibody VL4. Persistent virus infection was proven after 130 days p.i. in spleen and blood samples. C: Intracerebral LCMV-infection. Five mice of each genotype were infected intracerebrally with 1 x 10^3 pfu LCMV-WE. The results represent the median of two independent experiments with five mice/genotype. D: LCM virus specific foot pad swelling assay after subcutanous injection of 1.5 x 10^3 pfu LCMV-WE in

one foot pad. Foot pad thickness was determined at the indicated time points. The results represent the median of three independent experiments with five mice/genotype.

FIGURE 2: Foot pad swelling reaction after depletion of participating T-cells in wild type and NF-κB $p52^{-/-}$ mice. A: Swelling reaction in non depleted wild type (black squares) and NF-κB $p52^{-/-}$ mice (open triangles) after local inoculation of LCMV. B: DTH reaction in $CD8^+$ T-cells depleted wild type (black squares) and NF-κB $p52^{-/-}$ mice (open triangles). NF-κB $p52^{-/-}$ mice showed no measurable swelling C: Foot pad swelling after depletion of $CD4^+$ T-lymphocytes in wild type (black squares) and NF-κB $p52^{-/-}$ mice (open triangles). Both mice genotypes had a decreased foot pad thickness. D: A minimal swelling in wild type (black squares) and no swelling in NF-κB $p52^{-/-}$ mice (open triangles) was observed after depletion of both T-cell subtypes. The results represent the median of two independent experiments with four mice/genotype.

FIGURE 3: A: T-cell cytotoxicity assay after LCMV-WE infection. Wild type mice (black squares) and $p52^{-/-}$ mice (open triangles) were infected intravenously at day zero with 1.5 x 10^2 pfu LCMV-WE. At day eight spleen cells were isolated, incubated with ^{51}Cr-labelled and LCMV-WE infected MC57 target cells. B + C: Humoral immune response after VSV infection of wild type and $p52^{-/-}$ mice. Total Ig levels (B) were slightly reduced in $p52^{-/-}$mice (white triangles) compared to wild type animals (black squares). NF-κB $p52^{-/-}$ mice (white triangles) showed a strong reduction of IgGs (C), whereas wild type mice (black squares) had a normal IgG response to VSV. The results represent the median of two independent experiments with three mice/genotype.

FIGURE 4: Humoral immune response 4, 8 and 20 days after LCMV infection (10^6 pfu) of wild type and $p52^{-/-}$ mice. IgM (A), total IgG (B) and the IgG subclasses IgG1 (C), IgG2a (D), IgG2b (E) and IgG3 (F) were determined. NF-κB $p52^{-/-}$ mice (white triangles) showed similar IgM levels after LCMV infection compared to wild type animals (black squares). However, IgG was reduced in the sera of NF-κB $p52^{-/-}$ mice compared to wild type mice. The results represent the median of two independent experiments with three mice/genotype.

FIGURE 5: Immunohistochemistry of spleen sections of wild type and NF-κB $p52^{-/-}$ mice after LCMV infection (10^6 pfu). A + B: Staining of $CD4^+$ T-cells in uninfected and LCMV infected wild type (A) NF-κB $p52^{-/-}$ (B) mice and mice 4, 8 and 20 days past infection C + D:

Localization of $CD8^+$ T-cells in uninfected wild type (C) and NF-κB $p52^{-/-}$ (D) 21 mice and mice 4, 8 and 20 days past LCMV infection. White arrows indicate T-cells in the white pulp, red arrows show T-cells in the red pulp, black arrows show germinal centres. The figure represents the results from two independent experiments with four mice/genotype and time point.

FIGURE 6: FACS-analysis of spleen samples from wild type and NF-κB $p52^{-/-}$ mice after LCMV infection (1 x 10^6 pfu): Wild type (black bars) and $p52^{-/-}$ mice (white bars) were infected intravenously in the tail vein. On day 0, 4, 8 and 20 days p.i. spleen cells were analyzed using BD Bioscience FACSCALIBUR and Cell Quest software. The amount of cells 4, 8 and 20 days p.i. was given as a relative percentage to the amount of cells prior to infection. A: $CD4^+$ T-cell rate and B: $CD8^+$ T-cell rates in spleens of infected wild type (black bars) and $p52^{-/-}$ mice (white bars). The results represent the median of two independent experiments with three mice/genotype. *: Significant differences in the amount of $CD4^+$ ($p = 0.0069$) as well as $CD8^+$ T-cells ($p = 0.0051$) between wild type and NF-κB $p52^{-/-}$ mice were observed at day eight post-infection.

FIGURE 7: Adoptive transfer experiment in wild type and NF-κB $p52^{-/-}$ mice. A: Transfer of lymphocytes from NF-κB p52 deficient mice into wild type recipients leads to T-cell mediated meningitis and death after i.c. inoculation of LCMV. B: Transfer of wild type lymphocytes to NF-κB p52 deficient mice caused no T-cell mediated meningitis. The animals survived the i.c. LCMV infection.

FIGURE 8: Concentration of CCL21 in the spleens of wild type and NF-κB $p52^{-/-}$ mice. The amount of the T-cell chemoattractant CCL21 in spleens of uninfected and LCMV-infected wild type (black circles) and NF-κB $p52^{-/-}$ mice (white circles) was investigated by ELISA. NF-κB $p52^{-/-}$ mice showed reduced amounts of CCL21 compared to wild type animals. The results represent the median of two independent experiments with three mice/genotype.

Table

TABLE 1 : Summary of the adoptive transfer experiment

		No. of dead	time of	Virus titer at day 8 p.i. [a)]			
recipient	**donor**	**mice**	**death**	**blood**	**spleen**	**brain**	**liver**
C57Bl/6	C57Bl/6	3/4	9.0 ± 1.0	4.4 ± 0.1	4.2 ± 0.1	3.7 ± 0.1	1.8 ± 0.2
C57Bl/6	NF-κB52$^{-/-}$	3/4	10.3 ± 0.6	1.9 ± 0.3	1.7 ± 0.2	2.9 ± 0.3	2.7 ± 0.2
NF-κB2$^{-/-}$	NF-κB52$^{-/-}$	0/4	-	5.5 ± 0.1	4.3 ± 0.5	5.1 ± 0.1	4.8 ± 0.3
NF-κB2$^{-/-}$	C57Bl/6	0/4	-	5.7 ± 0.1	4.5 ± 0.4	5.1 ± 0.1	4.3 ± 0.1

[a)] virus titer is given as (log_{10})pfu/ml organ homogenate

Figures

Figure 1

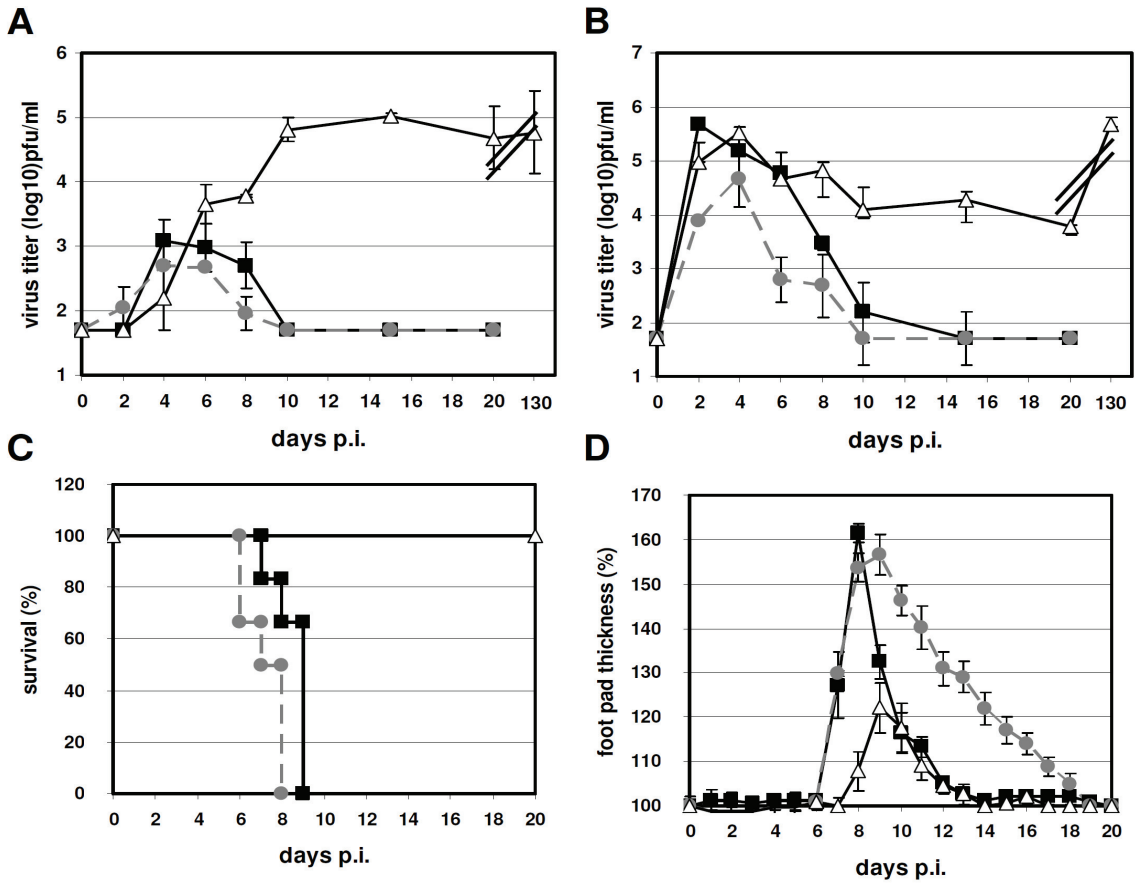


Figure 2

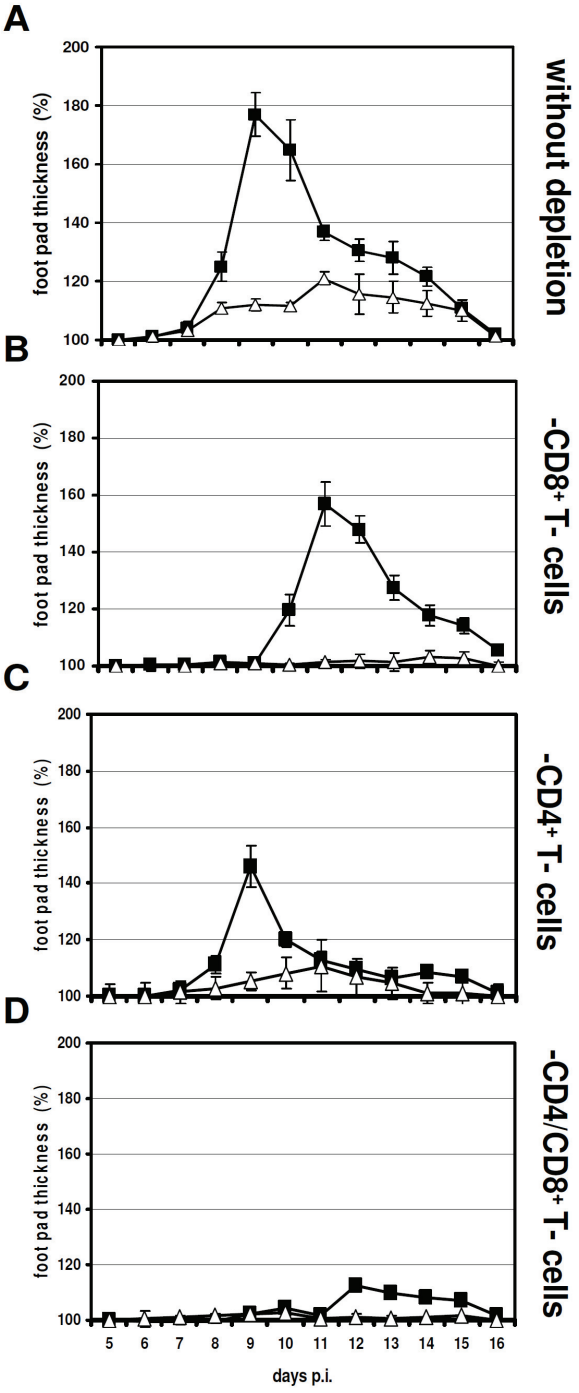

A
B
C
D
foot pad thickness (%)
200
180
160
140
120
100
without depletion
-CD8+ T- cells
-CD4+ T- cells
-CD4/CD8+ T- cells
5
6
7
8
9
10
11
12
13
14
15
16
days p.i.

Figure 3

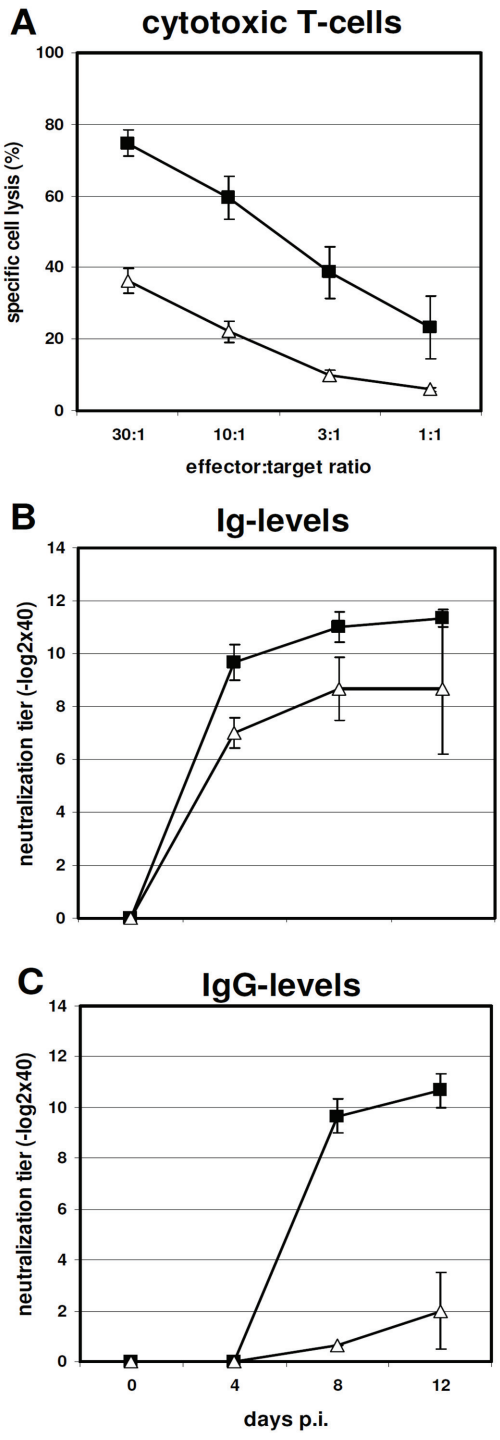

A
cytotoxic T-cells
specific cell lysis (%)
100
80
60
40
20
0
30:1
10:1
3:1
1:1
effector:target ratio
B
Ig-levels
neutralization tier (-log2x40)
14
12
10
8
6
4
2
0
C
IgG-levels
neutralization tier (-log2x40)
14
12
10
8
6
4
2
0
0
4
8
12
days p.i.

Figure 4

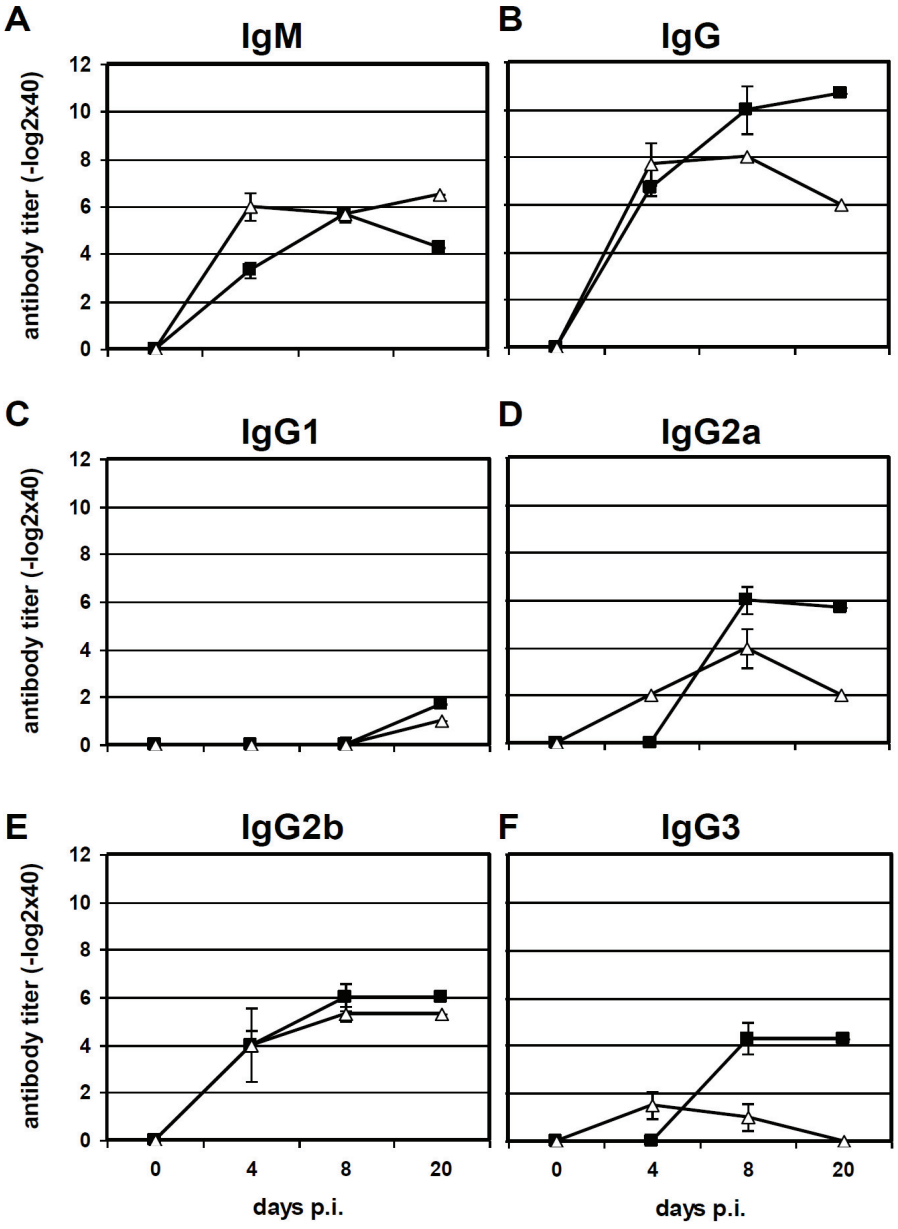

A
IgM
B
IgG
C
IgG1
D
IgG2a
E
IgG2b
F
IgG3
antibody titer (-log2x40)
0
2
4
6
8
10
12
0
4
8
20
days p.i.

Figure 5

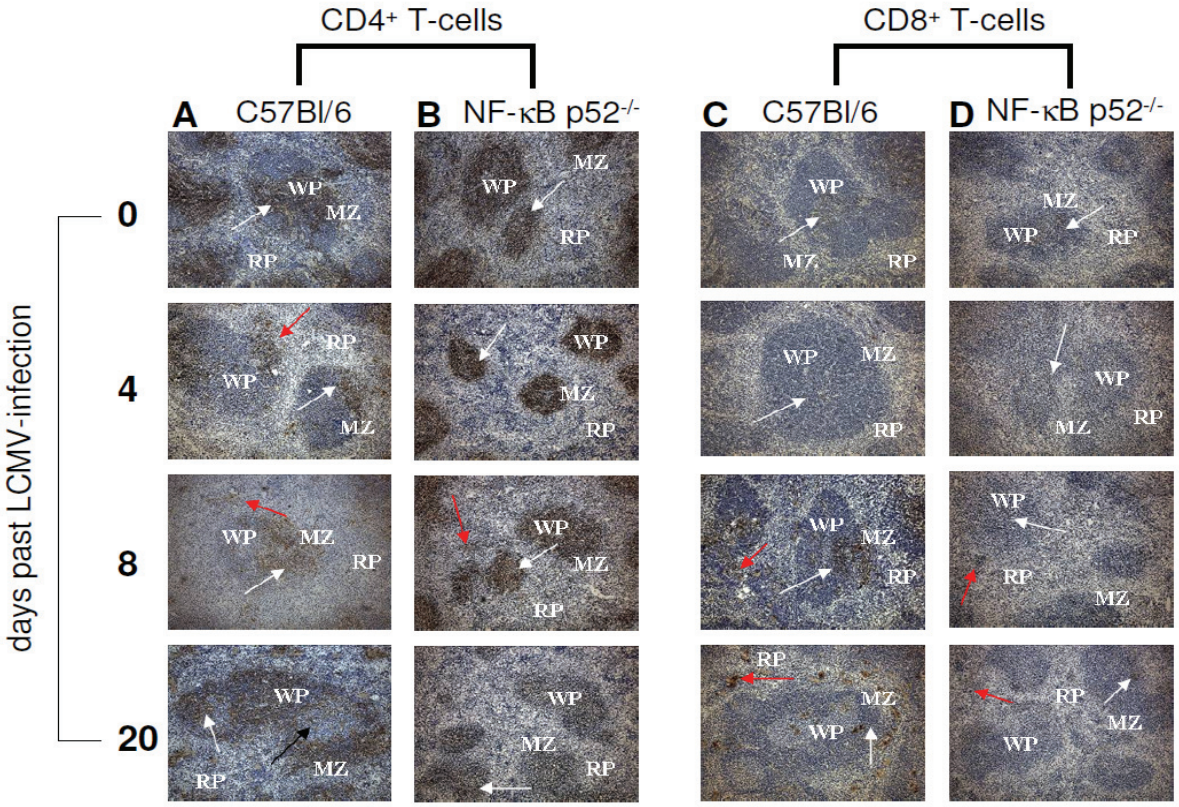


Figure 6

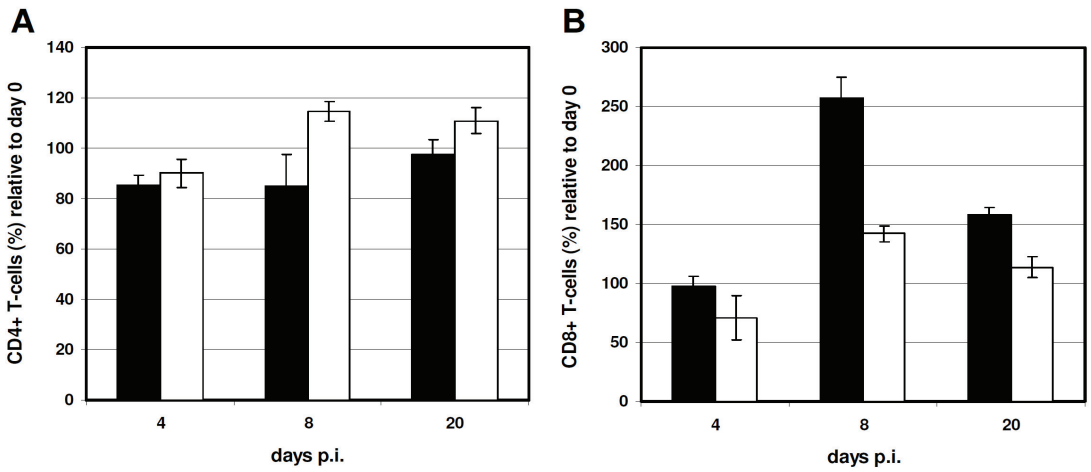


Figure 7

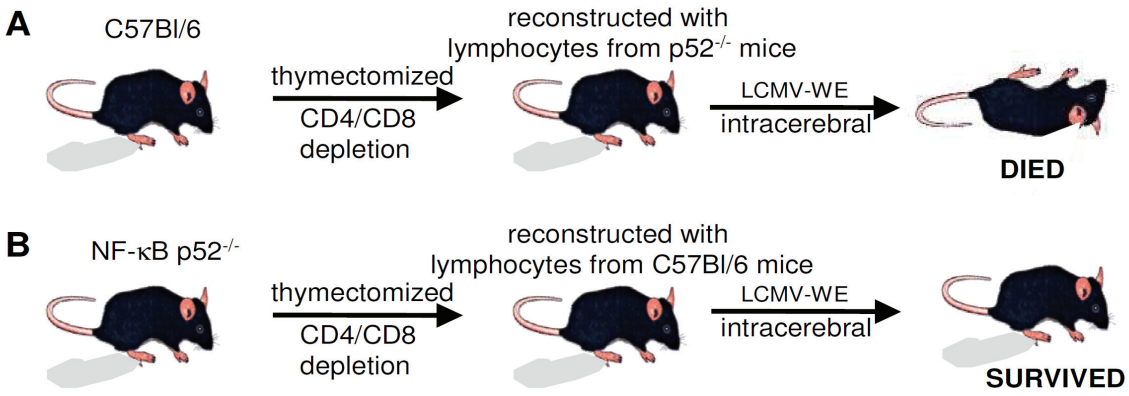
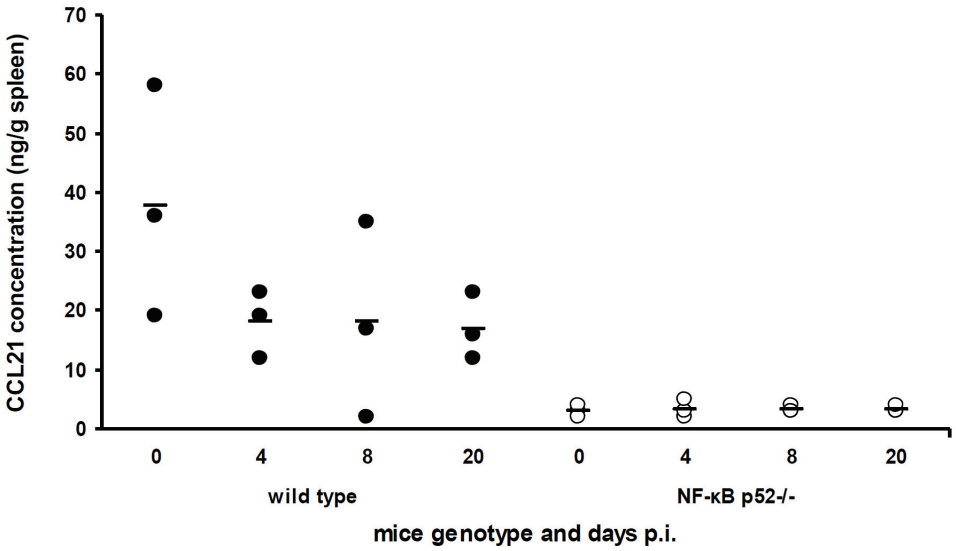


Figure 8



PUBLICATION #2:

Role of hypercytokinemia in NF-κB p50 deficient mice after H5N1 influenza A virus infection

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Abstract:

During H5N1 influenza virus infection, proinflammatory cytokines are markedly elevated in the lungs of infected hosts. The significance of this dysregulated cytokine response in H5N1-mediated pathogenesis remains to be determined. To investigate the influence of hypercytokinemia, or "cytokine storm," a transgenic mouse technology was used. The classical NF-κB pathway regulates the induction of most proinflammatory cytokines. Deletion of the p50 subunit leads to a markedly reduced expression of the NF-κB-regulated cytokines and chemokines. Here we show that H5N1 influenza virus infection of this transgenic mouse model resulted in a lack of hypercytokinemia but not in altered pathogenesis.

Main Body

Infections with H5N1 influenza A viruses cause severe illness and high mortality rates in mammalian hosts (15, 21, 23, 30). It has been described previously that this critical disease outcome is associated with a dysregulation of the immune system involving elevated levels of proinflammatory cytokines (cytokine storm or hypercytokinemia). This observation is based on a large variety of studies demonstrating a marked increase of cytokines and chemokines in the lungs of humans and animals during H5N1 influenza A virus infection (2, 8, 12, 19). In addition, the combination of acute respiratory distress syndrome, lymphopenia, and multiorgan failure characteristic of H5N1 disease in humans has previously been associated with cytokine dysregulation (6). It has been demonstrated in vitro that H5N1 infection causes significantly greater expression of cytokines than that induced by seasonal H1N1 or H3N2 infections (5). Nevertheless, hypercytokinemia was also found in macaques infected with the H1N1 influenza A virus responsible for the "Spanish flu" pandemic (11). In contrast, recent studies gave rise to a more controversial view on the role of hypercytokinemia in H5N1-mediated pathogenesis. Studies using transgenic mice lacking one cytokine revealed no altered pathogenesis after lethal H5N1 influenza virus infection (22, 25). However, hypercytokinemia is a multicytokine event (9, 24), so the effect of deleting only one cytokine might be of limited significance. The possibility to interfere with the majority of cytokines and chemokines is provided by the manipulation of the nuclear factor kappa B (NF-κB) pathway. The transcription factor NF-κB plays a central role in the regulation of a large variety of cellular events. It functions as a regulator of the expression of inflammatory cytokines, chemokines, immunoreceptors, and adhesion molecules. There are two different NF-κB pathways (4). The expression of many cytokines and chemokines involved in hypercytokinemia is regulated through the classical NF-κB pathway, which operates through

p50 and p65 subunits (18). Thus, using NF-κB p50 knockout mice for H5N1 infection represents a useful model to study the influence of hypercytokinemia in the pathogenesis after H5N1 infection.

To examine the local cytokine production in the lung after H5N1 influenza virus infection of C57BL/6 wild type (NF-κB $p50^{+/+}$), NF-κB $p50^{+/-}$, and NF-κB $p50^{-/-}$ mice, multiple cytokine assays were performed at 6 days post-H5N1 infection.

In our studies, we used a natural H5N1 field isolate, MB1 (A/mallard/Bavaria/1/2006) that is highly pathogenic to mice without adaptation. An upregulation of IP-10, gamma interferon (IFN-γ), interleukin-6 (IL-6), MIG, macrophage inflammatory protein 1β (MIP-1β), RANTES, IFN-ß, tumor necrosis factor alpha (TNF-α), IL-2, and IL-10, but not IL-4, was found in the lungs of infected wild type mice (Fig. 1A to L). These elevated mRNA levels correlated with protein expression of the different chemokines and cytokines in the lung (data not shown). NF-κB $p50^{+/-}$ (Fig. 1, gray bars) and NF-κB $p50^{-/-}$ (Fig. 1, white bars) mice revealed a strong reduction of most cytokines and chemokines after H5N1 infection. The amounts of IL-2, RANTES, MIG, and IP-10 were more reduced in NF-κB $p50^{+/-}$ mice than in NF-κB $p50^{-/-}$ mice (Fig. 1), without an influence on the pathogenesis. The levels of MIP-1β (Fig. 1K) and IP-10 (Fig. 1L) were equal to or even higher than those in wild type controls. One might argue that the high levels of IP-10 and MIP-1 in wild type and NF-κB p50-deficient mice are responsible for the severity of disease or pathogenesis. It was previously demonstrated that MIP-1-deficient mice showed no significant difference in mortality compared to wild type animals after H5N1 infection (25). Experiments with mice deficient in the IP-10 receptor ($CXCR3^{-/-}$ mice) demonstrated identical virus replication and mortality rates after H1N1 infection (27). Taken together, these results indicate that no hypercytokinemia was found in NF-κB $p50^{+/-}$ and NF-κB $p50^{-/-}$ mice after infection with the natural H5N1 field isolate. We next asked how the lack of hypercytokinemia would influence the pathogenesis in these animals after H5N1 influenza virus infection.

First, the 50% lethal infectious dose was determined (20) for wild type, $p50^{+/-}$, and $p50^{-/-}$ mice. Interestingly, H5N1 infection of wild type mice and both NF-κB p50-deficient strains revealed similar 50% lethal dose titers, of 1.7×10^3 PFU for $p50^{+/+}$ mice, 2.1×10^3 PFU for $p50^{+/-}$ mice, and 1.0×10^3 PFU for $p50^{-/-}$ mice. For further experiments, all three mouse strains were infected via the intranasal route with 5×10^3 PFU MB1 (H5N1). The mice were weighed and monitored every day. According to German animal protection law, mice were sacrificed when they lost 25 to 30% of their initial body weight. In addition to weight progress and survival rates, the onset and severity of disease were monitored. Surprisingly, there were no

differences in the onset of disease between the three mouse strains. All mice developed the first disease symptoms by day 7 postinfection (p.i.). Within a few days, the symptoms became severe and resulted in high disease scores for all three mouse strains (Fig. 2A). Furthermore, all groups of mice showed similar changes in body weight. The animals lost weight immediately after infection. Within 9 days, all mice had lost 30% of their initial body weight (Fig. 2B). The severe symptoms accompanied by weight loss finally resulted in death. Wild type and $p50^{-/-}$ mice died by day 9 p.i., and $p50^{+/-}$ mice succumbed to the infection within 8 to 10 days after H5N1 inoculation (Fig. 2C).

Next, we questioned whether viral tropism and virus loads were altered after H5N1 infection. Therefore, we performed virus titration of the organs by the Avicel method (16). Mice were infected with 5 x 10^3 PFU, and the distribution of the virus in the organs was investigated 6 days after infection. Nearly the same virus loads were detected in the lungs, blood, livers, spleens, hearts, and kidneys of infected animals. Interestingly, no virus was detected in the brains (Table 1). This experiment revealed no differences in viral load and viral tropism in H5N1-infected NF-κB $p50^{+/-}$ or NF-κB $p50^{-/-}$ mice compared to wild type controls. As reported previously, influenza A viruses need activation of the NF-κB signaling pathway, resulting in activation of TRAIL and FasL. Furthermore, caspase-3 activity is required for efficient nuclear export of the viral RNP complex (13, 28, 29). Using real-time reverse transcription-PCR, we demonstrated that TRAIL- (Fig. 3A) and FasL- (Fig. 3B) specific nucleic acids were found in all mouse strains after H5N1 infection, even though the amounts of TRAIL and FasL were slightly reduced in NF-κB $p50^{+/-}$ and NF-κB $p50^{-/-}$ mice. Nevertheless, TRAIL and FasL expression was sufficient to induce caspase-3 activity in the lungs of NF-κB $p50^{+/-}$ and NF-κB $p50^{-/-}$ mice, as indicated by Western blot analysis (Fig. 3C). These results demonstrate that the NF-κB p50 subunit has no influence on TRAIL, FasL, and caspase-3, all of which are required for efficient viral replication *in vivo*.

Since the transcription factor NF-κB is involved in the regulation of innate and adaptive immunity, we questioned whether a lack of NF-κB would influence the immune cell population after H5N1 infection. Therefore, we investigated the numbers of lymphocytes in the blood of H5N1 influenza virus-infected wild type and NF-κB p50-deficient mice. Whole blood samples were stained with fluorescent antibodies to detect $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$monocytes. The number of cells at 6 days p.i. was given as a relative percentage of the number of cells prior to infection. One hallmark of influenza A virus infection is an alteration in the lymphocyte population, also known as lymphopenia (7, 26). Consequently, one might expect a reduction of lymphocytes already in wild type mice

after H5N1 infection. All three mouse strains showed similar reductions of $CD4^+$ T cells after the infection. Even though NF-κB $p50^{+/-}$ (Fig. 3, gray bars) and $p50^{-/-}$ (Fig. 3, white bars) mice had increased numbers of $CD4^+$ T cells compared to wild type controls (Fig. 3, black bars), Student's *t* test revealed no significant differences in the numbers of $CD4^+$ T cells. Similar reductions of $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ cells (Fig. 3D) were observed in all three mouse strains 6 days after H5N1 infection. To summarize the data on disease outcome, viral load, and lymphopenia, there was hardly any difference in the pathogenesis between wild type and NF-κB p50-deficient mice after infection with the H5N1 natural field isolate.

The results revealed no involvement of hypercytokinemia or cytokine storm in H5N1-mediated pathogenesis. From our data, one might argue that inhibition of the local cytokine response in the lung is not sufficient to reduce the lethality and morbidity after infection with highly pathogenic avian influenza virus. Consequently, our data support the view that early interference with viral replication might be a more promising therapeutic strategy in supporting host survival after infection with a highly pathogenic influenza virus than inhibition of the local cytokine response in the lung by glucocorticoid treatment (22). Moreover, glucocorticoids inhibit the proliferation of antigen-specific plasma B cells (1), which are crucial for controlling the viral spread to organs.

Using a transgenic mouse technology that allows inhibition of the cytokine response and a natural H5N1 field isolate for our investigations, we cannot totally exclude the influence of cytokine storm on H5N1-mediated pathology in humans, in particular because influenza viruses easily mutate. Thus, an antiviral approach that inhibits virus replication and interferes with cytokine expression might be very promising for future therapeutic strategies. Previously, we published a study showing that influenza virus replication is dependent on the activation of the NF-κB signaling pathway (28) and that the NF-κB inhibitor ASA efficiently blocks influenza virus replication both in vitro and in vivo (17). In addition, ASA interacts with a second immunoregulatory pathway. It induces the proteasomal degradation of TNF receptor-associated factor 2 (TRAF2) and TRAF6, leading to diminished cytokine production (14). A recent publication identified the involvement of the Toll-like receptor 4–TRIF–TRAF6 pathway in cytokine production and lung injury after inoculation with inactivated H1N1 or H5N1 viruses, revealing a potential role for oxidative stress and the innate immune response as key regulators of lung injury. Blocking the Toll-like receptor 4–TRIF–TRAF6 pathway resulted in minor lung pathology (10). Thus, antiviral drugs that also target cellular components, in particular the NF-κB signaling pathway might be candidates for future therapeutic strategies against human infection with highly pathogenic influenza A viruses.

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Figure Legends

FIGURE 1: mRNA levels of cytokines and chemokines at 6 days p.i. with 5 x 10^3 PFU H5N1 in NF-κB $p50^{+/+}$ (black bars), NF-κB $p50^{+/-}$ (gray bars), and NF-κB $p50^{-/-}$ (white bars) mice after reverse transcriptase real-time PCR performed according to the manufacturer's protocol (Qiagen). (A) IL-2; (B) IL-4; (C) IL-10; (D) IL-6; (E) RANTES; (F) TNF-α; (G) IFN-α; (H) IFN-β; (I) IFN-γ; (J) MIG; (K) MIP-1β; (L) IP-10. The bars represent the mRNA levels for three infected mice compared to those for uninfected controls according to Boeuf et al. (3). This experiment was performed twice with similar results. *****, Student's *t* test revealed no significant difference between the three NF-κB mouse strains ($P > 0.1$). The amounts of cytokines were also analyzed at the protein level by use of Bio-Plex arrays, except for MIG, IP-10, and IFN-β, revealing similar increases of expression (data not shown).

FIGURE 2: Disease indexes (A), weights (B), and survival rates (C) after H5N1 infection. Five female NF-κB $p50^{+/+}$ (black squares), $p50^{+/-}$ (gray triangles), or $p50^{-/-}$ (open circles) mice at the age of 6 to 8 weeks, obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tübingen, Germany, were infected intranasally with 5 x 10^3 PFU H5N1 (A/mallard/Bavaria/2006) virus. For the estimation of the disease index, animals were scored with different points depending on their health status. Healthy animals scored 0, animals with one symptom (ruffled fur, reduction of normal activity rate, unnatural posture, or fast breathing rate) scored 1, animals with two symptoms scored 2, animals with three or more symptoms scored 3, and dead animals scored 4 for the rest of the 14-day observation period. The graphs represent the mean values for five animals. This experiment was performed twice with comparable results.

FIGURE 3: mRNA levels of TRAIL (A) and FasL (B) in lungs of NF-κB $p50^{+/+}$ (black bars), $p50^{+/-}$ (gray bars) and $p50^{-/-}$ (white bars) mice at 6 days p.i. with 5 x 10^3 PFU H5N1 virus relative to those in uninfected mice after a reverse transcriptase real-time PCR performed according to the manufacturer's protocol (Qiagen). (C) Western blot analysis for the detection of cleaved caspase-3 (anti-cleaved caspase-3 antibody [Cell Signaling]) in lungs of uninfected (left) and MB1 (H5N1)-infected (right) NF-κB $p50^{+/+}$, $p50^{+/-}$, and $p50^{-/-}$ mice at 6 days p.i. Detection of ERK (anti-ERK; Santa Cruz Biotechnology) was used as a loading control. (D) Lymphopenia after H5N1 infection. Whole blood samples were stained with fluorescent antibodies against CD3, CD4, CD8, CD19, and CD11b according to the manufacturer's protocol (BD Pharmingen). Reductions of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and

$CD11b^+$ cells in the blood of infected NF-κB $p50^{+/+}$, (black bars), $p50^{+/-}$ (gray bars), and $p50^{-/-}$ (white bars) mice at 6 days p.i. with 5 x 10^3 PFU H5N1 virus are shown relative to the levels in uninfected mice. Numbers of lymphocytes in the blood of uninfected mice were set at 100%. The bars represent the mean values for four animals. There were no significant differences in the reduction of lymphocytes between the NF-κB p50-deficient mouse strains and C57BL/6 controls ($P > 0.1$). This experiment was performed twice with similar results.

Table

Table 1: Distribution of MB1 virus after infection of NF-kB p50 mice

d6 p.i.	lung	brain	kidney	liver	spleen	blood	heart
$p50^{+/+}$	4.8 ± 0.3	< 1.7	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	4.2 ± 0.2	2.4 ± 0.7
$p50^{+/-}$	5.2 ± 0.3	< 1.7	3.1 ± 0.2	3.4 ± 0.1	3.5 ± 0.1	4.0 ± 0.2	2.6 ± 0.1
$p50^{-/-}$	5.2 ± 0.1	< 1.7	2.9 ± 0.4	2.8 ± 0.2	3.6 ± 0.1	3.6 ± 0.2	2.1 ± 0.4

Figures

Figure 1

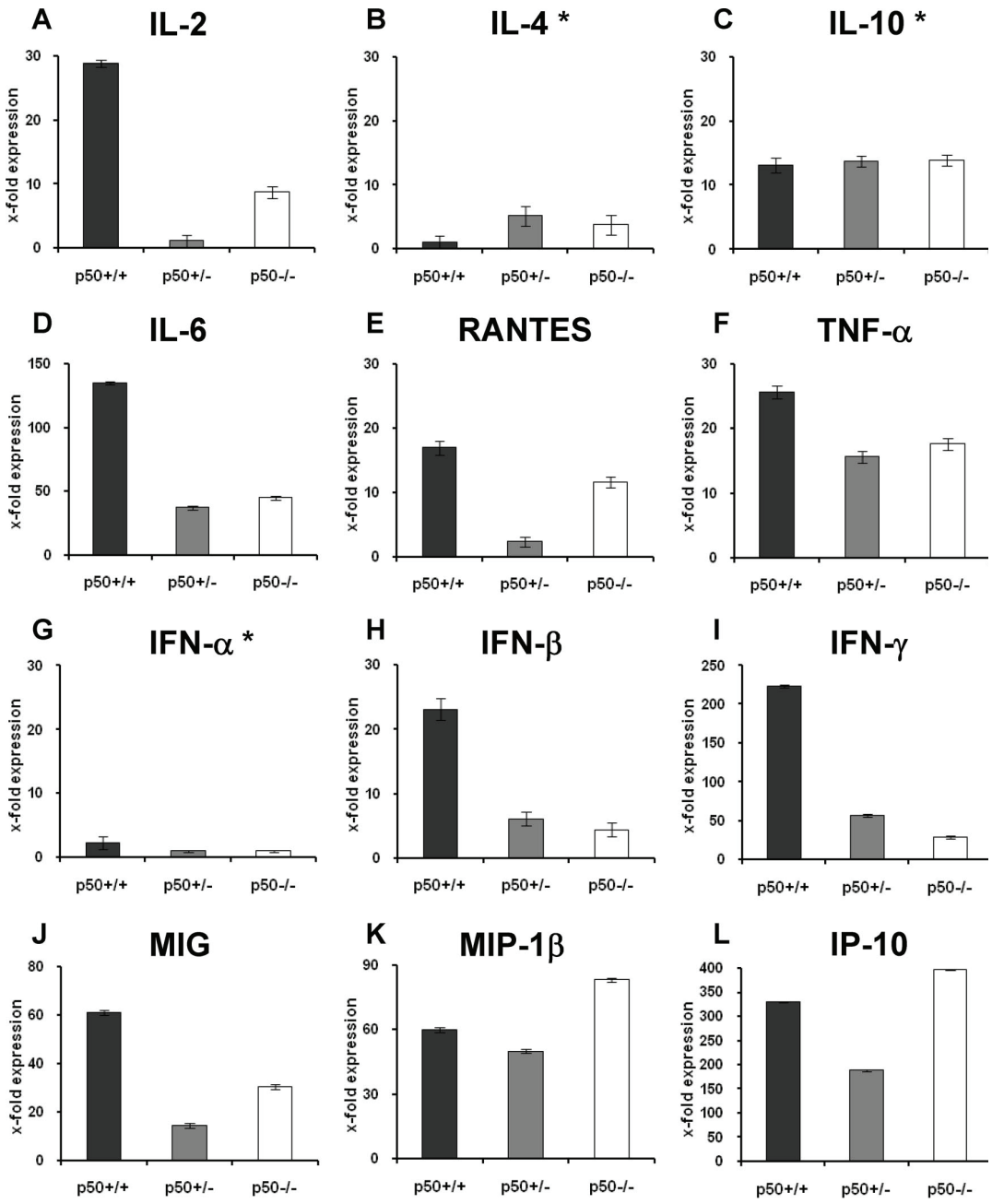


Figure 2

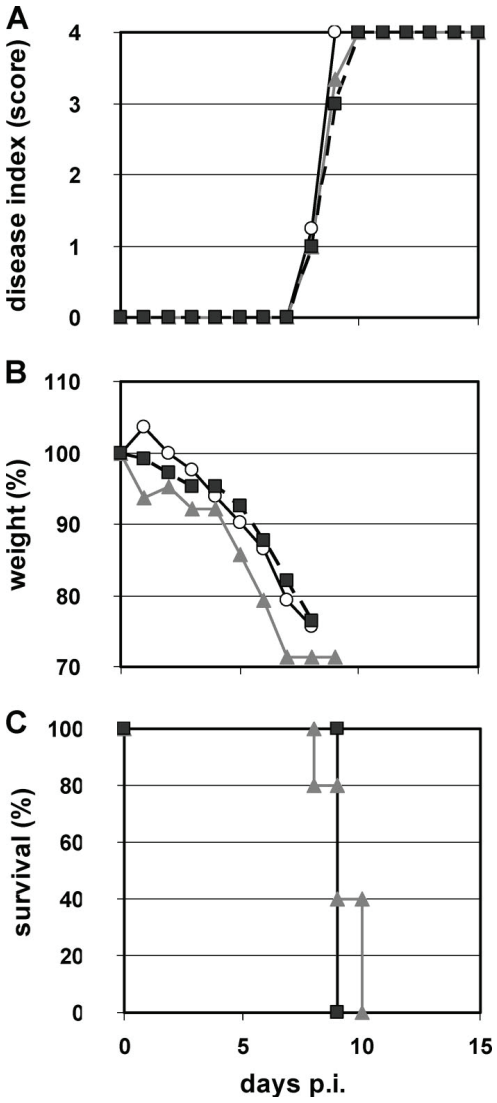
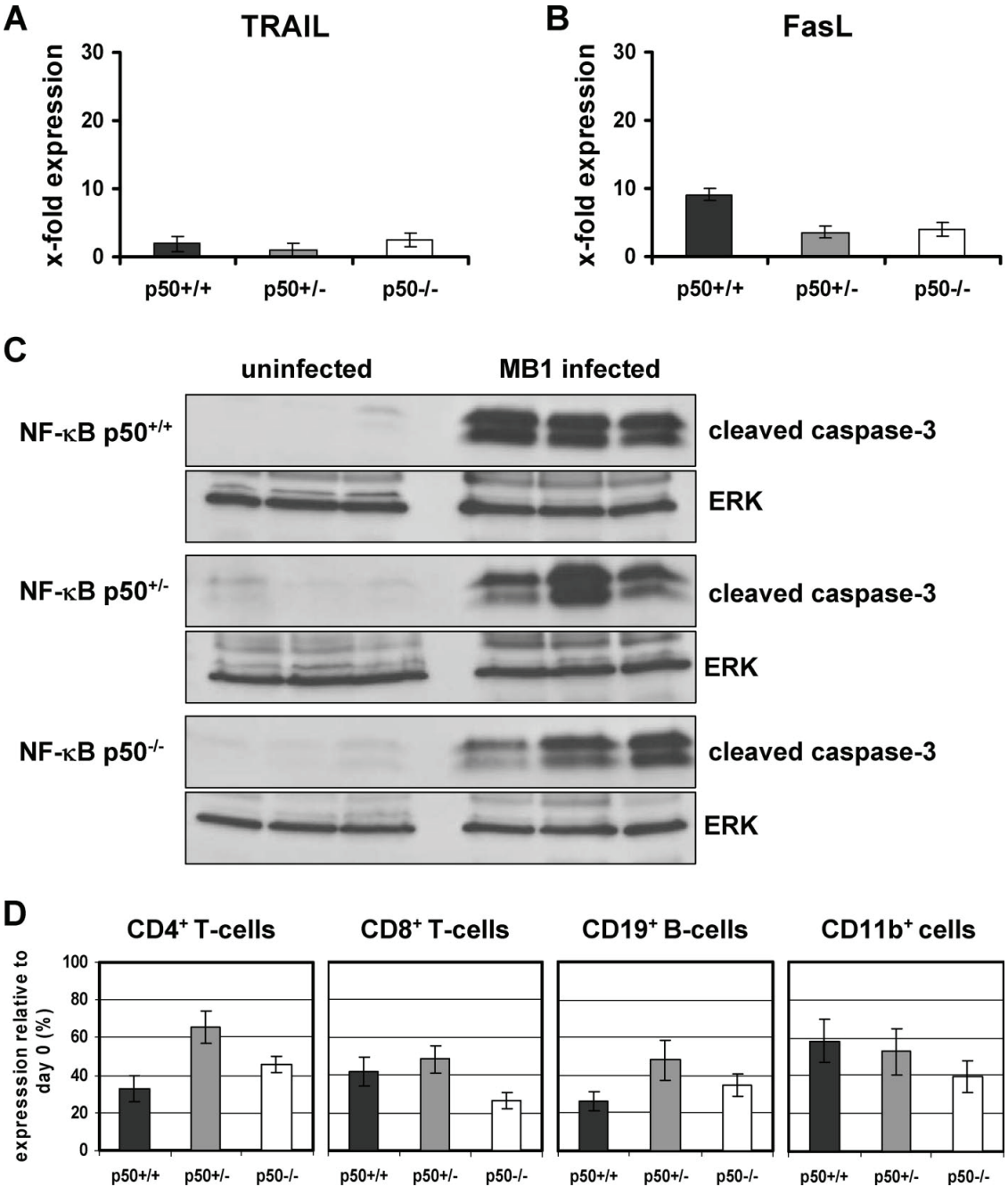

A
disease index (score)
4
3
2
1
0
B
weight (%)
110
100
90
80
70
C
survival (%)
100
80
60
40
20
0
0
5
10
15
days p.i.

Figure 3


A
TRAIL
x-fold expression
30
20
10
0
p50+/+
p50+/-
p50-/-
B
FasL
x-fold expression
30
20
10
0
p50+/+
p50+/-
p50-/-
C
uninfected
MB1 infected
NF-κB p50+/+
cleaved caspase-3
ERK
NF-κB p50+/-
cleaved caspase-3
ERK
NF-κB p50-/-
cleaved caspase-3
ERK
D
CD4+ T-cells
CD8+ T-cells
CD19+ B-cells
CD11b+ cells
expression relative to day 0 (%)
100
80
60
40
20
0
p50+/+
p50+/-
p50-/-

PUBLICATION #3:

Highly pathogenic influenza virus infection of the thymus interferes with T lymphocyte development

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Running Title: HPAIV infection of DCs modulates the adaptive immune response

Keywords: H5N1 influenza A virus, pandemic H1N1v, DC, thymus, lymphopenia

Abstract

Highly pathogenic avian influenza A viruses (HPAIV) cause severe disease in humans. Still the basis for their increased pathogenesis particularly with regards to the younger population remains unclear. Additionally, the recent pandemic H1N1v outbreak in 2009 demonstrated the urgent need for a better understanding about influenza virus infection. In the present study we demonstrated that HPAIV infection of mice led not only to lung destruction but also to functional damage of the thymus. Moreover, respiratory dendritic cells (RDCs) in the lung functioned as targets for HPAIV infection being able to transport infectious virus from the lung into the thymus. In addition the pandemic H1N1v influenza virus was able to infect RDCs without a proper transport to the thymus. Especially the strong interference of HPAIV with the immune system is devastating for the host and can lead to severe lymphopenia. In summary, from our data we conclude that highly pathogenic influenza viruses are able to reach the thymus via dendritic cells and to interfere with T lymphocyte development. Moreover, this exceptional mechanism might not only be found in influenza virus infection but also might be the reason of the increased immune evasion of some new emerging pathogens.

Introduction

Influenza A virus infection causes severe disease in humans and is a major topic in clinical health. While epidemic outbreaks of human influenza viruses cause over < 300.000 death worldwide per year (1), the recent swine origin influenza virus (SOIV) H1N1v pandemic outbreak demonstrates the full threat of influenza virus infection. So far SOIV infection in humans worldwide causes rather mild clinical symptoms though over 18.000 deaths in humans have been reported (WHO). In contrast, infection with the highly pathogenic avian influenza virus (HPAIV) H5N1 is more fatal to humans. Till today, 499 confirmed cases have been reported while 295 of them were lethal mainly in South East Asia (WHO). This fatality rate of roughly 59 percent demonstrates the urgent need to get a closer inside into the pathogenesis of HPAIV infections in humans, since H5N1 influenza virus is still considered as a potential pandemic strain. Moreover, a reassortant between H5N1 and pandemic H1N1v influenza virus might be a candidate for a serious pandemic influenza virus strain with high morbidity and more devastating high fatality rate.

While annual epidemic outbreaks typically affect people with an impaired immune system (e.g. elderly, infants and immunosuppressed people), severe influenza after HPAIV infection is mostly found in young and middle-aged people (2-3). Human infection cluster of H5N1

outbreaks with an average age of 13.7 and 15.4 years have been reported (4-5). The same phenomenon was found for the "Spanish Flu" pandemic (6) and the 2009 pandemic H1N1v strain (7). The reason for this altered host specificity remains unclear. One hallmark of H5N1 infection in humans is a strong reduction of lymphocytes, also known as lymphopenia. Lymphopenia is a common feature of some infectious diseases, in particular with new emerging pathogens (8-9). The mechanism of lymphopenia induced after H5N1 influenza virus infection is largely unknown. Remarkably, H5N1 influenza virus seems to reduce the effector function of $CD8^+$ T cells by an insufficient perforin expression (10). Moreover, it was reported that H5N1 viruses are able to infect cells of the immune system, leading to an impaired adaptive immune response (11). As a consequence the unbalance between innate and adaptive immune response might lead to a second hallmark of H5N1 infection, namely hypercytokinemia which is an increase of proinflammatory cytokines and chemokines in the lung (1).

The thymus, where T cell development takes place, presents an organ that is highly active during childhood, but stays functionally in young adults. The human thymus has its highest activity phase in an age group from 0 to 10 years and shows a major activity drop in the population older than 39 years. Nevertheless the thymus remains still active in the elderly population (12-13).

Bone marrow originated common lymphoid progenitor cells reach the cortical area of the thymus and become $TCR^+CD4^+CD8^+$ thymocytes. At this stage, the positive selection takes place. During this process thymocytes with a TCR that does not recognize self major histocompartibility complex (MHC) get lost. Surviving thymocytes migrate into the medulla of the thymus were self-peptides are presented. Thymocytes that recognize presented antigen undergo apoptotic processes and will be eliminated. Only thymocytes which do not recognize MHC class I or II presented self-peptide become naive T lymphocytes and migrate to the periphery. During the highly active life phase, the T lymphocyte production rate of the thymus ranges from 2×10^8 to 1.8×10^{10} cells per day in humans (14). Macrophages and dendritic cells in the thymus are also involved in the process of negative selection. It was a given fact for a long time that both cell types have their origin in the bone marrow and migration out of the thymus is an one way event. However, recent reports demonstrated that dendritic cells with extrathymic origin migrate also from the periphery into the thymus (15). The uptake of foreign antigens by DCs and the transport of these antigens into the thymus results in a development of a T cell tolerance against foreign antigens (16).

Fatal H5N1 influenza virus infection in mice results in massive lung damage originated by infiltrating immune cells namely macrophages and neutrophiles (17). T lymphocytes only play a secondary role for this damage. Moreover, $CD8^+$ T lymphocytes have a poor cytotoxic profile after influenza virus infection (18) and lymphopenia can be observed (1). We hypothesize that the influenza virus itself plays an active role in these findings. A conceivable mechanism could be the direct interference with the thymus as the primary lymphoid organ for T lymphocyte development.

In the present study we were able to demonstrate that highly pathogenic influenza viruses abuse the homing process of DCs into the thymus after infecting respiratory DCs (RDCs) in the lung. RDCs migrated into the thymus after activation by the infection with HPAIV and thereby interfere with the T lymphocyte selection processes. That way a reduced cellular immune response against the invading pathogen and the predominantly increased disease symptoms in younger people might be explained. Moreover, the atrophic process of the thymus observed during HPAIV infection, is a common feature of severe diseases like AIDS, Rabies and Chagas disease (19). Our results give rise to the assumption that this mechanism of increased pathogenesis is not only due to influenza viruses with pandemic potential, but also to infections with other new or reemerging pathogens like FMDV, Chikungunya, SARS and West Nile Virus (20-24).

Materials and Methodes

Mice

Inbred C57BL/6 and C57BL/6-Tg(ACTB-EGFP)1Osb/J (C57BL/6-GFP^+) mice at the age of 8 to 10 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Tuebingen, Germany and were used throughout all the experiments. The C57BL/6-Tg(ACTB-EGFP)1Osb/J mouse strain was originally obtained from Charles River Germany (Wiga).

Virus and infection

Two different highly pathogenic avian influenza virus strains were used throughout this study. The mouse-adapted avian influenza A/FPV/Bratislava/79 (H7N7) virus was grown on Madin-Darby canine kidney (MDCK) cells. The Bratislava strain of the H7N7 avian influenza A virus was originally obtained from the Institute of Virology, Justus-Liebig University, Giessen, Germany. The avian influenza A H5N1 virus A/mallard/Bavaria/1/2006 grown in embryonated chicken eggs was originally obtained from the Bavarian Health and Food Safety

Authority, Oberschleissheim, Germany. Like the H5N1 strain, the low pathogenic avian influenza virus (LPAIV) strain A/mallard/Bavaria/1/2005 (H5N2) was also obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany and propagated on MDCK cells (25). The SOIV strain A/Regensburg/D6/09 (H1N1v) was obtained from the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Riems, Germany was grown on MDCK cells.

All animal studies were approved by the Institutional Animal Care and Use Committee of Tuebingen. For infection of mice, the animals were anaesthetized by intraperitoneal injection of 200 µl of a ketamine (Sanofi) -rompun (Bayer)-solution (equal amounts of a 2%-rompun-solution and a 10%-ketamin-solution were mixed at the rate of 1:10 with PBS) and infected intranasal (i.n.) with a 10-fold MLD_{50} specific for each HPAI virus strain ($2x10^3$ pfu for H7N7, $2x10^4$ pfu for H5N1). For both, the low pathogen H5N2 and the H1N1v strain, $1x10^4$ pfu (< MLD_{50}) was used for infection.

Antibodies and flow cytometry analysis

The following fluorochrome conjugated antibodies were purchased from BD Bioscience: mAbs to mouse CD4 (L3T4), CD8a (Ly-2), CD11C (Integrin αx chain), CD3 (CD3ε chain), Siglec-F and CD103. Additionally, goat anti- influenza A antibody (AbD Serotec) and donkey anti-goat Alexa Fluor 647 (Invitrogen) were used. For flow cytometry analysis, a single-cell suspension was stained in FACS-buffer (PBS, 2% FCS, EDTA, pH 7.5) with required fluorochrome conjugated antibodies for 30 min at 4°C. After the incubation time, cells were washed and analyzed by the FACSCalibur (BD Bioscience).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the cell homogenate of organs using TRIZOL reagent (Invitrogen). 1 µg RNA was used for qRT-PCR to determine the expression of Gapdh, CXCL12, CCL25, ICAM-1, and V-CAM using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's protocol and the SmartCycler™ System and software (Cepheid, Sunnyvale, CA). The following specific primer for qRT-PCR were used: Mm_Gapdh_2_SG, Mm_Cxcl12_1_SG, Mm_Ccl25_1_SG, Mm_Icam1_1_SG and Mm_Vcam1_1_SG (Qiagen). The protocol for qRT-PCR was an initial incubation at 50°C for 10 min after that 95°C for 3 min, followed by 40 cycles of 95°C for 10s, 60°C for 30 sec. Afterwards, melt curve data were collected from 40°C to 90°C at a ramping rate of 0.2°C per second and finally cooling to 40°C was performed. The relative expression values were

normalized to the expression value of the housekeeping gene Gapdh.

Detection of viral M-protein RNA was performed as previously described (26) using the following oligonucleotides: 5-AGA TGA GTC TTC TAA CCG AGG TCG-3' (forward), 5'-TGC AAA AAC ATC TTC AAG TYT CTG-3' (reverse) and 5'-Tet-TCA CCC CTC AAA GCC GA-BHQ-1-3' (sonde; Metabion, Germany).

In vitro cytotoxicity assay

Effector cytotoxic $CD8^+$ lymphocyte activity was measured by in *vitro* cytotoxicity assay. Therefore, five mice were infected with a 10-fold MLD_{50} of H7N7 or H5N1 virus. 6 days p.i., mice were sacrificed and leukocytes from the lung and thymus were isolated. Meanwhile, $H2b^b$ MC57 target cells were loaded with the immunodominant peptide $NP_{366-374}$ in a concentration of 10 µg/ml and labeled with 0.2 mCi of ^{51}Cr at 37°C for 1 h. For all avian influenza virus strain the peptide ASENMEAM was used, for pandemic H1N1v the peptide ASNENVEIM was utilized. Target cells were co incubated with the isolated leukocytes at various effector-to-target ratios in a final volume of 200 µl/well. After 5 h, supernatant was collected and measured for the presence of released ^{51}Cr using the micro-beta counter (Wallace). The percentage of ^{51}Cr release was calculated according to the formula previously described (27).

Influenza virus titration (AVICEL® plaque assay)

The titration in 96 well plates was performed as described previously (25). Briefly, organs from sacrificed mice were homogenized in a ten percent homogenate in saline buffer and target cells were incubated with the homogenate. For H5N2 and H1N1v, trypsin was added during the incubation period (Gibco). Thereafter, cells were immunostained by incubating for one hour with a monoclonal antibody specific for the influenza A virus nucleoprotein (AbD Serotec) followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA). Labelling was visualized by 10 min incubation with True BlueTM peroxidase substrate (KPL). Stained plates were scanned on a flat bed scanner and the data were acquired by PhotoStudio5.5 (ArcSoft) software. Stained foci were counted. The virus titer is given as the logarithm to the base 10 of the mean value. The detection limit for this test was <1.7 log 10 ffu/ml.

In situ histology

For *in situ* hybridization various organs including lung, thymus, brain, heart and kidney were removed, fixed over night in 4% buffered paraformaldehyde and embedded in paraffin. 5 µm thick tissues sections were used for *in situ* stainings.

In situ hybridization

Influenza RNA in tissues was detected using single-stranded 35S-labeled RNA probes which were synthesized from a bluescript KS+ vector containing a fragment of the NP gene (nt.1077 to nt.1442) of A/FPV/Rostock/34 (H7N1) as previously described (28). Control RNA probes were obtained from a transcription vector containing the plasmid of coxsackievirus B3 (pCVB3-R1; (29). Pretreatment, hybridization, and washing conditions of dewaxed paraffin tissue sections were performed as described previously (29). Slide preparations were subjected to autoradiography, exposed for three weeks at 4°C and counterstained with hematoxylin/eosin.

Preparation of single cell suspension for DC isolation

Isolation of DCs was performed as described previously (30). Mice were sacrificed and lungs were perfused with 10 ml phosphate-buffered saline (PBS). Afterwards lungs or thymi were minced and digested in RPMI 1640 medium (Gibco) together with 0.125% collagenase II (Roche) for 30 min at 37°C in 5% CO_2. Thereafter, the minced and digested fractions were passed through a 70 µm BD cell strainer (BD Bioscience) and washed three times. The cell pellets were resuspended in PSA containing 2 mM EDTA and 0.5% bovine serum albumin for the following DC purification steps.

Purification of RDCs and DCs

RDCs were isolated as previously described (30). Briefly, alveolar macrophages were removed by incubating the counted cells with a PE-labeled anti-SiglecF antibody. Thereafter, magnetic microbeads were used for cell separation according to the manufacturer's protocol (Miltenyi Biotec GmbH). Cells were labeled with a magnetic anti-PE-microbead and separated by negative selection using an Auto-MACS Separator (Miltenyi Biotec GmbH). In the next step, the $SiglecF^-$ fraction was incubated with anti-CD11c magnetic microbeads and $CD11c^+$ $SiglecF^-$ cells were obtained by positive selection according to the manufacturer's protocol (Miltenyi Biotec GmbH). Isolation of thymus DCs was accomplished similar to the

isolation steps of RDCs. In contrast to the RDC purification protocol, SiglecF$^+$ macrophages were not depleted in the thymus.

Adoptive transfer

Donor C57BL/6-GFP$^+$ mice were infected with the H7N7 influenza virus and scarified 3 days after infection. 4.5x10^6 CD11c$^+$SiglecF$^-$ cells were isolated from the lung of C57BL/6-GFP$^+$ mice and were injected i.n. into C57BL/6 wildtype recipient mice. Two days later, C57BL/6 recipient mice were also scarified and both lung and thymus were harvested. Single cell suspensions were generated and CD11c$^+$ cells were isolated using magnetic microbeads according to the manufacturer's protocol (Miltenyi Biotec GmbH). Purification was analyzed with a FACSCalibur (BD Bioscience). Cells were surveyed by immunofluorescence.

Immunofluorescence

Isolated CD11c$^+$ cells were fixed for 30 min in 4% buffered paraformaldehyde at 4°C. The fixed cells were washed twice with PBS and incubated with DAPI. Afterwards, cells were washed again, mounted with ProLong Gold Antifade Medium (Invitrogen) and were analyzed by the Apotome Care Invert Microscope Labovert FS (Leitz) and the AxioVision Rel 4.5 (Carl Zeiss Imaging).

Immunohistology

Two-micrometer-thin slides were obtained from paraffin embedded organs. Sialic acid staining was performed as previously described (31). Briefly, the staining of sialic acid α-2.6 linked to galactose was performed with *Sambucus nigra* agglutinin, while for sialic acid α-2.3 linked to galactose *Maackia amurensis* agglutinin was utilized (Vector Laboratories). Signal detection was visualized by ABC- and DAB kit according to the manufacturer's protocol (Vector Laboratories). For viral detection, slides were incubated with the goat α-influenza A antibody (AbD Serotec) and the ImmPRESS™ REAGENT Anti-Goat Ig Kit (Vector Laboratory) was used for secondary staining. Substrate reaction was accomplished with DAB kit (Vector Laboratory) and counterstaining was performed with hematoxylin Gill II (Carl Roth GmbH). The staining was evaluated by microscope (Leitz) and photographed with the DC 300 (Leica).

Statistical analysis

Error bars are given as the standard error of the mean. For the investigation of significant differences (p-value < 0.05), paired *t-test* was performed.

Results

H5N1 influenza virus interferes with T lymphocyte development

To investigate the mechanism of influenza virus mediated lymphopenia, mice were infected for 6 days with either H7N7 or H5N1, two highly pathogenic avian influenza virus strains, the pandemic H1N1v human influenza strain or with a low pathogenic H5N2 virus of avian origin. Thereafter, the animals were sacrificed and the morphology of the thymus was analyzed. During infection with either H7N7 or H5N1 virus the thymus underwent a strong atrophic process that led to a drastically reduced size already 6 days after virus infection (Fig. 1A, B lower panel) which was not found after H1N1v or H5N2 influenza virus infection (Fig. 1C,D lower panel). H7N7 infection led to an 84% reduction of the total leucocyte count (Fig. 1E white bar). After H5N1 infection the total leucocyte amount was even more drastically reduced to less than 5% compared to total leucocytes of the thymus of uninfected mice (Fig. 1E vertical line bar). No significant leucocyte depletion could be observed after H5N2 or H1N1v infection (Fig. 1E, grey and horizontal line bar). Analysis of cells within the thymus revealed that especially the $CD4^+CD8^+$ thymocytes population was nearly absent after HPAIV infection. While $CD4^+CD8^+$ thymocytes of control, H5N2 and H1N1v infected mice presented about 80% to 85% of the cells in the thymus and extinguished about 1×10^8 cells. This population was reduced to 5% dropped to around 1×10^6 thymocytes after H7N7 but also H5N1 infection (Fig. 1F; Supplementary Fig. 1). These results suggested that HPAIV infection of mice interferes with T lymphocyte development or thymocyte progenitor cells. Since the bone marrow is the source for thymocytes progenitor cells, we next investigated whether highly pathogenic avian influenza virus is present in the bone marrow using quantitative real-time PCR to detect viral RNA. However, no viral nucleic acid could be found in the bone marrow in mice 3 and 6 days after infection with either H7N7 or H5N1 (data not shown). These results indicated that the bone marrow itself is not targeted by HPAIV influenza virus infection. To further investigate the influence of influenza virus infection of the thymus, expression of chemokines (CXCL12, CCL25) and cell adhesion molecules (ICAM-1, VCAM-1) that are involved in T lymphocyte development were analyzed. Infection of mice with H7N7 led to a strong down regulation of mRNA encoding for CXCL12 and CCL25 which are produced by thymic epithelial cells in the cortex and the

medulla (Fig. 2A). The mRNA encoding for either CXCL12 or CCL25 was less reduced after H5N1 infection. In contrast ICAM-1 mRNA expression at day 6 was even slightly increased (Fig. 2B). No strong alterations of the thymus or thymocyte development were found after H5N2 or H1N1v influenza virus infection (Fig. 2C and D). These results indicate that the thymus T lymphocyte development is altered during an HPAIV infection and give rise to the assumption that the function of thymic epithelial cells is impaired. Thus HPAIV infection affects the thymus and could interfere with the thymic homeostasis.

Presence of influenza virus specific $CD8^+$ T cells in the thymus after HPAIV infection

The previous results allowed the idea that the altered function of the thymic epithelial cells might have an influence on the impaired T lymphocyte development in the thymus after HPAIV infection including the dramatic loss of $CD4^+CD8^+$ thymocytes. Therefore, we next questioned whether the activation status of the immune lymphocytes in the thymus was altered after influenza virus infection. While in the thymus of uninfected mice the amount of activated thymocytes is less than 5% within this population, the number increased up to about 50% after H5N1 or H7N7 infection. No significant change of the thymocyte activation was observed after H5N1 or H1N1v influenza virus infection. However, HPAIV infection also changed the activation status based on CD69 expression of the single positive lymphocyte populations in the thymus. While there was no strong increase of the activated $CD4^+$ T lymphocyte population, the activated $CD8^+$ T lymphocyte fraction rose from 23% of uninfected mice up to 47% of H5N1 infected mice (Supplementary Fig 2). To determine whether activated $CD8^+$ T lymphocytes in the thymus were specific for influenza virus, an *in vitro* cytotoxicity assay was performed using target cells labeled with the immunodominant peptide $NP_{366-377}$ derived from the viral nucleoproteins (Fig. 3). As a control, influenza virus specific $CD8^+$ T lymphocytes could be detected in the lung of mice after either H5N1, H7N7, H1N1v or H5N2 influenza virus infection (Fig. 3 A-D, black solid line). Most surprisingly after H7N7 and H5N1 infection of mice nucleoprotein specific $CD8^+$ T lymphocytes were also found in the thymus (Fig. 3A,B grey solid line). In contrast, no $NP_{366-377}$ specific $CD8^+$ T cells were notable in the thymus after H5N2 or H1N1v infection (Fig. 3C,D grey solid line). These results demonstrated that after infection with both HPAIV strains functional influenza virus specific cytotoxic $CD8^+$ T lymphocytes were present in the thymus to recognize infected target cells in an MHC class I restricted manner.

Infectious H5N1 and H7N7 influenza viruses are present in the thymus

The above results indicate a direct interaction of H5N1 and H7N7 influenza virus with the thymus. Avian influenza viruses require Siaα2-3gal as a receptor. Siaα2-6gal is a prerequisite for infection with human influenza viruses. To determine whether the thymus of mice and humans are sensible for avian or human influenza virus infection, immunohistology was performed to scrutinize the distribution of Siaα2-3gal and Siaα2-6gal. In addition to lung and mediastinal lymph nodes, Siaα2-3gal and Siaα2-6gal expression was found in the thymus of both species, whereas especially the medulla region showed positive signals for Siaα2-3gal and Siaα2-6gal (Fig. 4A, B). To analyze whether infectious influenza virus is present in the thymus of mice, virus infectivity assays were performed. H7N7 and H5N1 influenza virus was found to high titers in the lung and to moderate titers in the med. LN's and in the thymus. In addition virus was present in the spleen after H7N7 but not after H5N1 infection. In the lung of H1N1v infected mice, comparable titers to H5N1 and H7N7 infected mice were found in the lung. In contrast only low amounts of infectious virus were present in the med. LN's. In only one out of four thymi few infectious virus particles could be detected. After H5N2 influenza virus infection low amounts of virus were found in the lung and only one out of four mice revealed some infectious particles in the med. LN's. No virus was found in the thymus. (Table I). Quantitative real-time PCR (qRT-PCR) was used to confirm the data achieved by virus infectivity assay after HPAIV infection and to observe the progression of viral load in the lymphoid organs (Fig. 5A). The results demonstrate viral nucleic acid in the thymus of mice after infection with the HPAIV virus strains. In addition, viral RNA was imaged by *in situ hybridization* in the lung and thymus (Fig. 5B). The *in situ* hybridization experiments confirm the existence of viral footprints in both organs after H5N1 and H7N7 influenza virus infection and localized the infectivity in the thymus to the cortico-medullary junctions. By imaging the regions of the thymus which are virus positive, the question appears which route of infection the virus uses to reach these specific thymus cells.

Infection of lung dendritic cells by H5N1, H7N7 and human pandemic H1N1v influenza virus

Circulating DCs in addition to mature peripheral T lymphocytes can re-enter the thymus after the uptake of antigenic material in the periphery (16). Next to this DC attribute, it was demonstrated that DCs are a target for influenza virus infection (30). To investigate whether DCs harbor influenza virus, mice were sacrificed and respiratory DCs were isolated 3 days after infection. DCs were identified by $CD11c^+$ and $SiglecF^-$ staining. CD11c is found on DCs and macrophages. SiglecF, a sialic acid binding immunoglobulin-like lectin, is expressed by

macrophages but not by dendritic cells. Flow cytometry analysis demonstrated a high infection rate of DCs either after H5N1 or H7N7 influenza virus and to a lower extends also after pandemic H1N1v infection (Fig. 6A,B). H5N1 virus infected about 19.0% of DCs in the lung, while 13.5% of DCs were infected with H7N7 influenza virus. Only 9.2% DCs were infected with the H1N1v virus strain. In contrast, after H5N2 influenza virus infection of mice no $CD11c^+$ cells harbored viral footprints. To define the DC subpopulation being the target for influenza virus in more detail, DCs were stained against CD103 which is a marker for mucosal DCs (Fig. 6A; middle row). The flow cytometry analysis demonstrated that the $CD11c^+/CD103^+/SiglecF^-$ DC population is a preferred target for influenza virus. Nevertheless $CD11c^+/CD103^+/SiglecF^-$ DC's present roughly only 13% a of the dendritic cell population (data not shown). Our results indicate that macrophages are not the obvious main target for influenza virus infection. These findings suggest a potential role of migrating DCs in the viral spread to the thymus.

To investigate whether lung DCs migrate to the thymus after infection with HPAIV, DCs from H7N7 infected C57BL/6-GFP^+ mice were isolated and transferred into the lungs of GFP^- mice (Fig. 7A). Three days after the transfer, recipient mice were sacrificed and GFP^+ DCs were isolated from the lung and thymus. Isolated DCs were characterized by immunofluorescence. The analysis clearly demonstrated that GFP^+CD11c^+ cells were detectable in lung and most interesting in the thymus of recipient wildtype mice, providing evidence that respiratory DCs migrate to the thymus after infection with HPAIV (Fig. 7B). These results strongly propose a model by which HPAIV hitchhike DCs in the lung for trafficking into the thymus, leading to alterations of the antigen presentation for developing thymocytes.

Discussion

A common feature of human infection with pandemic avian influenza virus is a strong reduction of T lymphocytes. This feature, called lymphopenia, is also found in some other infectious diseases, in particular with new emerging pathogens (8-9). Nevertheless, the reason and mechanism for the declining number of lymphocytes remains unclear (32). To investigate a possible mechanism for lymphopenia after influenza virus infection we focused on the thymus as a primary lymphoid organ. We hypothesized that highly pathogenic influenza A virus infection of mice interferes with T lymphocyte development and selection processes in the thymus which consequently leads to an impaired cellular immune response. The hypothesis was developed, since the morphology of the thymus revealed massive destruction

after H5N1 and H7N7 influenza virus infection of mice but not after pandemic H1N1v and low pathogenic H5N2 influenza virus infection. As a consequence reduced or even missing naive T lymphocytes outflow could lead to the observed lymphopenia. We speculate a direct involvement of the highly pathogenic avian influenza A virus. Two reasons for such a dysregulation followed by the destruction of the T lymphocyte producing site might be possible. First, the virus stops the influx of T lymphocyte progenitor cells directly by infecting the bone marrow which is the source of T cell progenitors. Second, influenza virus infection interferes with T lymphocyte development directly in the thymus. Since quantitative real-time RT-PCR experiments revealed no traces of viral RNA in the bone marrow, we can exclude that the observed phenomenon is due to infection of the bone marrow. The hypothesis that HPAIV interferes with T lymphocyte development was supported by the findings that the expression of chemokines (CXCL12, CCL25) and cell adhesion molecules (ICAM-1, VCAM-1) involved in T cell development was drastically reduced after HPAIV but only slightly after H1N1v or H5N2 influenza virus infection. CXCL12, which is recognized by the CXCR4 receptor, is an essential prerequisite for functional thymocytes migration (33). CXCL12 is produced by both cortical and medullar epithelial cells and recent studies underline the urgent need for CXCL12/CXCR4 pathway in T lymphocyte development *in vivo* (34). In single positive $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ T lymphocytes the expression rate of CXCR4 is reduced which supports the outflow of naive T lymphocytes into the periphery (35). The importance of this signal pathway becomes apparent in studies with *Trypanosoma cruzi* which is the causative agent of Chagas disease and also infects the thymus. Here, the up regulation of CXCL12 in the thymus seems to lead to an outflow of immature T lymphocytes into the periphery (36). The surprising finding that highly pathogenic avian influenza A virus infection interferes with this important signal gave reason to believe that the virus affects the thymus structure and function directly. Therefore, the question was raised whether HPAIV is able to infect the thymus. Using immunohistochemistry, we clearly demonstrated that especially the thymus medulla is positive for both Siaα2-3gal and Siaα2-6gal, a prerequisite for influenza virus infection. Moreover, we were able to demonstrate infectious virus and viral RNA in the thymus, particularly in the cortico-medullary junction and medulla, the place where negative selection occurs. After H1N1v infection virus was found in the thymus of one mouse to a low amount. This result gives rise to the assumption that this new emerging human pandemic influenza virus strain has the ability to infect the thymus. Nevertheless, from our data we speculate that a so far unknown “viral-piece” is still absent in this recent pandemic H1N1v strain to induce fatal disease. This observation could underline the fact, that

pandemic H1N1v has a high morbidity coming with a mild illness while fatale cases are fairly rare (37).

The amount of activated T cells after HPAIV infection was increased in the thymus after influenza virus infection including influenza virus NP-specific $CD8^+$ T cells. The existence of activated, influenza virus specific $CD8^+$ T lymphocytes in the thymus is crucial and might lead to organ destruction via an immunopathological mechanism. Whether these cells were primed in the thymus or moved into the thymus as active virus-specific T cells remains unclear. The fact that the expression of chemokines and chemoattractants involved in T lymphocyte development was altered might argue in favor for the possibility that already activated T cells might move back from the periphery into the thymus. The re-entry of mature and even activated T lymphocytes has been described elsewhere (38-39). Still, the question how the virus is being transported into the thymus is unanswered at this point. The former paradigm of the thymus as a "one-way street" is important for a functional development of T lymphocytes. By that way the organism assures that only self-peptides are presented on the MHC-complexes during thymocytes selection processes. However, recent studies revealed that selected lymphoid populations can migrate into the thymus within the cortico-medullary junction allowing foreign antigens to reach the thymus (40). This could be important for oral tolerance against nutrition antigens (41). Also, the evidences rise that an organism is able to develop a tolerance against an intravenous, intrathymic but also intranasal injected high dose antigen by deletion of antigen-specific thymocytes and induction of apoptosis of T lymphocytes in secondary lymphoid organs (42-43). By reaching the thymus, non-self antigens can generate a suppressive Treg population, which could also have an suppressive effect on the previously generated existing peripheral T cell precursor population (44). It also might be possible that highly pathogenic avian influenza viruses assemble a suppressive Treg population by migrating into the thymus. Because of the implausibility that the virus can pass the barrier between periphery and thymus by itself without causing a systemic infection, a carrier cell is more likely. In a recent publication Gabriel and colleagues demonstrated that a murine T cell line can be infected by influenza viruses *in vitro* (28). Moreover, the virus used in this study was also able to infect macrophages *in vitro* and *in vivo* (28). In our *in vivo* study, macrophages were not the major target for infection. More interestingly, it's been reported that dendritic cells can be infected by influenza viruses *in vitro* (30). Because of the DC function to migrate continuously into the LN but also in the thymus (15) with or without having contact and being activated by an antigen, thymic dendritic cells play a major role in establishing tolerance by negative selection of thymocytes and induction of Tregs (45). We

hypothesized that DCs are the carrier cell type for influenza viruses. We first tested whether different influenza viruses were able to infect DCs in the lung. Flow cytometry analysis was used to demonstrate a high rate of infection of DCs by the H5N1 and H7N7 strains and to lower extends by H1N1v infection. Low pathogenic H5N2 virus infection of DCs could not be detected. Our results demonstrate that the infection of DC's with HPAIV also includes the $CD103^+$ DC subpopulation for which an induction of peripheral Tregs has already been described (46). Since $CD103^+$ mucosal DC's only present 13% (data not shown) of the total DC population we cannot exclude that the virus strains used in this study are specialized on $CD11c^+/CD103^+$ subpopulations.

The adoptive transfer experiment using GFP^+ lung DCs further strengthen our hypothesis that DCs can migrate from the lung into the thymus. Flow cytometry and immunofluresence revealed the presence of GFP^+ cells in the thymus of HPAIV infected wild type mice. Nevertheless from our immunofluoresence data, we cannot exclude that these respiratory dendritic cells in the thymus undergo apoptotic processes either due to influenza virus infection or $CD8^+$ T cell response. From these results we hypothesize that activated, HPAIV infected DCs move from the lung into local, mediastinal lymph nodes where viral replication can take place. In a second step, DCs move further on into the thymus, where influenza virus infected DCs first reach the cortico-medullary junction and the medulla the place of negative selection processes. Thymic antigen presenting cells in the thymus, namely DCs but also thymic epithelial cells are responsible for the selection processes (47). We conclude that $CD45^-$ epithelial cells in the medulla are the first target for infection since viral footprints in the $CD45^+$ thymocyte population were absent (data not shown). Epithelial cells are critical for the development of T lymphocytes. These stroma cells give the structure for a working positive and negative selection of outgoing naive T lymphocytes. An interference with $CD45^-$ cells can lead to a dysregulation of T lymphocytes development especially in the younger population when the thymus is still highly active. One result could be a reduced or even missing immune response against new antigens that break through the mechanical barriers of our immune system. The pandemic H1N1v strain doesn't reach the thymus consistently which could be due to a missing pathogenesis factor, since virus titer is even higher in the lung of H1N1v infected mice compared to viral titers in the lung after H5N1 and H7N7 infection.

We propose a mechanism, where highly pathogenic avian influenza virus has the ability to infect dendritic cells in the lung. This migrating cell type can now transport the virus to secondary lymphoid organs like lymph nodes, but more important to the primary lymphoid organ: the thymus. In both cases, the infected cell can not only support the virus to spread to

selected targets but also present viral antigens as pseudo self peptides. That way, an additional clonal deletion of influenza specific T lymphocytes can take place in the thymus. The collapse of the thymocytes developing signal network could be due to the fact that stromal cells in the thymus are a target for the virus (Fig. 8). Additionally, influenza specific Tregs could be generated that suppress already existing T lymphocyte activity against viral epitopes. The existence of Tregs needs to be analyzed in further studies to assure this assumption.

Taken together, the infection of dendritic cells demonstrates a very effective way to evade the immune system and could be the reason for fatal influenza virus infection that leads to lymphopenia and the strong influx of innate immune cells (17). The fact that the Siaα2-3gal and Siaα2-6gal lectins that function as the receptor for influenza virus infection are also expressed in human thymus (Fig. 4), makes the transfer of the described mouse model to the pathogenic situation in humans likely. The resent pandemic H1N1v outbreak which also causes lymphopenia in severe clinical cases demonstrates that this described mechanism might not only be restricted to highly pathogenic avian influenza viruses but might be a general feature of severe influenza (48).

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Disclosures

The authors have no financial conflicts of interest.

Abbreviations used in this paper:

C, cortex; DC, dendritic cell; ffu, focus forming unit; HPAIV, highly pathogenic avian influenza virus; J, corticomedullary junction; LN, lymph node; M, medulla; MDCK, Madin-Darby canine kidney; MLD_{50}, 50% mouse lethal dose; NP, nucleoprotein; qRT-PCR, quantitative real-time RT-PCR; RDC, respiratory dendritic cell; SOIV, swine origin influenza virus; Treg, regulatory T cell.

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Figure Legends

FIGURE 1: Reduction of $CD4^+$ T lymphocytes (A) and $CD8^+$ T lymphocytes (B) in the mediastinal LN after 6 d of influenza virus infection. Two independent experiments with four mice per group were performed. Morphology of the thymus after influenza virus infection in mice (C–H).Visual atrophy of the thymus postinfection with high pathogenic H7N7 (C) or H5N1 (D) virus strains but not by low pathogenic H5N2 (E) or pandemic H1N1v (F) influenza virus. All mice were sacrificed 6 d postinfection. Images show uninfected control C57BL/6 thymus (top row) in comparison with thymus of infected mice (bottom row). G, Total number of counted leukocytes in the thymus after influenza virus infection. Number of animals: control, n = 8; H7N7, n = 9; H5N1, n = 9; H5N2, n = 4; H1N1v, n = 4. Paired t test revealed significant difference compared with uninfected controls. *p > 0.05; **p > 0.01. H, Changes of the thymocyte populations and single-positive T lymphocytes 6 d after HPAIV influenza virus infection analyzed by flow cytometry. Thymus population after H5N2 and H1N1v are not listed, but show comparable distribution to controls. Four mice per group were used in the experiments. Five independent experiments yielded consistent results.

FIGURE 2: Decreased mRNA levels of chemokines involved in T lymphocyte development after H7N7(A) or H5N1(B) but not after H5N2 (C) or H1N1v (D) infection using qRT-PCR. Analysis was performed 3 or 6 d postinfection, and $-\Delta\Delta$ct values compared with uninfected controls were evaluated. For each value, four mice were tested in three different experiments. Paired t test revealed significant difference to uninfected controls. *p > 0.05; **p > 0.01.

FIGURE 3: *In vitro* cytotoxicity assay of influenza virus specific $CD8^+$ lymphocytes. ^{51}Cr-labeled MC57 targets cells were loaded with the immunodominant influenza peptide $NP_{366-374}$. NP specificity of $CD8^+$ T lymphocytes isolated from lung and thymus 6 days after infection with (*A*) H7N7, (*B*) H5N1, (*C*) H5N2 or (*D*) H1N1v were determined by the $^{51}Chrom$ release of lysed target cells. Two experiments were performed independently with three mice.

FIGURE 4: Immunohistological detection of sialic acids in the lung, mediastinal lymph node and thymus of C57BL/6 mice (*A*) and the mesenterial lymph node and thymus of humans (*B*). Avian influenza viruses recognize Siaα2-3gal while human influenza viruses use Siaα2-6gal as a receptor for infection. Original magnification x 20. For each tissue, at least five slides were analyzed.

FIGURE 5: Detection of viral mRNA. A, Expression levels of viral matrix protein mRNA after H7N7 (left panel) or H5N1 (right panel) infection in the lung and lymphatic organs. Analysis was performed from days 0–6 postinfection. Cycle threshold values for every day are relative to the control cycle threshold value of each organ. For each value, three mice were tested in three different experiments. B, In situ hybridization for the localization of viral RNA in lung and thymus. Numerous alveolar and bronchial epithelial cells are positive in mice infected with H7N7 and H5N1. In the thymus, H7N7- and H5N1-infected cells are located in the corticomedullary junction. For each tissue, three slides were evaluated. Original magnification x 10. C, cortex; J, corticomedullary junction; M, medulla.

FIGURE 6: Detection of influenza virus-infected DCs in the lung. A, Samples were collected 3 d postinfection and determined by flow cytometry. Postdepletion of SiglecF+ cells, $CD11c^+$ cells were isolated and additionally stained for the mucosal $CD103^+$ DC subpopulation (middle row). Infection was confirmed by intracellular staining against influenza A Ags using a polyclonal Ab. B, Percentage of cell populations analyzed by flow cytometry. Data represent one of two independent experiments with groups of four mice.

FIGURE 7: Adoptive transfer of GFP^+ DCs into H7N7 (10-fold MLD_{50}) influenza virus-infected wild type mice. A, Experimental design: DCs from infected C57BL/6-GFP–expressing mice were isolated 3 d postinfection and transferred into lungs of wild type C57BL/6 mice. Recipient mice were sacrificed 2 d posttransfer, and GFP+ DCs found in the

lung and thymus (< 1%) were visualized by immunofluorescence (B). Experiment was performed twice with groups of two mice.

FIGURE 8: Proposed mechanism of how HPAIV and possibly pandemic H1N1v could reach the thymus without causing a systemic infection. Influenza virus is able to infect DCs in the lung. Activated and infected DCs migrate into the LN, where the antigen presentation and a first viral replication phase take place. Additionally, DCs migrate into the thymus, where they support the viral spread and the depletion of influenza peptide-specific thymocytes by Ag presentation. As a result, the amount of T lymphocytes is reduced.

Table

Table I: Distribution of the viral load 6 days postinfection of C57BL/6 mice

	Lung	Medistinal LN	Thymus	Spleen	Brain
H7N7	5.24 ± 0.19	3.71 ± 0.24	3.85 ± 0.23	3.61 ± 0.22	< 1.7
H5N1	5.29 ± 0.06	3.80 ± 0.35	2.84 ± 0.21	< 1.7	< 1.7

Virus titers are given as the logarithm in focus forming units per 1 ml organ homogenate.

[a]Low amounts of virus were detected in one out of four organs (H5N2 mediastinal LN: 3.12 ffu/ml; H1N1 thymus: 2.01 ffu/ml).

Figures

Figure 1

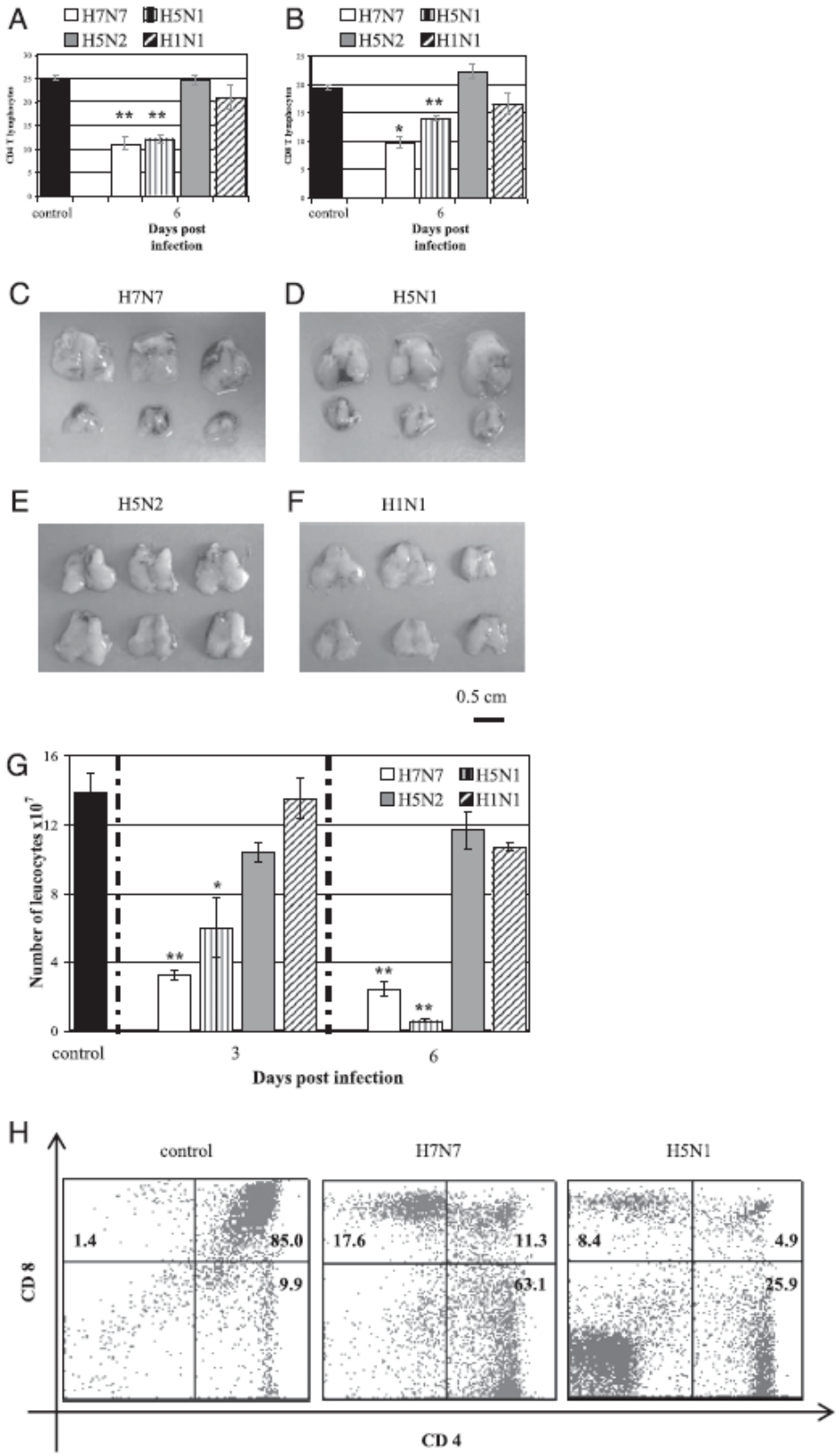


Figure 2

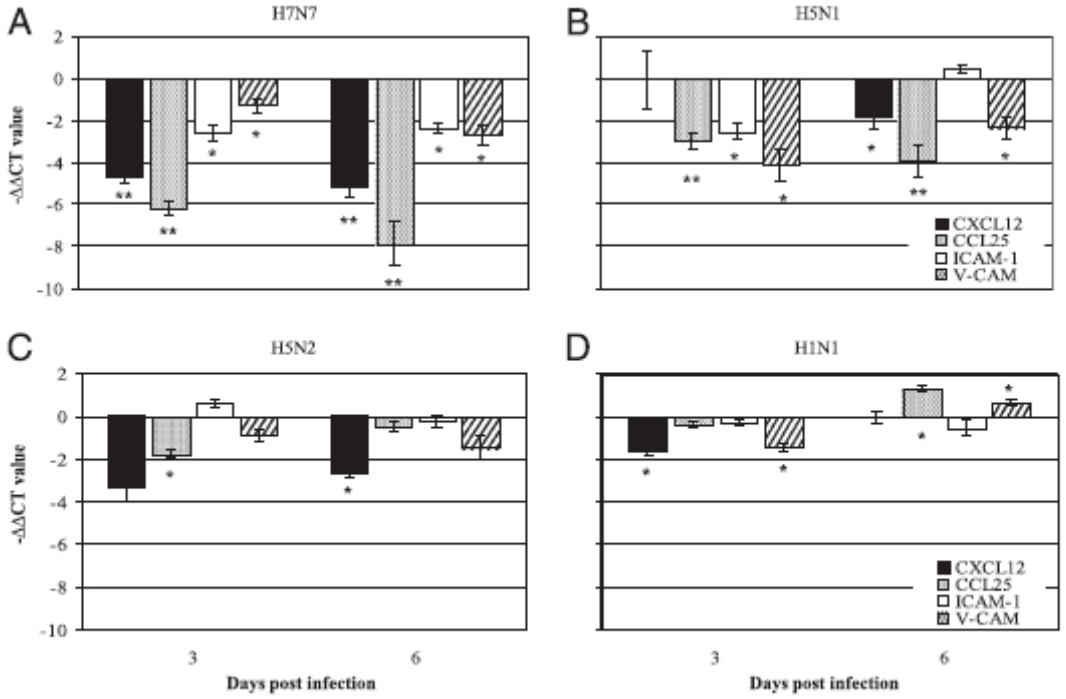


Figure 3

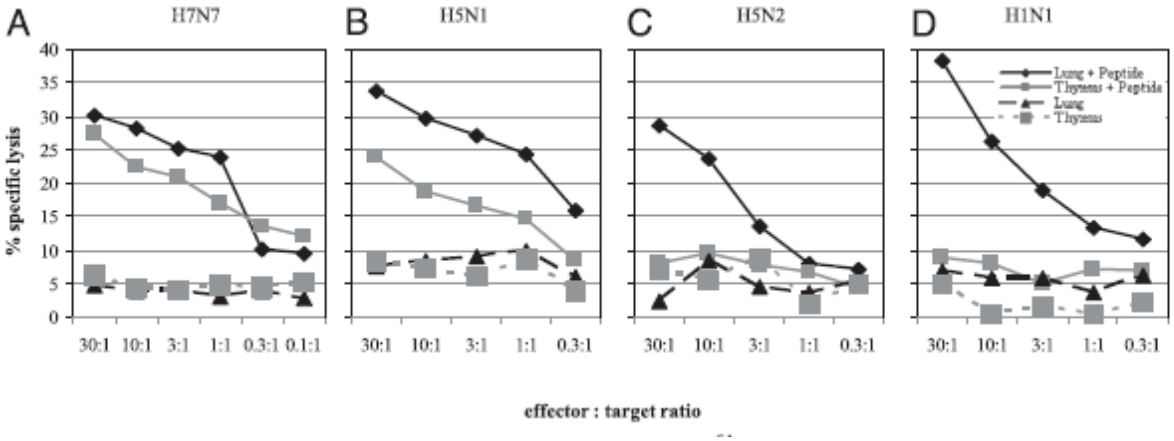


Figure 4

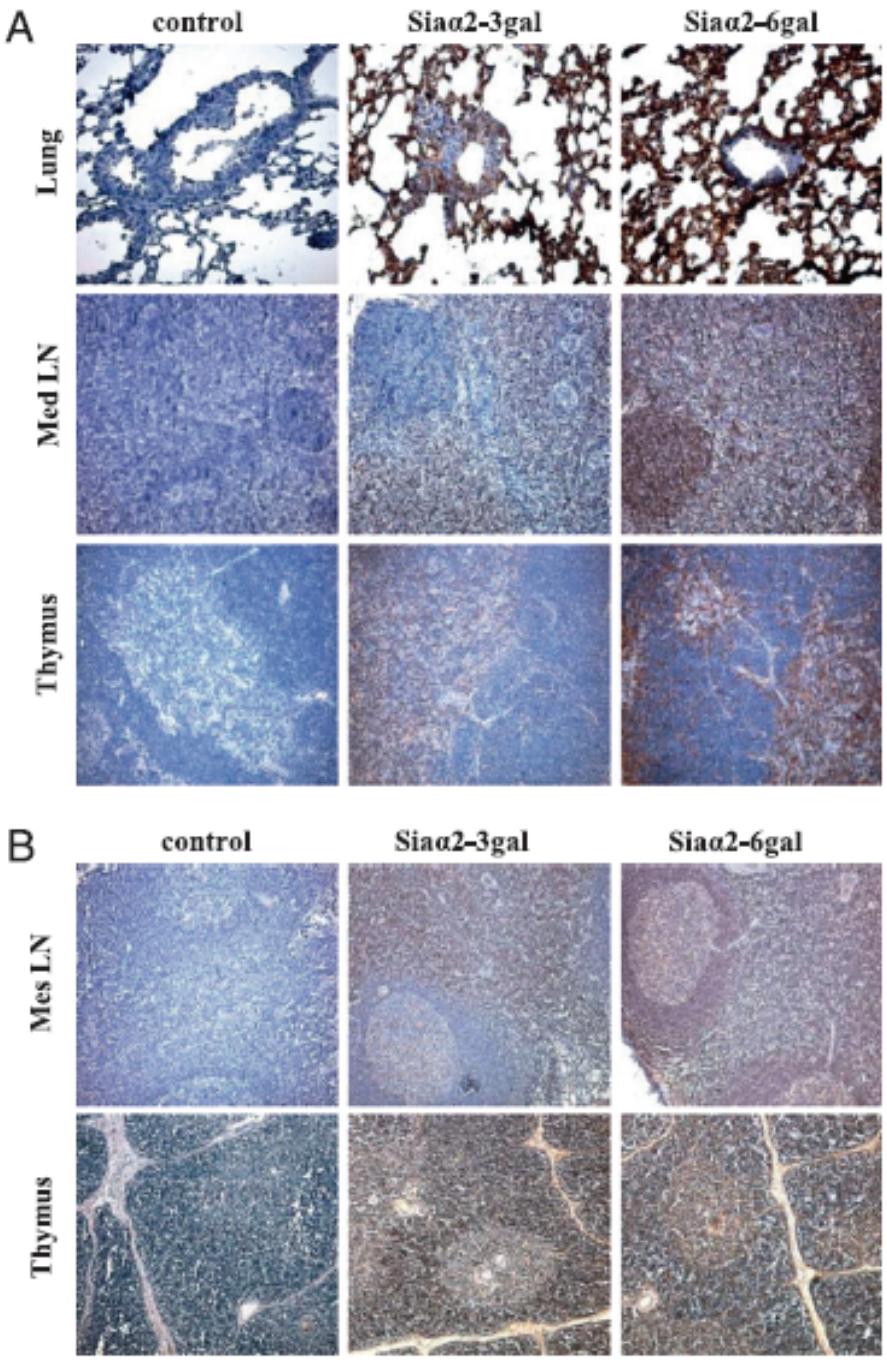

A
control
Siaα2-3gal
Siaα2-6gal
Lung
Med LN
Thymus
B
control
Siaα2-3gal
Siaα2-6gal
Mes LN
Thymus

Figure 5

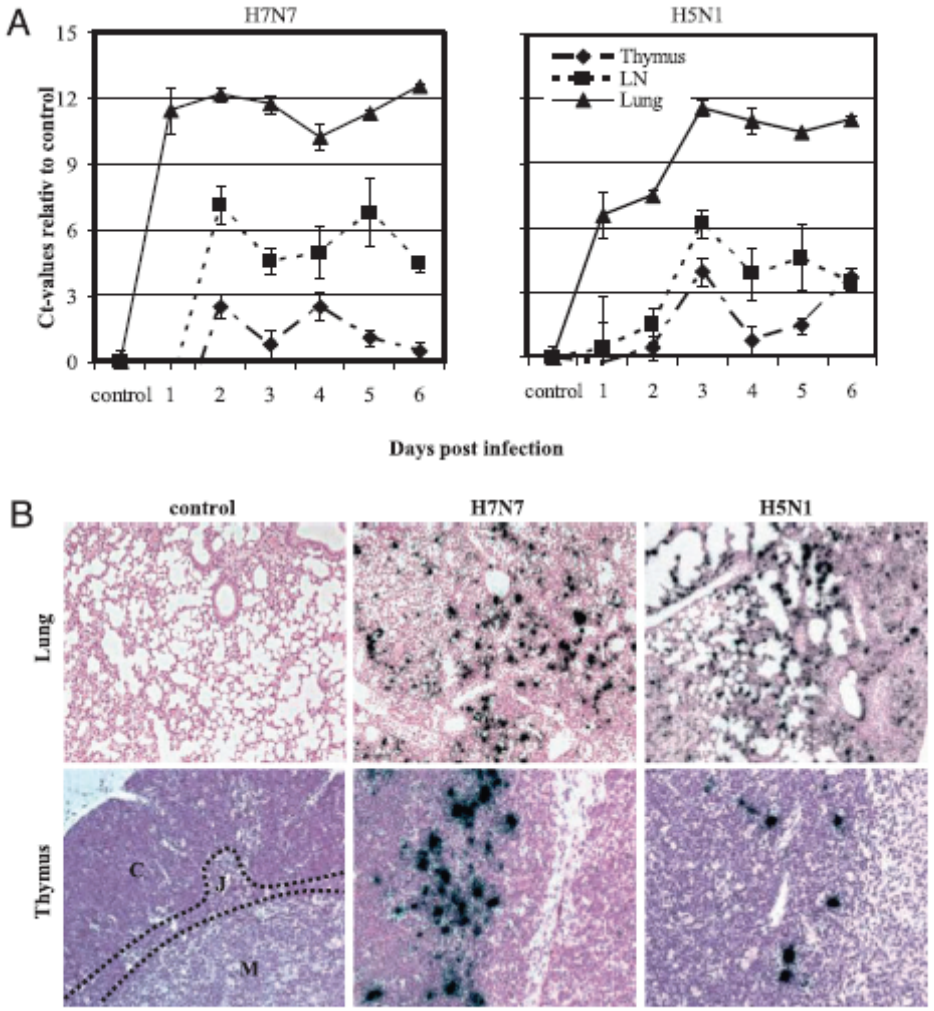

A
H7N7
H5N1
Thymus
LN
Lung
Ct-values relativ to control
15
12
9
6
3
0
control 1 2 3 4 5 6
Days post infection
B
control
H7N7
H5N1
Lung
Thymus
C
J
M

Figure 6

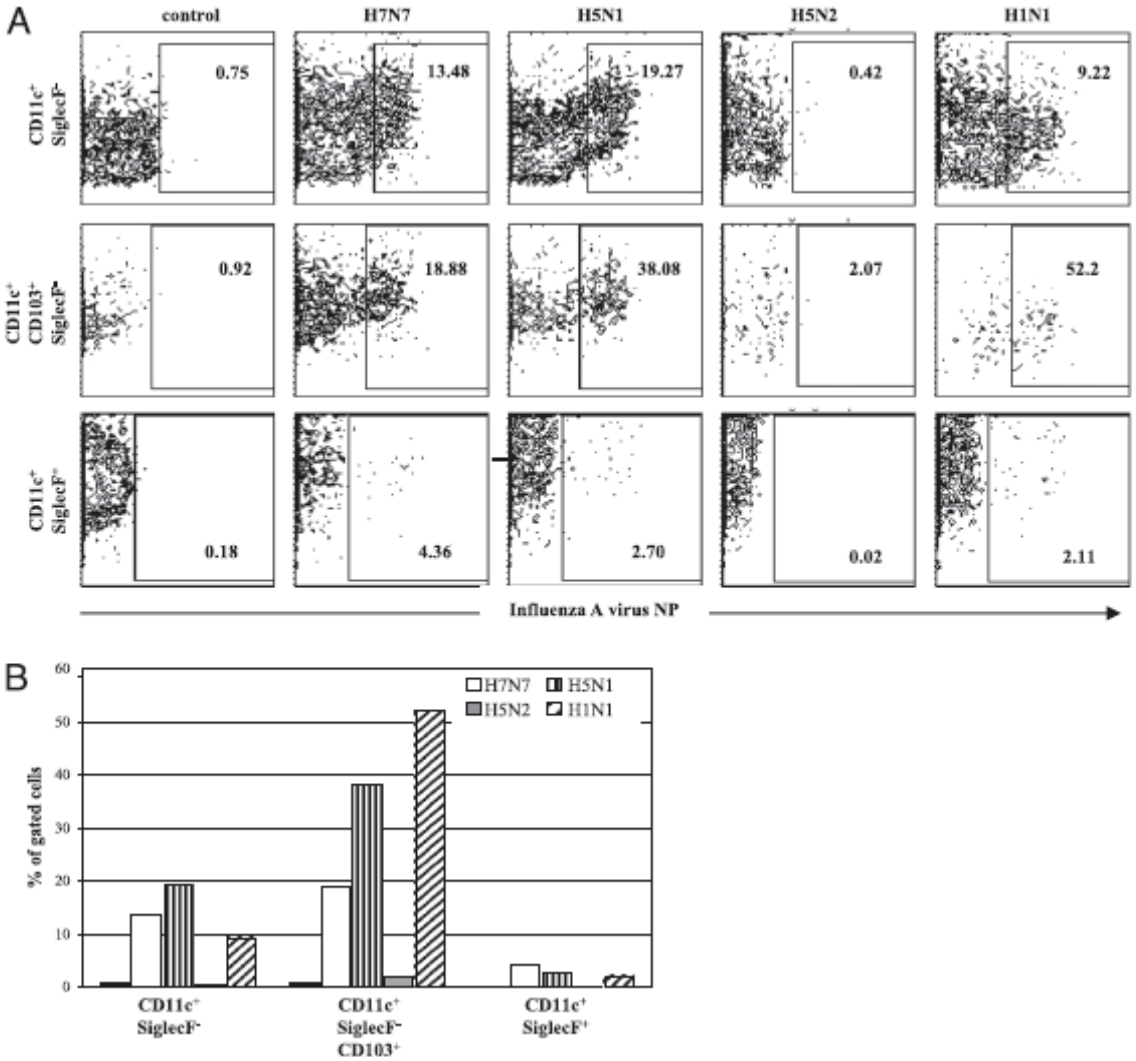

A
control
H7N7
H5N1
H5N2
H1N1
CD11c+ SiglecF-
CD11c+ CD103+ SiglecF-
CD11c+ SiglecF+
0.75
13.48
19.27
0.42
9.22
0.92
18.88
38.08
2.07
52.2
0.18
4.36
2.70
0.02
2.11
Influenza A virus NP
B
H7N7
H5N1
H5N2
H1N1
% of gated cells
60
50
40
30
20
10
0
CD11c+ SiglecF-
CD11c+ SiglecF- CD103+
CD11c+ SiglecF+

Figure 7

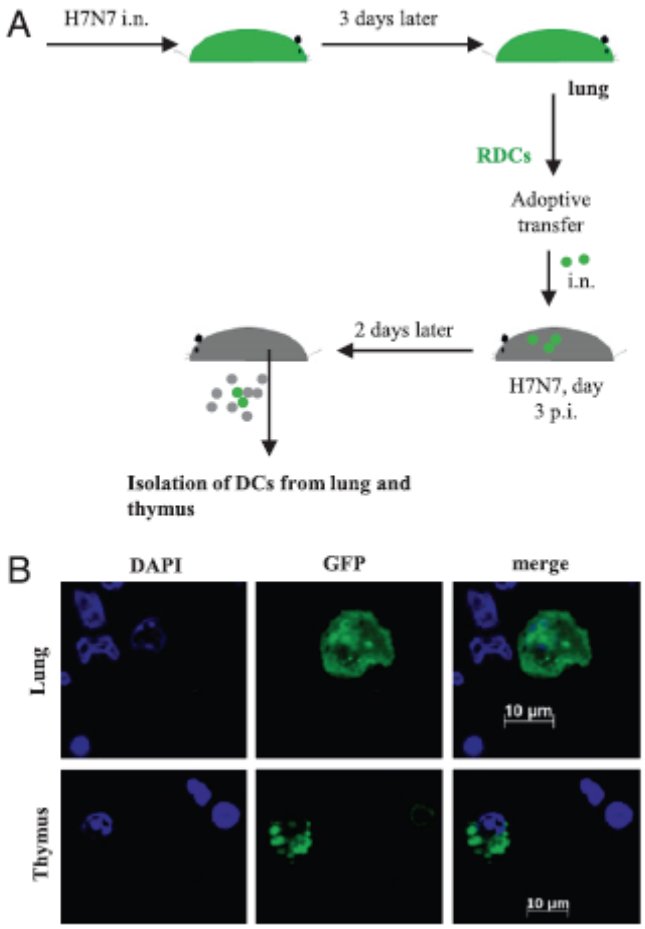
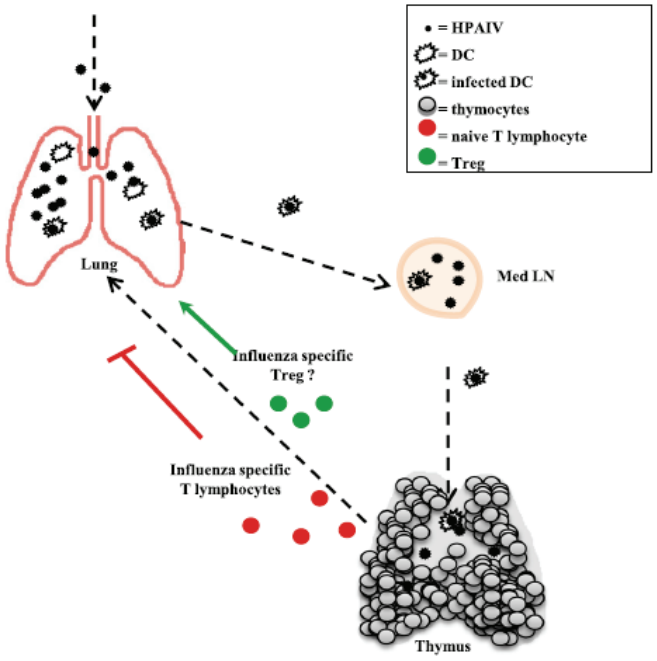
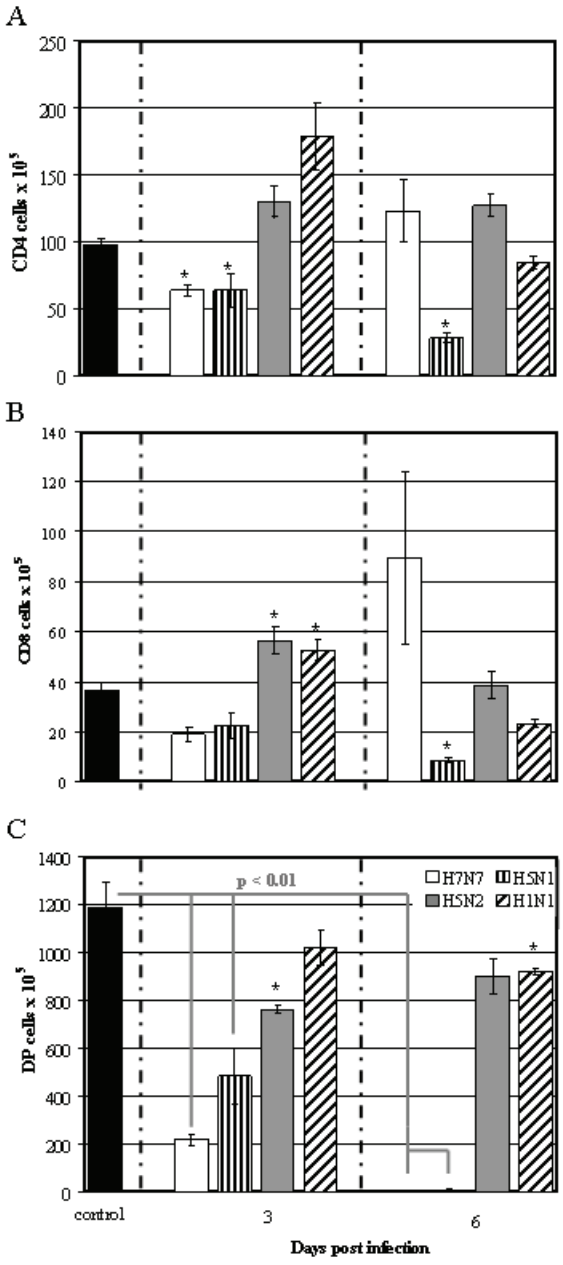


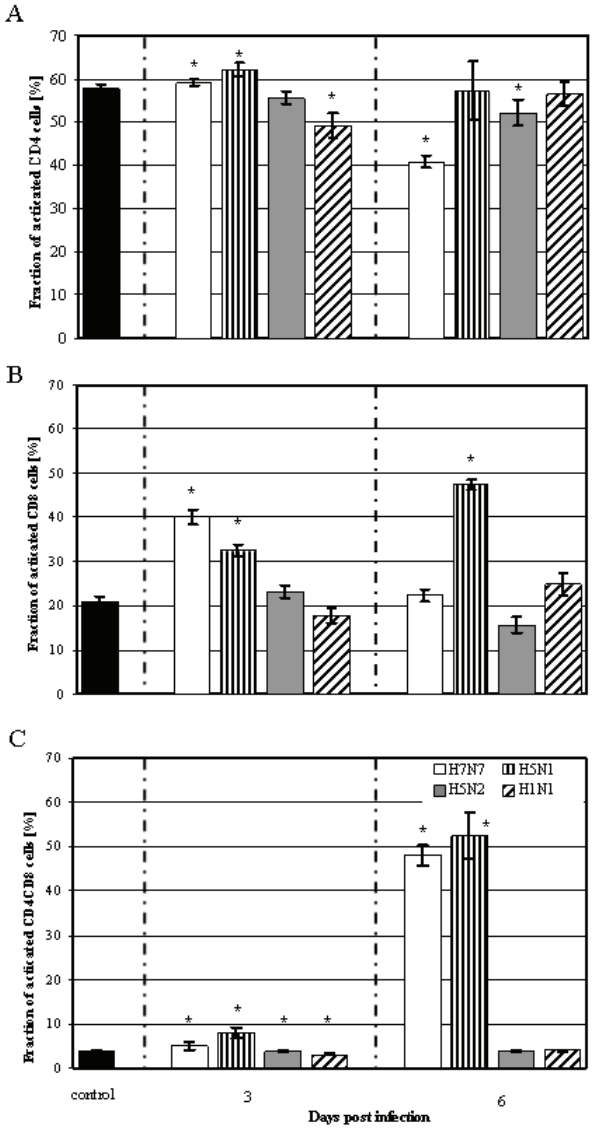
Figure 8



Supplementary Figure 1



Supplementary Figure 2



PUBLICATION #4:

Antibodies and $CD4^+$ T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain

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Keywords: cross-protection, H5N1 influenza virus, vaccine strategy

Abstract

A H5N2 low pathogenic avian influenza virus (LPAIV) was isolated from a natural reservoir in Bavaria during a routine screen and was used as a vaccine strain to scrutinize the immune response involved in cross-protection after challenge infection with a H5N1 highly pathogenic avian influenza virus (HPAIV). The challenge virus was also isolated from a natural reservoir in Bavaria. Wild type, antibody deficient (μMT), $CD4^{-/-}$ and $CD8^{-/-}$ mice were infected with the apathogenic H5N2 vaccine strain and challenge infection with a 100-fold MLD50 of the H5N1 strain was performed 80 days later. While 100% of the wild type and 100% of the $CD8^{-/-}$ mice stayed healthy, only 50% of the $CD4^{-/-}$ and none of the antibody deficient mice were protected. These results support the view that the humoral immune response and to certain extends the $CD4^{+}$ T helper cells are a prerequisite for cross-protective immunity against H5 influenza virus.

Introduction

Unlike the common seasonal influenza A viruses, infection with H5N1 influenza viruses cause severe pathology with high lethality. A sporadic human-to-human transmission of H5N1 viruses is suspected, but not finally proven. Nevertheless, through multiple mutations which might lead to effective transmission between humans, the virus could acquire the ability to cause a new pandemic [1, 2].

Vaccination is the most efficient method for preventing influenza and its severe complications. However, antigenic drift has a major impact on the vaccine effectiveness. Therefore, there is an urgent need for new vaccines that protect against seasonal and in addition potentially pandemic influenza virus strains. Various vaccine approaches are currently under investigation [3]. The ultimate goal is developing a universal influenza vaccine that can also protect against possible pandemic strains like the lethal avian influenza virus A/H5N1. The viral surface proteins hemagglutinin (HA) and neuraminidase (NA) are highly antigenic but also undergo antigenic drift. In order to create a universal vaccine the use of genetically conserved influenza virus antigens as immunogens like M2 and nucleoprotein (NP) is investigated [4–6].

However, at present the development of a universal influenza vaccine remains at a preclinical or clinical phase. Therefore, conventional vaccines with a strong focus on cross-protective immunity are the first choice for prophylactic intervention. Different approaches using either inactivated influenza vaccines (IIVs) or live-attenuated vaccines (LAIVs) were successful in inducing cross-protective immunity in various animal models [7–9]. Furthermore, LAIVs are

able to induce cross-protective immunity in healthy adults and children against mutated influenza virus strains [10]. It is under discussion whether broad protection that has been observed using LAIV is due to antibodies against the M2 protein. Moreover, it has also been suggested that the cellular immune response and the mucosal immune response may be involved in protection. Although innate immune reactions play a decisive role, recovery from primary influenza virus infection requires adaptive immunity, but the relative importance of the humoral and the cellular immune mechanisms is controversial [11,12]. Beside reports indicating that the influenza virus-specific IgG response is $CD4^+$ T-cell dependent there are also investigations describing $CD4^+$ T-cell independent antibody responses [13]. The cytotoxic T-cell (CTL) response is mainly directed against the M and NP proteins [14]. There are some reports indicating that CTL does not seem to contribute significantly in preventing infection [15, 16]. In contrast other investigations point out that a CTL response in addition to strong antibody formation will be more effective in preventing disease when hetero-subtype cross-protection is demanded [17–19]. Taken together, the puzzle of immunological mechanisms that can lead to robust hetero-subtype specific immunity after influenza vaccination is not completely understood.

In the present study, we wanted to scrutinize the role of the humoral and cellular immunity in cross-protection after vaccination with a non-pathogenic H5N2 virus from a naturally infected wild bird. Challenge infection was performed with a lethal dose of a H5N1 highly pathogenic avian influenza virus (HPAIV). Vaccination of wild type and $CD8^+$ T-cell deficient mice was protective against H5N1 lethal infection, whereas vaccination of antibody deficient mice and $CD4^+$ T-cell deficient mice failed to protect against H5N1 infection.

2. Material and methods

2.1. Mice

Inbred female C57Bl/6, μMT and CD8 knockout mice on a C57Bl/6 background, sv129 and CD4 knockout mice on a sv129 background at the age of 6–8 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Tübingen, Germany and were used throughout all the experiments.

2.2. Virus

Avian influenza A/mallard/Bavaria/1/2005 (H5N2) (DB1, gene bank accession number, see Table 1) virus grown on Madin–Darby canine kidney (MDCK) cells and A/mallard/Bavaria/1/2006 (H5N1) (MB1, gene bank accession number, see Table 1) virus,

grown in embryonated chicken eggs were used throughout this study. Both isolates of the avian influenza A virus were originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany and further propagated at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Tübingen,Germany.

2.3. In vitro infection

MDCK II and A549 cells were grown in minimal-essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). For infection cells were grown overnight in 96-well plates ($1x10^5$ cells/well). Immediately before infection the cells were washed with PBS and subsequently incubated with the differe virus at a multiplicity of infection (MOI) of 0.001 diluted in PBS/BA (0.2% BA) supplemented with 1mM $MgCl_2$, 0.9mM $CaCl_2$, penicillin and streptomycin for 30min at 37 ◦C. After 30min incubation period the inoculum was aspirated and cells were incubated with 1ml MEM containing 0.2% BA, 200 U/ml penicillin and 0.2 mg/ml streptomycin. For the infection with DB1 1.2µl/ml trypsine was added to the medium. At the given time points (8, 24, 32 and 48 h past infection) supernatants were collected.

2.4. Infection of mice

For infection of mice, the animals were anaesthetized by intraperitoneal injection of 150 µl of a ketamine (Sanofi)/rompun (Bayer) solution (equal amounts of a 2% rompun solution and a 10% ketamine solution were mixed at the rate of 1:10 with PBS) and infected intranasally (i.n.) with $2x10^3$ pfu/50 µl DB1 (H5N2) or $2x10^3$ pfu/50 µl MB1 (H5N1). Virus challenge experiments were performed with $2x10^2$ pfu/50 µl DB1 and $2x10^5$ pfu/50µl MB1 (100-fold MLD_{50} (mouse lethal dose 50%)). According to the German animal-protection law, the mice were sacrificed as soon as they lost 25–30% of their weight. All animals were monitored for 14 days after infection.

2.5. Influenza virus titration (AVICEL® plaque assay)

To assess the number of infectious particles (plaque titers) in organs and cell culture supernatants a plaque assay using Avicel® was performed in 96-well plates as described by Matrosovich et al. [20]. Virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec) followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA) and 10 min incubation with True Blue™ peroxidase substrate (KPL). Stained plates were scanned on a

flat bed scanner and the data were acquired by Microsoft®Paint software. The virus titer is given as the logarithm to the base 10 of the mean value. The detection limit for this test was <1.7 log 10 pfu/ml.

2.6. Hemagglutination inhibition assay

Hemagglutination inhibition assays were carried out in V-bottomed microtiter plates using 50µl of 2.5% suspensions of chicken red blood cells in PBS. Fresh chicken blood was supplemented with 1.6% sodium citrate in sterile H_2O and was centrifuged at 800x *g* for 10min at room temperature to separate red blood cells. Thereafter, the cells were washed three times with PBS. Hundred microliter of serum from infected mice was added and serially diluted in PBS. Thereafter, 50µl of a 1:64 virus dilution ($5x10^5$ pfu/well) was added to the serum. The plate was incubated for 1h at 37◦C and 5% CO_2. After the incubation period, 50µl of chicken erythrocytes were added to the wells. Assays were analysed following 1 h incubation on ice.

An inhibition of the hemagglutination was indicated, when red blood cells precipitated to the bottom of the plate, while red blood cells incubated with influenza virus or non-reactive serum showed a diffuse distribution on the microtiter plates. The results were given as reciprocal of the highest dilution causing detectable inhibition of hemagglutination.

2.7. Statistical analysis

For investigation of the significance of the data, statistical analysis was performed by using the Kaplan–Meier survival analysis and the Cox-regression test from WinStat®.

2.8. Neutralization assay

Fifty µl of influenza virus (DB1 or MB1) were pre-incubated for 30 min at room temperature with 50 µl of a serum pool from three mice. Sera from uninfected, DB1 infected (14 days p.i.) and challenged mice (14 days past MB1 infection), were used for this test. For control virus was incubated with PBS. The numbers of infectious particles (plaque titers) were assessed using the Avicel® plaque assay described above.

2.9. Flow cytometry

Naive C57Bl/6, µMT and $CD4^{-/-}$ mice or DB1 (H5N2) prechallenged C57Bl/6, µMT and $CD4^{-/-}$ mice (80 days p.i.) were infected with $2x10^3$ pfu of MB1 (H5N1) virus. Mice were sacrificed at the indicated times after infection, and tissues were removed and mechanically

disrupted by passage through a 70 µm cell strainer (BD Falcon). Cells ($1x10^6$ cells/sample) were stained with monoclonal antibody to CD8a (clone 53-6.7, BD Bioscience) and MHC class I tetramers (H-2Db). The MHC class I tetramers containing NP366–374 peptide predicted for MB1 (ASNENMEAM) or DB1 (ASNENMETM) were used to identify influenza-specific T-cells. Before FACS analysis, erythrocytes were lysed and cells were fixed with FACS lysing solution (BD Bioscience). Flowcytometry was performed on a dual laser FACSCalibur and analysed with Cell Quest software (BD Biosciences).

3. Results

3.1. DB1 (H5N2) and MB1 (H5N1) showed similar virus growth properties in vitro

Vaccines based on live-attenuated low pathogenic avian influenza viruses (LPAIVs) might be promising candidates for effective protection against H5N1 influenza A virus infections. In order to estimate, whether the LPAIV A/mallard/Bavaria/1/2005 (DB1; H5N2) and HPAIV A/mallard/Bavaria/1/2006 (MB1; H5N1), both isolated from naturally infected wild birds replicate in mammalian cell culture without adaptation *in vitro*, infectivity studies were performed to analyse the amount of progeny virus. MDCK cells that are highly permissive for influenza virus infection were used for infection with DB1 or MB1 viruses at an MOI of 0.001. To determine the amount of progeny virus cell culture supernatants from either DB1 or MB1 infected MDCK cells were collected 8, 24, 36 and 48 h after infection. Already 8 h p.i., DB1 ($3.2x10^2$ pfu/ml) but not MB1 was detectable in the cell culture supernatant (Fig. 1A). At the later time points both viruses showed similar growth curves whereas MB1 replicated to higher titer. Both viruses reached the maximum titer at 32 h p.i. (MB1 $9.3x10^7$ pfu/ml and DB1 $4.3x10^6$ pfu/ml). Thereafter, the amount of progeny virus declined in the cell culture supernatant (Fig. 1A). However, infection with DB1 only resulted in progeny virus production, when trypsine was present in the culture medium. In contrast, infection of MDCK cells with MB1 led to virus production in absence of trypsine and yielded similar titers in five consecutive passages. The sequence analyses of both HAs revealed a monobasic cleavage site for DB1 HA, while a multibasic cleavage site was found in the HA of MB1 (Fig. 1B).

3.2. Immunocompetent and immunodeficient mice were susceptible to DB1 or MB1 infection

In vitro infection with DB1 and MB1 led to progeny virus production in MDCK cells. We next questioned whether infection of mice with DB1 or MB1 would result in productive virus infection and in manifestation of disease. For *in vivo* studies in mice with either DB1 or MB1 the MLD_{50} for both influenza virus subtypes was determined. The MLD50 for the highly

pathogenic H5N1 isolate MB1 in C57Bl/6 mice was $2x10^3$ pfu (Table 1). Using this dose for i.n. infection all mice lost weight and developed clinical symptoms like ruffled fur. A general reduction of their normal activity rate starting 6 days after infection was found (Fig. 2A, open triangle). Lethality in this group was 40%; two out of five animals died (Fig. 2B, open triangles). Interestingly, mice infected with the low pathogenic H5N2 isolate DB1 neither lost weight (Fig. 2A, black squares) nor developed any apparent symptoms even at an infectious dose of $5x10^5$ pfu (Table 1). Furthermore, none of the DB1 infected mice died (Fig. 2B, black squares).

Since immunocompetent C57Bl/6 wild type mice showed no symptoms of disease after infection with DB1, we raised the question whether mice that lack an antibody response (μMT) or $CD8^+$ T-cell deficient mice, would also be able to control DB1 infection without clinical symptoms such as immunocompetent mice. Five female wild type, μMT and $CD8^{-/-}$ mice were either infected i.n. with $2x10^3$ pfu DB1 or MB1 virus. The animals were weighted daily and the health status was monitored twice a day. Infection of μMT mice with DB1 did not result in reduction of the body weight. All of the DB1 infected μMT mice gained weight during the 14 days observation period (Fig. 2C, black squares). None of the infected animals developed signs of disease or even died spontaneously (Fig. 2D, black squares).

Infection of μMT mice with the highly pathogenic MB1 virus was accompanied by weight loss and appearance of disease symptoms starting by day 5 p.i. (Fig. 2C, open triangles). The severity of disease symptoms increased until day 8 after infection. By day 10 p.i., four out of five (80%) of the infected μMT mice died, demonstrating an increased susceptibility to the infection (Fig. 2D, open triangles).

Infection of $CD8^+$ T-cell deficient mice with DB1 did not result in weight loss and the occurrence of clinical symptoms during the infection period (Fig. 2E, black squares) and none of the animals died after the infection (Fig. 2F, black squares). In contrast, after infection with MB1 all $CD8^{-/-}$ mice lost weight and developed disease symptoms (Fig. 2E, open triangles). The first clinical symptoms like ruffled fur, an unnatural posture and a general reduction of the normal activity rates were observed 6 days past infection in this group of animals. Ten days p.i. three out of five mice died (60%) while the other mice survived infection, recovered from clinical symptoms and regained weight (Fig. 2F, open triangles).

Taken together, the infection experiments revealed an increased susceptibility to the influenza A virus infection with the highly pathogenic MB1 in antibody deficient mice (μMT) and in mice lacking $CD8^+$ T-cells, whereas infection with DB1 showed no pathogenic effects nor changes in behaviour of these mice in direct comparison to infection of wild type mice.

3.3. DB1 infection was restricted to the respiratory tract

Because DB1 infected animals did not develop disease symptoms, the question raised whether DB1 infection is productive in mice and whether infectious virus would be found in various organs. To determine whether productive virus was detectable after infection with low pathogenic DB1, we decided to examine the organs of infected animals to assess the virus dissemination and the viral tropism in wild type, µMT and $CD8^{-/-}$ mice. Five female mice from each strain were either infected with $2x10^3$ pfu DB1 or MB1. Six days after infection all mice were sacrificed and virus titration from suspensions of different organs was performed as described in Section 2. High viral titers were detected in the lungs of C57Bl/6, µMT and $CD8^{-/-}$ mice six days after DB1 infection, indicating efficient replication without causing any clinical symptoms. C57Bl/6 and µMT mice showed almost the same viral titers in the lungs with 4.9 ± 0.5 log 10 pfu/ml or 4.8 ± 0.4 log 10 pfu/ml, respectively. In $CD8^{-/-}$ mice DB1 virus titer was slightly increased in the lungs (5.2 ± 0.5 log 10 pfu/ml, Table 2). This experiment clearly demonstrated that DB1 efficiently replicated in the lungs of mice, without causing any apparent clinical symptoms.

MB1 virus was detected in the lungs of C57Bl/6 mice showing high titers (4.9 ± 0.3 log 10 pfu/ml). A high viral titer was also found in the blood (4.0 ± 0.3 log 10 pfu/ml) of infected animals. Interestingly, in addition to its presence in the lungs and blood, MB1 virus was also found to lower extend in livers (2.7 ± 0.3 log 10 pfu/ml) and spleens (2.6 ± 0.3 log 10 pfu/ml) of wild type mice (Table 3). In contrast, infectious virus was detected only in the lung (5.1 ± 0.2 log 10 pfu/ml) and in the blood (3.4 ± 0.3 log 10 pfu/ml) of MB1 infected $CD8^{-/-}$ mice. The amount of infectious virus in those animals was not significantly higher when compared to the amount of MB1 detected in the lungs and blood of wild type mice. While the viral load in the lungs of µMT mice (4.9 ± 0.2 log 10 pfu/ml) was identical to wild type mice, no virus was detected in the livers, blood or spleens of antibody deficient mice. Interestingly, MB1 virus was detected in the brain of µMT mice (3.3 ± 0.2 log 10 pfu/ml) (Table 3). Until now we cannot explain the absent viral tropism in spleen and liver of µMT mice. To confirm this observation, this experiment was performed three times with an overall amount of 22 mice per strain. All experiments showed similar results.

3.4. Vaccination of wild type C57Bl/6 mice with DB1

Intranasal infection with DB1 did not lead to any apparent clinical symptoms but virus replicated in the lungs of infected mice. Therefore, we questioned whether infection of mice with an LPAIV from a natural reservoir would lead to solid immunity against a challenge

infection with a highly pathogenic H5N1 influenza A virus. Thus, six wild type mice were infected with $2x10^2$ pfu DB1. As demonstrated before, neither clinical symptoms nor weight loss were found after this primary infection. Eighty days later, mice were challenged with $2x10^5$ pfu (100-fold MLD_{50}) of MB1 virus. As a control, a group of naive, age-matched mice received the same lethal dose of MB1 virus. The animals were weighted daily and the health status was monitored twice a day for 14 days. As expected, wild type mice receiving only MB1 lost 20% of their initial body weight within 7 days after infection (Fig. 3A, open triangles) and died within days 8 and 12 past infection (Fig. 3B, open triangles). Mice that were vaccinated with DB1 and challenged with MB1 showed a moderate weight loss during the first 7 days of the infection (Fig. 3A, black squares), but showed no outward signs of illness. All animals survived the infection with the lethal H5N1 virus (Fig. 3B, black squares); thus, vaccination with DB1 significantly (p = 0.000653) protected C57Bl/6 mice against a lethal infection with MB1.

3.5. Vaccination and challenge infection of immunodeficient mice

To gain more information about the role of the immunological mechanisms that were involved in protection against H5N1 virus infection μMT, $CD8^{-/-}$ and in addition $CD4^{-/-}$ mice were also immunized with DB1.

When antibody deficient μMT mice were used for H5N1 challenge infection both the vaccinated (Fig. 4A, black squares) and the naive animals (Fig. 4A, open triangles) started losing weight immediately after challenge infection. Five days past MB1 infection mice of both groups lost already 20% of their body weight. Severe signs of clinical symptoms accompanied the decrease in body weight. Within 11 days past infection vaccinated (Fig. 4B, black squares) and unvaccinated (Fig. 4B, open triangles) μMT mice succumbed to infection. In contrast, when $CD8^+$ T-cell deficient mice were used for challenge infection the vaccinated mice showed a milder reduction of their body weight (10–12%) until day 5 past infection (Fig. 4C, black squares) and two out of six mice developed mild clinical symptoms (ruffled fur). Nevertheless, all vaccinated $CD8^{-/-}$ mice survived the 100-fold MLD_{50} H5N1 challenge infection (Fig. 4D, black squares). Naive $CD8^{-/-}$ mice lost body weight (Fig. 4C, open triangles) developed disease symptoms and all mice died (Fig. 4D, open triangles). Vaccination with DB1 significantly (p = 0.001) protected $CD8^{-/-}$ mice from lethal H5N1 infection.

In order to investigate the role of $CD4^+$ T-cells in protection against H5N1 influenza virus $CD4^{-/-}$ mice were used for vaccination with $2x10^2$ pfu DB1 (H5N2). After a 50-day recover

period the mice were challenged with 100-fold MLD_{50} ($2x10^5$ pfu) of MB1 virus. As a control, naive littermates were infected with the same lethal dose of MB1 virus. The vaccinated and unvaccinated group of $CD4^{-/-}$ mice lost a significant but similar amount of body weight during the first 7 days of the lethal infection (Fig. 4E). While all unvaccinated animals infected only with MB1 died by day 9 after lethal H5N1 inoculation (Fig. 4F, open triangles), 50% of the DB1 vaccinated mice recovered from the H5N1 infection, gained weight and survived (Fig. 4F, black squares). Since the $CD4^{-/-}$ mice belong to the sv129 genetic background wild type sv129 mice were used for vaccination experiments, showing similar susceptibility as C57Bl/6 mice to both influenza virus strains (data not shown). These data demonstrated that B-cells and to a lower extend $CD4^+$ T-cells but not $CD8^+$ T-cells are crucial for cross-protective primary immunity against H5 influenza virus.

To examine whether vaccination with DB1would lead to induction of cross-reactive antibodies against the hemagglutinin surface protein, serum from DB1 vaccinated mice was collected at different time points after infection. A hemagglutination inhibition assay was performed with the collected sera. Immunization with DB1 resulted in the production of HA-specific antibodies directed against DB1. First anti-HA antibodies were present in the sera already 3 days after the infection. The serum titers increased until day 14 past infection. Thereafter, the titer decreased, but anti-HA antibodies were detectable until challenge infection 80 days p.i. Similar pattern of anti-HA-antibodies were found after infection of $CD8^{-/-}$ and $CD4^{-/-}$ mice (Fig. 3C, black bars). Six days past challenge infection vaccinated wild type, $CD8^{-/-}$ and $CD4^{-/-}$ mice showed a rapid increase in serum titers compared to unvaccinated controls against DB1 (Fig. 3C, white bars). The highest anti-HA titers were reached by day 14 past infection in the vaccinated wild type, $CD8^{-/-}$ and $CD4^{-/-}$ mice, a time point where all control animals already had succumbed to infection (Fig. 3C, asterisk). These results demonstrated that an increased antibody response was induced in vaccinated mice. In addition to the presence of HA-specific antibodies, a virus neutralization assay revealed the presence of neutralizing antibodies. The antibody specificity after MB1 challenge infection was directed against DB1 and MB1 virus indicating specificity against DB1 and MB1 influenza virus (Fig. 5A and B). To quantify the cross-reactive antibodies elicited in immunized mice the HI titers in the sera of challenged wild type, $CD8^{-/-}$ and $CD4^{-/-}$ mice were compared towards DB1 (Fig. 5C, white bars) and MB1 (Fig. 5C, black bars). Comparable titers against DB1 and MB1 were found in the mice sera.

3.6. Detection of influenza virus-specific CD8$^+$ T-cells after challenge infection

In order to analyse the role of cellular immune response after challenge infection, tetramer staining was performed to investigate the CD8$^+$ T-cell response against the challenge influenza virus infection using the immunodominant epitope ASNENEAM from the viral NP. Interestingly, this epitope was found in the NP of MB1, while one amino acid change was detected in DB1 (ASNENETM). Nevertheless, both epitopes were recognized in similar intensity by virus-specific T-cells generated either after DB1 or MB1 infection (data not shown).

Six days after MB1 infection an increase of the amount of CD8$^+$ T-cells was found in μMT and CD4$^{-/-}$ mice compared to wild type mice, whereas no difference in the amount of CD8$^+$ T-cells was found in the spleen 6 days after challenge infection (Fig. 5D). In contrast, no difference in the amount of CD8$^+$ T-cells in wild type, μMT and CD4$^{-/-}$ mice was found in the lung either after primary MB1 infection or challenge infection (Fig. 5D).

Interestingly, most ASNENEAM specific CD8$^+$ T-cells were detected in μMT mice, either in spleen or lung after primary or challenge infection, while the amount of ASNENEAM specific T-cells in wild type and CD4$^{-/-}$ mice was similar in spleen or lung after primary or challenge infection (Fig. 5E).

4. Discussion

The emergence of highly pathogenic avian influenza viruses from the H5N1 subtype and its ability of crossing species barriers and infecting humans raised the strong need for new influenza vaccine approaches, in particular since H5N1 shows a broad antigenic heterogenicity. The development of a vaccine against potentially pandemic influenza will require a substantially different procedure than current strategies that are based on influenza vaccines made from a reassortant seed strain containing the viral hemagglutinin and neuraminidase of the circulating viruses [3]. Safe live-attenuated vaccine strains that provide broader cross-protective immunity against antigenic distinct H5N1 viruses are good candidates for protection covering also avian influenza viruses with pandemic potential [21]. Nevertheless, there are several questions to be answered on the way to design a successful live-attenuated vaccine with broad cross-protective properties. First, the live-attenuated vaccine strain needs to be safe, genetically stable and highly immunogenic. In addition, the vaccine should be designed in away to allow an optimal induction of cross-protective immune responses.

To bring a new light into this discussion, we wanted to scrutinize the immune cell populations involved in cross-protection by the use of immunodeficient mice for vaccination and challenge infection. The mouse model of influenza A virus infection is the best developed experimental system to investigate the immune response after infection, even though experimental influenza virus infection of mice does not resemble the natural infection found in humans and susceptible animal species. Nevertheless, there is increasing support that the findings in the mouse model are pertinent to the immune-mediated mechanisms found in humans during influenza [22, 23]. In the present work, the primary vaccinations and the challenge infections were performed with field isolates from birds without prior adaptation to the mammalian host. The avian influenza virus strain DB1 (A/mallard/Bavaria/1/2005; H5N2) was isolated from a healthy duck during a routine monitoring screen. The strategy of using H5N2 subtype viruses as inactivated vaccines is practised worldwide [24]. Here, infection with apathogenic H5N2 led to solid protection and to reduced virus shedding of MB1, a highly pathogenic H5N1 virus (A/mallard/Bavaria/1/2006) which was isolated from a dead mallard [25]. Interestingly, both viruses replicated in cell culture and in the lung of infected mice to similar titers. However, sufficient progeny virus after DB1 infection of MDCK cells was only found in the presence of trypsine after the first culture passage. This might be due to the monobasic cleavage site of the DB1 hemagglutinin. Thus, it was surprising that in the mouse model DB1 and MB1 replicated to similar titers in the lungs. While MB1 infection resulted in severe clinical symptoms and death, no signs of disease were found after DB1 infection. This was a prerequisite for DB1 to be used as a live-attenuated vaccine in the present study.

The challenge infection of DB1 infected/vaccinated wild type mice with 100 fold MLD_{50} of MB1 resulted in protection, while control littermates without vaccination developed disease or even died. This demonstrates that infection with the H5N2 virus acts like modified live vaccination and confers solid cross-protection against H5N1. Nevertheless, the H5 protein of both viruses is highly conserved and shares B-cells epitopes (Table 1, accession number) In contrast, when DB1 vaccinated μMT mice were challenged with MB1 ($2x10^5$ pfu; 100-fold MLD_{50}) all animals developed disease and died. This experiment reveals the important role of the humoral immune response for cross-protective vaccination. It is generally believed that isotype-switched antibodies are neutralizing and protective in challenge infections with influenza A virus and that B-cells are not able to produce neutralizing isotype-switched, influenza-specific antibodies in the absence of $CD4^+$ T-cell help [26–29]. However, other reports demonstrate that B-cells can produce protective isotype-switched antibodies in

response to different bacterial and viral infections including influenza A without $CD4^+$ T-cell collaboration [13, 30–32]. Thus, we questioned whether DB1 vaccination would protect mice deficient in $CD4^+$ T-cells against lethal H5N1 infection. Our results demonstrate that only 50% of the $CD4^+$ T-cell deficient mice were protected against H5N1 challenge, indicating a particular role for $CD4^+$ T-cells either by T-cell help for antibody production or by other effector functions. Nevertheless, the fact that similar amounts of HA-specific antibodies could be detected after challenge infection in $CD4^{-/-}$ mice supports the view for $CD4^+$ T-cell independent antibody response. There are data available that reveal a complex response for influenza virus-specific $CD4^+$ T-cells, which results in multiple effector phenotypes. These data imply that the $CD4^+$ T-cells and the memory cells derived from them can display a broad spectrum of functional potentials [33]. Thus, further research is mandatory to understand the role and function of $CD4^+$ helper T-cells during influenza virus infection and in cross-protective immunity in more detail.

After intranasal influenza A virus infection of mice the expansion of naive $CD8^+$ T-cells is tightly depended on antigen localization. Initial activation of $CD8^+$ T-cells occurs during the first 3 days after infection exclusively within the draining mediastinal lymph nodes [34]. Thereafter, the division of the influenza virus primed $CD8^+$ T-cells within the draining lymph nodes occurs between days 3 and 4 after infection and they migrate to the lung and to other regions of the respiratory tract where antigen is present [35]. At the site of virus infection the $CD8^+$ T-cells lyse the infected host cells and exert other effector functions like production of antiviral cytokines [36]. Influenza A virus-specific $CD8^+$ T-cells recognize multiple viral epitopes on infected target cells, whereas an epitope from the viral NP366–374 resembles an immunodominat epitope in C57Bl/6 mice (H2-Db) [37]. A lot of our knowledge was gained in the experimental mouse model but also broad information on common and immunodominant HLA restricted epitopes of circulating non-avian strains is available. Since influenza A virus activates dendritic cells that support the development of $CD8^+$ T-cells via innate immune mechanisms; T helper cells are not necessarily essential for a primary $CD8^+$ T-cell response [38]. These findings suggest that $CD4^+$ T-cell help is not required at the site of the pathological changes if the infection induces a sufficient amount of other immune stimuli. So the question raises, whether $CD8^+$ T-cells are mandatory for cross-protective immunity after vaccination with live-attenuated vaccine candidates. When DB1 vaccinated $CD8^+$ T-cell deficient mice were challenged with MB1 ($2x10^5$ pfu), all mice were protected. While it is of common knowledge that antibodies are required for protective immunity after vaccination, we were surprised by the result that $CD8^+$ T-cells seem not to be necessary for protection against

H5N1 challenge infection. It was reported that in μMT mice, the total number of virus-specific CTLs in the alveolar lavage after influenza virus infection is almost comparable to wild type mice [39]. Our results show that the amount of NP366–374 epitope-specific $CD8^+$ T-cells in lung and spleen was even increased compared to wild type mice after primary and challenge infection. Thus, $CD8^+$ T-cells are unable to counterbalance a missing humoral immune response.

From the present data one might question whether $CD8^+$ T-cells are dispensable for the control of an influenza virus infection? Despite evidence that live-attenuated influenza vaccines and inactivated influenza vaccines induce a cross-reactive cellular immune response in mice and humans, re-infection with homologous or heterotypic influenza viruses can be demonstrated [40, 41]. Cellmediated immunity in particular mediated by $CD8^+$ T-cells can promote viral clearance but in general it will not prevent infection. Already more than 25 years ago it could be shown that the amount of anti-influenza CTLs correlates with viral clearance but not with altered susceptibility to infection or re-infection [41].Nevertheless, Ulmer et al. were able to induce influenza virus nucleoprotein-specific $CD8^+$ T-cells by DNA vaccination that were able to protect against hetero-subtype lethal virus challenge in mice [42]. Moreover, vaccines based on immune stimulating complexes (ISCOMs) are able to induce CTL responses in addition to a strong antibody response in humans, but the contribution of $CD8^+$ T-cells for protection in primary infection was not investigated [43].

Taken together, our results support the mandatory role of humoral immune response in protection against influenza virus infection. Consequently, vaccine strategies including live-attenuated vaccines should focus on the induction of an effective antibody-mediated immune response accompanied by the helper function of $CD4^+$ T-cells. From our results one might speculate a minor role for $CD8^+$ T-cells in cross-protective primary immunity to influenza virus after vaccination, but their essential contribution for controlling the infection at the place where pathological changes occur, namely in the lung, is indisputable.

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Figure Legends

FIGURE 1: (A) Progeny virus titer of high pathogenic MB1 (H5N1) (open triangles) and low pathogenic DB1 (H5N2) (black squares) influenza viruses after infection of MDCK cells with MOI of 0.001. Detection limit was <1.7 log 10 pfu/ml. This experiment was performed three times. (B) Nucleic acid sequence analyses. The MB1 isolate contains a cleavage site with multiple basic amino acids. This polybasic cleavage site is an attribute for HPAI viruses. DB1 revealed a monobasic cleavage site, characteristic for LPAI viruses.

FIGURE 2: Susceptibility of immunocompetent and immunodeficient mice to DB1 or MB1 infection. Five mice from each group were infected i.n. with $2x10^3$ pfu of the low pathogenic H5N2 strain DB1 or with $2x10^3$ pfu of the highly pathogenic H5N1 strain MB1. Course of average body weight of wild type (A), µMT (C) and $CD8^{-/-}$ (E) animals after infection with DB1 (black squares) or MB1 (open triangles). Survival rates of wild type (B), µMT (D) and $CD8^{-/-}$ (F) mice. The graph represents one experiment with five mice. The experiment was performed twice with similar results.

FIGURE 3: Immunization of C57Bl/6 mice. Groups of six C57Bl/6 mice were infected i.n. with $2x10^2$ pfu DB1. Eighty days past DB1 infection, the animals were challenged with 100-fold MLD_{50} of the highly pathogenic MB1 virus (black squares). Controls (littermates) were only infected with MB1 (open triangles). (A) Weight progress. (B) Survival rates. (C) Production of anti-HA-specific antibodies directed against DB1 in C57Bl/6, $CD4^{-/-}$ and $CD8^{-/-}$ mice. Serum samples were collected at different time points past infection from immunized (black bars) and control (white bars) animals. *: at this time point all animals succumbed to infection. The results are given as the reciprocal of the highest dilution causing detectable inhibition of hemagglutination. This experiment was performed twice with similar results.

FIGURE 4: Immunization of immunodeficient mice. Six animals of each group were immunized i.n. with $2x10^2$ pfu DB1. Eighty days past DB1 infection, the animals were challenged with 100-fold MLD_{50} of the highly pathogenic MB1 virus. Controls (littermates) were infected with MB1. Weight progress from µMT (A), $CD8^{-/-}$ (C) and $CD4^{-/-}$ (E) mice infected with DB1 (black squares) or MB1 (open triangles). Survival curves from µMT (B), $CD8^{-/-}$ (D) and $CD4^{-/-}$ (F) mice. For statistical analysis the Cox-regression test was used.

FIGURE 5: *In vitro* neutralization assay. To test the ability of different sera to inhibit DB1 (A) or MB1 (B) virus an *in vitro* neutralization assay was performed with sera from C57Bl/6 mice. Virus was incubated for 30min at room temperature with serum from uninfected mice (white bars), DB1 infected mice 14 days past infection (pale gray bars) and challenged mice 14 days past infection (dark gray bars). For control virus was incubated with PBS (black bars). The numbers of infectious particles were assessed by plaque titration. The bars represent the mean values of three different experiments. (C) Comparison of the HI titers in the serum pool (n = 5) of immunized C57Bl/6, $CD8^{-/-}$ and $CD4^{-/-}$ mice 14 days past challenge infection towards DB1 (white bars) and MB1 (black bars). The results are given as the reciprocal of the highest dilution causing detectable inhibition of hemagglutination. Flow cytometry for $CD8^+$ T-cells and NP-specific $CD8^+$ T-cells in the lungs and spleens of MB1 infected (n = 4) C57Bl/6, µMT and $CD4^{-/-}$ mice 6 days past MB1 infection and challenged C57Bl/6, µMT and $CD4^{-/-}$ (n = 3) also 6 days past MB1 infection. Cells were obtained from spleens and lungs of infected mice and assayed for the presence of CD8+ T-cells (D) and NP366–374-specific H2Db restricted $CD8^+$ T-cells determined by tetramer staining (E).

Tables

Table 1
Overview of the used avian influenza A virus strains

Species	Subtype	Abbreviation	Accession-No.[a]	MLD_{50}
A/mallard/Bavaria/1/2006	H5N1	MB1	DQ458992	$2x10^3$
A/mallard/Bavaria/1/2005	H5N2	DB1	DQ387854	$> 5x10^5$

[a] Accession number based on hemagglutinin

Table 2
Distribution of DB1 virus after infection of mice

d6 p.i.	lung	brain	kidney	liver	spleen	blood	heart
C57Bl/6	4.9 ± 0.5	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
µMT	4.8 ± 0.5	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
$CD8^{-/-}$	5.2 ± 0.5	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7

Virus titer is given as log 10 pfu per 1ml 10% organ homogenate

Table 3
Distribution of MB1 virus after infection of mice

d6 p.i.	lung	brain	kidney	liver	spleen	blood	heart
C57Bl/6	4.9 ± 0.3	< 1.7	< 1.7	2.7 ± 0.3	2.6 ± 0,3	4.0 ± 0.3	< 1.7
µMT	4.9 ± 0.2	3.3 ± 0.2	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
CD8$^{-/-}$	5.1 ± 0.2	< 1.7	< 1.7	< 1.7	< 1.7	3.4 ± 0.3	< 1.7

Virus titer is given as log 10 pfu per 1ml 10% organ homogenate

Figures

Figure 1

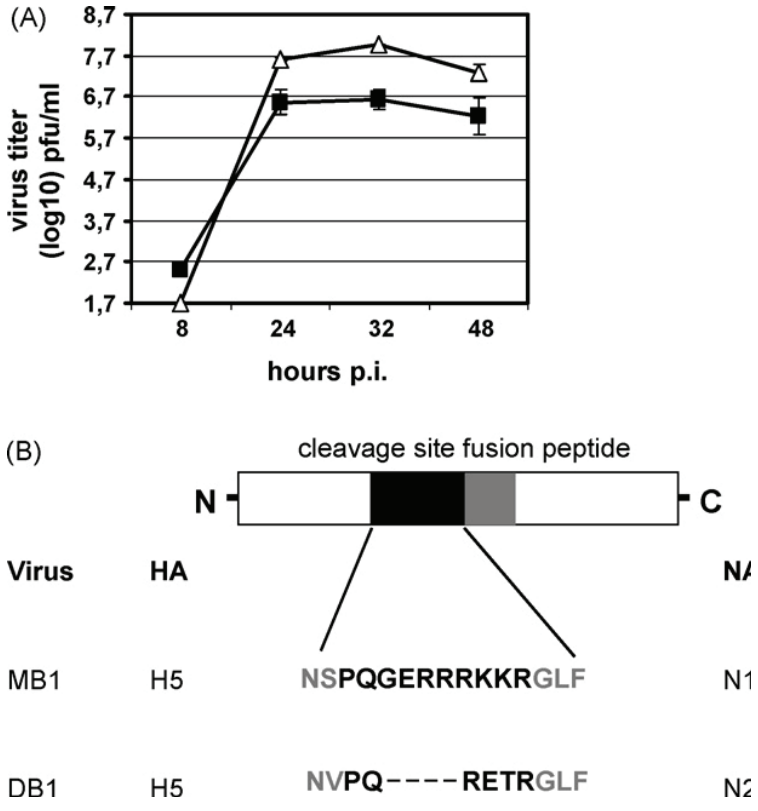


Figure 2

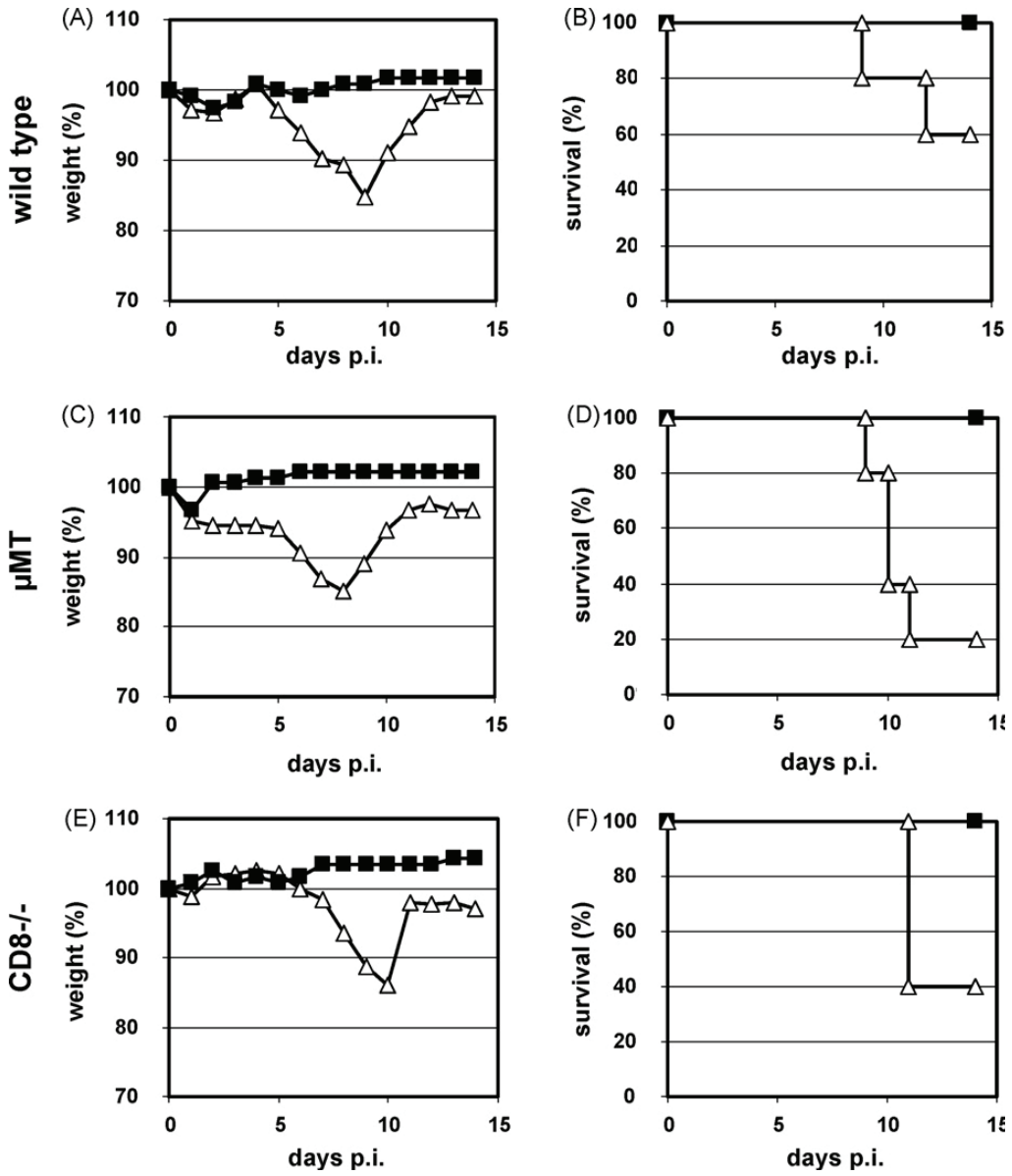

(A)
wild type
weight (%)
110
100
90
80
70
0
5
10
15
days p.i.
(B)
survival (%)
100
80
60
40
20
0
0
5
10
15
days p.i.
(C)
µMT
weight (%)
110
100
90
80
70
0
5
10
15
days p.i.
(D)
survival (%)
100
80
60
40
20
0
0
5
10
15
days p.i.
(E)
CD8-/-
weight (%)
110
100
90
80
70
0
5
10
15
days p.i.
(F)
survival (%)
100
80
60
40
20
0
0
5
10
15
days p.i.

Figure 3

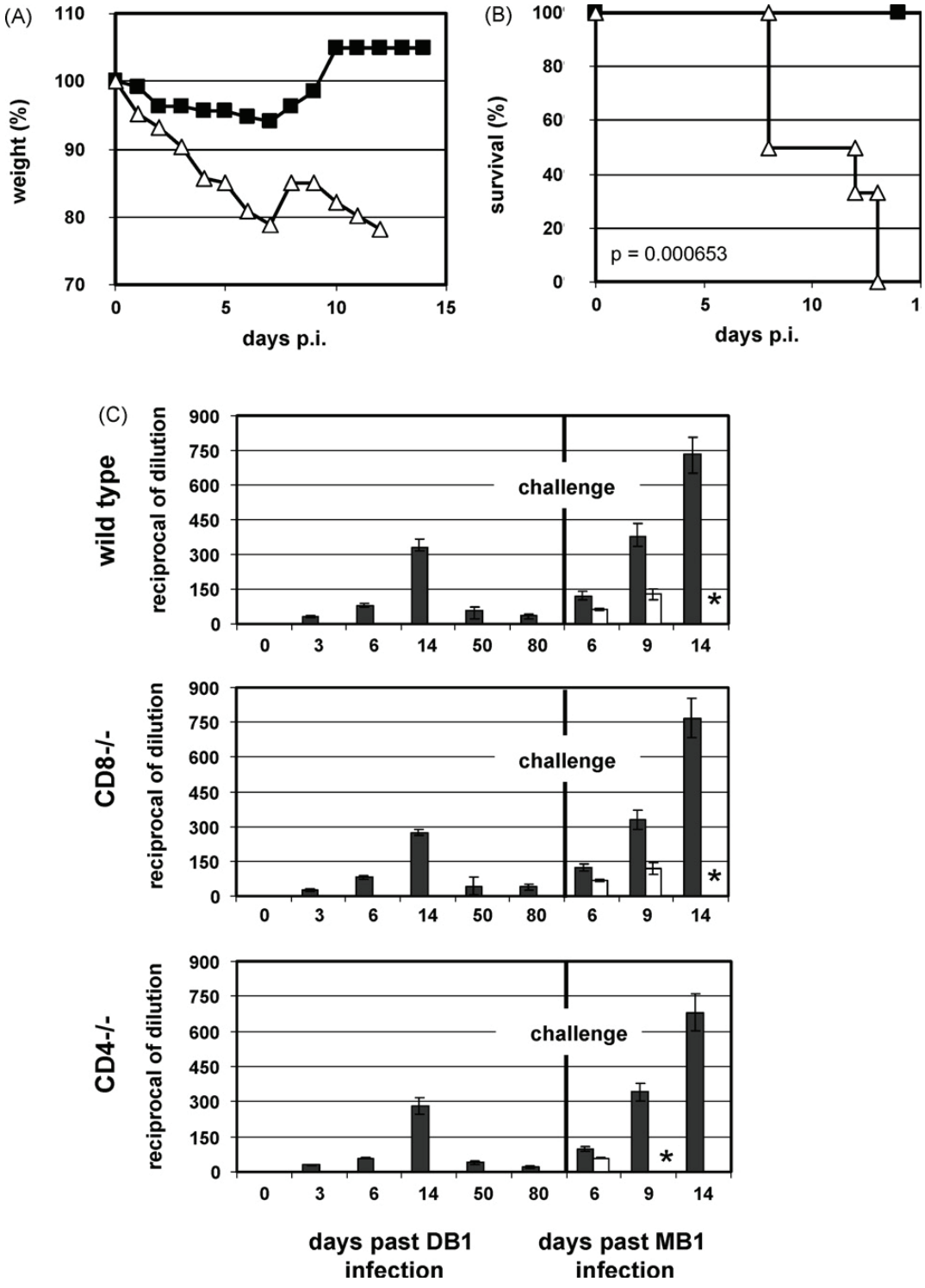

(A)
weight (%)
110
100
90
80
70
0
5
10
15
days p.i.
(B)
survival (%)
100
80
60
40
20
0
p = 0.000653
0
5
10
1
days p.i.
(C)
wild type
CD8-/-
CD4-/-
reciprocal of dilution
900
750
600
450
300
150
0
challenge
0
3
6
14
50
80
6
9
14
*
days past DB1 infection
days past MB1 infection

Figure 4

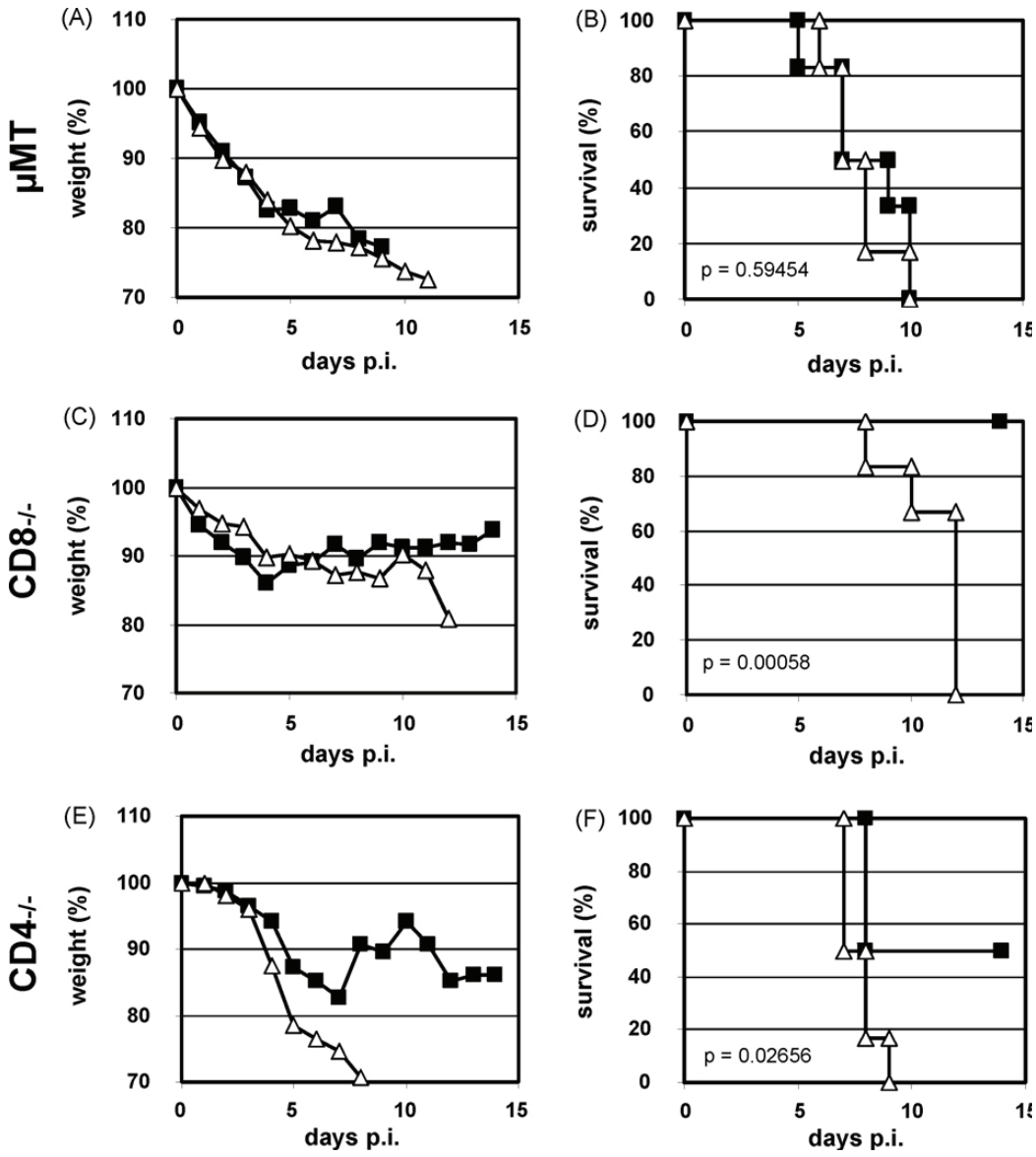
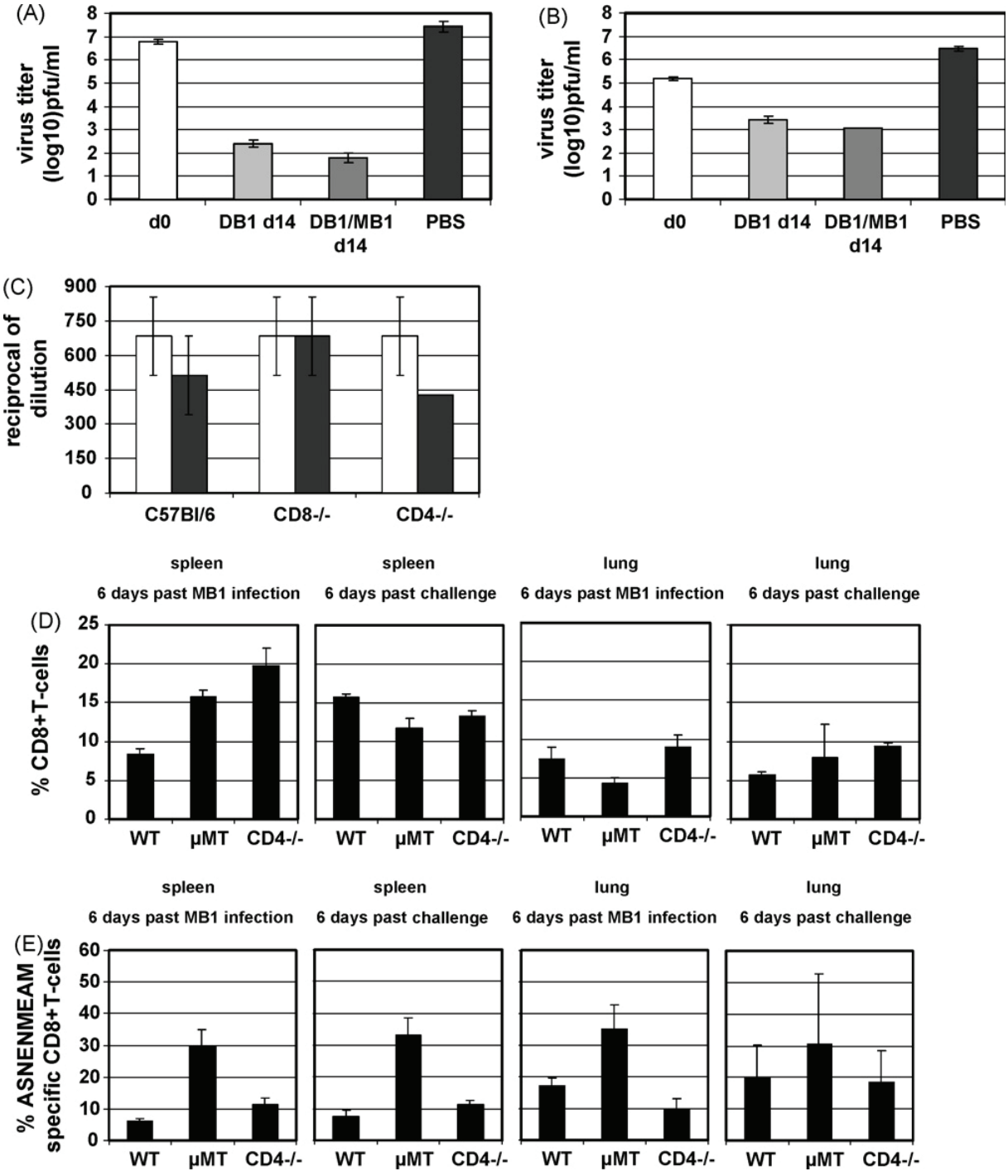

(A)
μMT
weight (%)
days p.i.
(B)
survival (%)
p = 0.59454
days p.i.
(C)
CD8-/-
weight (%)
days p.i.
(D)
survival (%)
p = 0.00058
days p.i.
(E)
CD4-/-
weight (%)
days p.i.
(F)
survival (%)
p = 0.02656
days p.i.

Figure 5


(A)
virus titer (log10)pfu/ml
d0
DB1 d14
DB1/MB1 d14
PBS
(B)
virus titer (log10)pfu/ml
d0
DB1 d14
DB1/MB1 d14
PBS
(C)
reciprocal of dilution
C57Bl/6
CD8-/-
CD4-/-
spleen
6 days past MB1 infection
spleen
6 days past challenge
lung
6 days past MB1 infection
lung
6 days past challenge
(D)
% CD8+T-cells
WT
µMT
CD4-/-
(E)
% ASNENMEAM specific CD8+T-cells

PUBLICATION #5:

A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance

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Abstract
Infections with influenza A viruses still pose a major threat to humans and several animal species. The occurrence of highly pathogenic avian influenza viruses of the H5N1 subtype capable to infect and kill humans highlights the urgent need for new and efficient countermeasures against this viral disease. Here we demonstrate that a polyphenol rich extract (CYSTUS052) from the Mediterranean plant *Cistus incanus* exerts a potent anti-influenza virus activity in A549 or MDCK cell cultures infected with prototype avian and human influenza strains of different subtypes. CYSTUS052 treatment resulted in a reduction of progeny virus titers of up to two logs. At the effective dose of 50 µg/ml the extract did not exhibit apparent harming effects on cell viability, metabolism or proliferation, which is consistent with the fact that these plant extracts are already used in traditional medicine in southern Europe for centuries without any reported complications. Viruses did not develop resistance to CYSTUS052 when compared to amantadine that resulted in the generation of resistant variants after only a few passages. On a molecular basis the protective effect of CYSTUS052 appears to be mainly due to binding of the polymeric polyphenol components of the extract to the virus surface, thereby inhibiting binding of the hemagglutinin to cellular receptors. Thus, a local application of CYSTUS052 at the viral entry routes may be a promising approach that may help to protect from influenza virus infections.

1. Introduction

Influenza A viruses are negative strand RNA viruses with a segmented genome that belong to the family of *orthomyxoviridae* (Lamb and Krug, 2001). While the natural reservoir of these viruses is within wild living water fowl, influenza A viruses also infect humans and several animal species (Webster et al., 1992). In fact, influenza A viruses still pose a major burden to human health and cannot be eradicated due to their large natural reservoir. Thus, introduction of avian virus genes into the human population can happen at any time and may give rise to a new pandemic. There is even the likeliness of a direct infectionof humans by avian viruses, as evidenced by the emergence of highly pathogenic avian influenza viruses of the H5N1 subtype that were capable to infect and kill humans (Shortridge et al.,2000; Webby and Webster, 2001).

Although vaccination is the best option to protect from influenza virus infections, this approach is difficult with regard to pathogenic avian strains or human reassortants with avian glycoprotein genes. To date only two classes of anti-influenza drugs have been approved; inhibitors of the M2 ion channel such as amantadine and rimantadine or neuraminidase inhibitors such as oseltamivir or zanamivir (De Clercq, 2004). Treatment with amantadine and

derivatives rapidly results in the emergence of resistant variants and is not recommended for a general and uncontrolled use (Fleming, 2001; Hayden and Hay, 1992). Among H5N1 isolates from Thailand and Vietnam 95% of the strains have been shown to harbor genetic mutations associated with resistance to the M2 ion channelblocking adamantine derivatives, amantadine and rimantadine (Cheung et al., 2006). Furthermore influenza B viruses are not sensitive to amantadine derivatives (Pinto and Lamb, 2006). Recent reports have described that resistance development also occurs to neuraminidase inhibitors (Hatakeyama and Kawaoka, 2006; Hurt et al., 2007). According to a recent study, oseltamivirresistant mutants in children being treated for influenza with oseltamivir appear to arise more frequently than previously reported (Kiso et al., 2004). The authors concluded that children can be a source of viral transmission, even after 5 days of treatment with oseltamivir (Kiso et al., 2004). In addition, there are several reports that suggest that resistance of H5N1 viruses can emerge during the currently recommended regimen of oseltamivir therapy and may be associated with clinical deterioration (de Jong et al., 2005). Thus, it has been stated that the strategy for the treatment of influenza A (H5N1) virus infection should include additional antiviral agents. This highlights the urgent need for new and amply available anti-influenza agents.

Polyphenols are a large family of natural compounds widely distributed in plants and characterized by the presence of more than one phenol group per molecule. Thus, polyphenols constitute one of the most numerous groups of substances with more than 8000 phenolic structures currently known. The structure can vary from simple molecules, such as phenolic acids, to highly polymerized compounds (as reviewed by Harborne, 1980). Natural polyphenols have been attributed to exert an array of biological effects. Plant derived polyphenols have been shown to be strong antioxidants with potential health benefits (Urquiaga and Leighton, 2000). Reports on an antiviral and antibacterial potency of polyphenolic compounds are numerous (reviewed by Cos et al., 2003, 2004; Scalbert, 1991; Taguri et al., 2006). A variety of studies described the antiviral action of natural flavonoids/flavanoids on rhinoviruses and other picornaviruses (reviewed by Conti et al., 1988; Selway, 1986; Vlietinck et al., 1988). This work has also led to the synthesis of derivatives of the natural products and their detailed examination as antiviral drugs (reviewed by Vlietinck and Vanden Berghe, 1991).

While a variety of mechanisms of action of different polyphenolic plant products have been proposed in different studies, it has been suspected quite early on that antiviral activities of many phenolic compounds may be due to direct interactions with the viral particle (reviewed by Haslam, 1996). In an early study by Konishi and Hotta the authors showed that the

polyphenolic agent tannic acid inactivates Chikungunya virus (CHIKV), a positively stranded enveloped RNA virus, most likely due to preferential binding to viral proteins on the particle (Konishi and Hotta, 1979). While anti-HIV activity of the biphenolic agent l-chicoric acid was initially thought to be due to integrase inhibition, it was later found that the compound targets the HIV glycoprotein gp120 at the surface of the particle (Pluymers et al., 2000). More recent data show that flavonoids and proanthocyanidins from *Crataegus sinaica* exhibit significant inhibitory activity against herpes simplex virus type I (HSV-1) due to an extracellular mechanism (Shahat et al., 2002).

Finally, it has been demonstrated that the polyphenols (-) epigallocatechin gallate and theaflavin digallate are able to bindhemagglutinin of influenza viruses and agglutinate the particles and therefore reduce their infectivity and binding at the cell surface (Nakayama et al., 1993).

Bioavailability differs greatly from one polyphenol to another (Manach et al., 2004; Urquiaga and Leighton, 2000). The current knowledge of absorption, biodistribution and metabolism of polyphenols is poor, however, in general it can be stated, that some low-molecular-weight polyphenols are absorbed and exhibit bioactive properties, while only few polymeric polyphenols are metabolized (Manach et al., 2004). Thus, it was suggested that polyphenols might exert direct effects when present in high concentrations (Halliwell et al., 2005).

CYSTUS052 is a plant extract that is very rich in highly polymeric polyphenols. Major constituents include flavan-3-ols and proanthocyanidins (Danne et al., 1993; Petereit et al., 1991). The total content of monomeric components is lower than 2% and the polymeric components, especially the proanthocyanidines with a molecular weight range of 500–1200 Da are dominant. The extract is a preparation of a selected variety of the biochemical polymorphic species *Cistus incanus* (Pink Rockrose). The genus *Cistus* consists of a group of about 20 shrub species found in wide areas throughout the whole Mediterranean region up to the Caucasus (Comandini et al., 2006). *Cistus* species are used as anti-diarrheic, as general remedies in folk medicine for treatment of various skin diseases, and as anti-inflammatory agents (Danne et al., 1993; Petereit et al., 1991).

Given that some purified polyphenolic compounds have been demonstrated to showanti-influenza virus activity (Nakayama et al., 1993; Palamara et al., 2005; Song et al., 2005) and that *Cistus* plant preparations have been attributed to exert bactericidal and fungicidal effects (Bouamama et al., 1999), we were prompted to analyze whether CYSTUS052 may have anti-influenza virus activity in cell culture.

2. Experimental/materials and methods

2.1. CYSTUS052 extract

CYSTUS052 Extract Charge-Number 40121T01B/04 was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (8-2004; Glandorf, Germany). The extract is a special preparation from a distinct variety of *Cistus incanus* (*Cistus incanus* PANDALIS). CYSTUS052 extract was defined by the producer to exhibit a polyphenolic content of more than 26% (determined after Singleton et al., 1999; Singleton and Rossi, 1965). The total content of monomeric components (gallic acid, gallocatechin, catechin, epicatechin) is lower than 2%.

CYSTUS052 was solved in sterile PBS (1mg/ml) at 100°C for 1 h and was employed from 1 to 100 μg/ml and added directly to the medium or virus-stock.

2.2. Viruses, cells and viral infections

Avian influenza virus A/FPV/Bratislava/79 (H7N7) (FPV) has been propagated and passaged in Madin Darby canine kidney (MDCK) cells. Human influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) was grown in 10-days-old embryonated chicken eggs. After incubation at 37°C for 2 days the allantois fluid was harvested and used for infection as described below (Ehrhardt et al., 2004, 2006; Wurzer et al., 2004).

The human influenza isolate A/Thailand/1(KAN-1)/2004 (H5N1) was provided by P. Puthavathana (Mahidol University, Bangkok, Thailand) (Puthavathana et al., 2005) and has been passaged in MDCK cells. Human Rhinovirus serotype 14 (HRV14) was purchased from ATCC (ATCC #284) and was amplified in HeLa cells. HRV was propagated at 33°C. For infection cells were washed with PBS, incubated with virus diluted in PBS/BA (PBS containing 0.2% BSA, 1mM $MgCl_2$, 0.9mM $CaCl_2$, 100U/ml penicillin and 0.1mg/ml streptomycin) for 30min at 33°C or 37°C at the indicated multiplicities of infection (MOI). The inoculum was aspirated and cells were incubated with MEM, DMEM or HAM's F12 containing 0.2% BSA and antibiotics. In the case of PR8-infection medium was supplemented with 2 μg/ml trypsin. MDCK cells were grown in MEM, human cervical carcinoma cells (HeLa) were grown in DMEM and the human lung epithelial cell line A549 was grown in HAM's F12, respectively. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). For proapoptotic or proinflammatory stimulation of cells staurosporine dissolved in DMSO (2.5 μM, 8 h) (Sigma) or recombinant TNFα (Sigma) (30 ng/ml, 15 min) were directly added into the medium.

2.3. Plaque titrations and resistance assays

Supernatants, collected at the indicated time points, were used to assess the number of infectious particles (plaque titers) in the sample. Briefly, MDCK-cells grown to 90% confluency in 6-well dishes were washed with PBS and infected with serial dilutions of the supernatants in PBS/BA for 30min at 37°C. The inoculum was aspirated and cells were incubated with 2ml MEM/BA (medium containing 0.2% BSA and antibiotics) supplemented with 0.6% Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech) and 1.5% $NaHCO_3$ at 37°C, 5% CO_2 for 2–3 days. Virus plaques were visualized by staining with neutral red. Generation of resistant virus variants to antiviral treatment was assessed as recently described (Ludwig et al., 2004). Briefly, MDCK cells were infected with the influenza A virus strain FPV (MOI = 0.01) and were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine (Sigma–Aldrich). At 24 h post infection supernatants were taken and employed for infection in the second round of investigation. After infection cells were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine again. This procedure was repeated six times. Supernatants were assayed for progeny virus yields by standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%

2.4. Hemagglutination-inhibition-test

Influenza viruses are characterized by their ability to agglutinate erythrocytes. This hemagglutinating activity can be visualized upon mixing virus dilutions with chicken erythrocytes in microtiter plates. The chicken erythrocytes (Lensing–Baeumer, Ibbenbueren) supplemented with 1.6% sodium citrate (Sigma) in sterile water were separated by centrifugation (800×*g*, 10 min, room temperature) and washed three times with sterile PBS (Invitrogen). The lowest amount of virus particles able to agglutinate the chicken erythrocytes was determined in a serial virus dilution and used to investigate the inhibitory effect of CYSTUS052 onto the hemagglutinating activity. Untreated erythrocytes precipitate to the bottom of the plate, while upon preincubation with virus the blood cells show an even and diffuse distribution. Briefly, CYSTUS052 was serially diluted as indicated. From virus stocks (H5N1 or H7N7, HA titers: 29) 1/128 dilutions were made, and 50 µl/well of this virus dilution was added as indicated. After preincubation of 45min, chicken erythrocytes (1/20 in PBS) were mixed with the solution. In the samples where viruses were preincubated with CYSTUS052, up to a certain dilution the viral particles were no longer capable of agglutinating erythrocytes, indicating an interaction of CYSTUS052 with the viral HA.

2.5. MTT-assay

A549 cells were left untreated or treated with the indicated amounts of CYSTUS052 for the indicated time points. Cell-proliferation/metabolism was measured in the MTT-assay. Briefly, cells were treated with 5 mg/ml thiazolylbluetetrazoliumbromid/PBS (Sigma/Invitrogen) and incubated for 3 h at 37°C. Reaction was blocked by DMSO (Sigma) and cells were further incubated for 20 min at 37°C. The color reaction was measured in an Emax precision microplate reader at 562 nm. The untreated control was arbitrarily set as 100%.

2.6. Western blotting

For Western blotting cells were lysed on ice with Triton lysis buffer (TLB; 20mM Tris–HCL, pH 7.4; 137mM NaCl; 10% Glycerol; 1% Triton X-100; 2mM EDTA; 50mM sodium glycerophosphate, 20mM sodium pyrophosphate; 5 $\mu g ml^{-1}$ aprotinin; 5$\mu g ml^{-1}$ leupeptine; 1mM sodium vanadate and 5mM benzamidine) for 30 min. Cell lysates were cleared by centrifugation and directly subjected to SDS-PAGE and subsequent blotting on nitrocellulose membrane. For protein detection PARP-specific mouse antibody (BD Transduction Laboratories)or an IκBα-specific rabbit antibody (Santa Cruz Biotechnologies) were employed and loading controls were performed with pan-ERK2 antiserum (Santa Cruz Biotechnologies). Protein bands were visualized in a standard enhanced chemiluminescence reaction.

2.7. Indirect immunofluorescence microscopy

A549 cells were directly seeded onto 15mm glass plates. Twenty-four hours later cells were infected with FPV (MOI = 200) for 1 h or (MOI = 50) for 1.5 h. Cells and/or virus were incubated with CYSTUS052 as indicated. Cells were washed twice with PBS and then fixed for 30 min with 3.7% paraformaldehyde (in PBS) at room temperature. After washing, cells were permeabilized with acetone, washed with PBS and blocked with 1% bovine albumin or 10% fetal bovine serum in PBS for 20 min at 37°C. Upon blocking, cells were incubated with a mouse monoclonal antibody against viral NP (Serotec) (1:400) in PBS for 30 min. Following several washing steps, cells were incubated with a nucleoprotein-specific mouse antibody and an Alexa fluor 488 chicken anti mouse IgG (H + L) (1:300) in PBS for 1 h. Finally, cells were washed. Fluorescence was visualized using a Leitz DMRB fluorescence microscope.

2.8. Flow cytometry analysis

For cell viability assays MDCK or A549 cells were grown to near confluency in 6-well or 12-well dishes and either left untreated or incubated with the indicated amounts of CYSTUS052 for the indicated time points. For analysis both adherent and attached cells were collected, washed with PBS and incubated with 250µl propidium iodide (PI) solution (50 µg/ml PI in PBS) for 10 min. The proportion of dead *versus* viable cells was then analyzed by flow cytometry analysis using a FACScalibur (BD Biosciences). All FACS analyses were repeated twice and revealed essentially similar results.

3. Results

3.1. CYSTUS052 treatment results in an efficient and concentration-dependent reduction of progeny virus titers

In a first set of experiments we attempted to assess whether CYSTUS052 may exert an antiviral effect on influenza virus propagation in cultured cells. We used A549 lung epithelial cells (A549) or Madine Darby canine kidney (MDCK) cells, both standard host cell lines for influenza virus propagation. These cells were incubated with the plant extract at various concentrations and subsequently infected with different influenza A virus strains. This included the human prototype isolate A/Puerto-Rico/8/34 (H1N1) (PR8), the highly pathogenic avian influenza virus (HPAIV) A/FPV/Bratislava/79 (H7N7) (FPV) as well as a human isolate of the HPAIV of the H5N1 subtype (A/Thailand/1(KAN-1)/2004 (H5N1)). The concentrations of the various extract dilutions were kept constant throughout the experiment in each sample (Fig. 1A and B) and showed a dose dependent reduction of progeny virus titers of FPV and PR8. Maximum reduction of progeny virus titers in the order of two logs was observed with both viruses in A549 cells using CYSTUS052 at a concentration of 50 µg/ml (Fig. 1A and B). This reduction is not due to a simple delay in virus replication since it can be consistently observed in growth kinetics of PR8 over a 36 h observation period (Fig. 1B). CYSTUS052 was also effective against the HPAIVof theH5N1subtype; however, compared to FPV, anHPAIV of the H7 subtype, a higher concentration was needed to achieve the same degree of reduction of progeny virustiters (Fig. 1C). These data indicate that CYSTUS052 has broad antiviral properties towards different influenza viruses.

3.2. CYSTUS052 treatment does not affect cell morphology and viability and does not negatively interfere with cellular proliferation and metabolism

A major prerequisite for an antiviral agent is safety. Thus, we tested whether CYSTUS052 in the concentrations used would have any harming effect on the healthy cell. In an initial approach cells treated with CYSTUS052 for different time points (up to 72 h) were examined morphologically (data not shown). No differences in cell shape or cell numbers could be observed compared to control cells. The same cells were stained with propidium iodide (PI) to detect the numbers of dead cells in each sample (Fig. 2A). Again no significant changes in the number of dead cells could be detected over a 72 h observation period. CYSTUS052 did also not negatively interfere with the proliferative and metabolic capacity of cells as determined in MTT assay (data not shown). In this assay the activity of a mitochondrial metabolic enzyme that is only active in proliferating healthy cells is monitored. Consistent with these data, CYSTUS052 did also not induce apoptosis as evidenced by a lack of cleavage of Poly-ADP-ribose-polymerase (PARP), a prominent substrate of apoptotic caspases (Fig. 2B).

While the extract did not show any effects on cell proliferation or cell survival, it may still be that cellular responses may be significantly altered in CYSTUS052 treated cell. Thus, the effect of CYSTUS052 on cellular gene transcription andtranslation was assessed in a reporter gene assay using cells that were transfected with a vector expressing luciferase under the control of a constitutively active CMV promoter element (data not shown). CYSTUS052 treatment did not alter transcription or translation in this system. Finally, it was analyzed whether CYSTUS052 may alter the response to ligands that bind to cellular receptors. As an example, the cellular response to the cytokine TNFα was analyzed (Fig. 2C). A549 cells were treated with CYSTUS052 or left untreated. Subsequently cells were stimulated with TNFα, a strong activator of the cellular transcription factor NF-κB (Fig. 2C, lanes 2, 4 and 5). Activation of NF-κB was then monitored in Western blots by examination of degradation of IκBα, the inhibitor of NF-κB (Fig. 2C). In these assays IκBα was nearly fully degraded in TNFα-stimulated cells regardless whether the cells were pretreated with CYSTUS052or not (Fig. 2C, lanes 4 and 5). This is indicative of an unimpaired response to TNFα stimulation and may stand as a general example for cellular ligand/receptor systems. In addition, these assays also demonstrated that CYSTUS052 alone is inert towards activation of NF-κB (Fig. 2C, lane 3) again indicating that the compound does not harm living cells.

3.3. CYSTUS052 affects early viral entry

In a next set of experiments we aimed to identify the step in the virus life cycle that is affected by CYSTUS052. Therefore, supernatants from cells that were treated with CYSTUS052 at different time points pre- and post-infection were analyzed for their content of progeny virus.

A titer reduction could only be observed if cells were either pre-incubated with CYSTUS052 orif the extractwas added simultaneously with infection (Fig. 3A). This argues for a very early step in the virus life cycle that is affected by CYSTUS052. Interestingly, a pronounced titer reduction of progeny virus could also be detected if untreated cells were infected with a virus lot that was preincubated with CYSTUS052. This is a first indication that components of the CYSTUS052 extract may interfere with the virus particle itself to prevent infection. Thus, we analysed whether CYSTUS052would affect viral uptake. Cells were infected with a very high multiplicity of infection (MOI) to detect incoming virus according to an established immunofluorescence protocol to study entry of influenza A virus (Ehrhardt et al., 2006; Sieczkarski et al., 2003). Briefly, the ribonucleoprotein (RNP) complexes of virus particles are stained with an antibody against the nucleoprotein, the major constituent of the viral RNPs. In untreated cells newly synthesized NP is already visible 2 h post infectionin the cell nucleus (Fig. 3C and D, left). If cells were pretreated with CYSTUS052 nuclear NP staining was more diffuse and there was enhanced detection of bright spots, which most likely represent RNPs of viral particles sticking to the cell surface or in intracellular compartments (Fig. 3C, middle). This effect was even more pronounced if the virus lot used for infection was pre-treated with CYSTUS052 (Fig. 3C, right) Here, newly synthesized NP is only poorly visible in the cell nucleus, however, a massive spot-like staining indicates the presence of virus particles that did not effectively enter the cell. For some cells a staining pattern could be observed that is consistent with virus particles lining-up in close proximity to the membrane without the ability to fully enter (Fig. 3D, right, white arrowheads).Taken together, CYSTUS052 treatment of virus particles appears to inhibit virus uptake.

3.4. CYSTUS052 blocks hemagglutinating activity of pre-treated virus particles

The data so far suggest that components of the CYSTUS052 extract directly interferes with the virus particle itself to inhibit infection. To study whether CYSTUS052 would prevent the ability of the virus particle to bind to cell surface receptors, we employed hemagglutination inhibition (HI) assays. Influenza A viruses are able to agglutinate red blood cells (RBC) *via* the viral glycoprotein, the hemagglutinin, that binds to N-acetylneuraminic acid at the cell surface. The RBC get crosslinked by the virus and will form a type of lattice in this case. This results in a diffuse appearance of theRBCin a round-bottom vial (Fig. 4A panel 3 and 6) in contrast to a spot-like appearance of precipitated RBC (or line shaped when plate is tilted) in the absence of any virus (Fig. 4A, panel 1, 4 and 7). CYSTUS052 pretreatment prevented binding of different viruses to RBC in this assay (Fig. 4A, panel 2 and 5) indicating that

components of the CYSTUS052 extracts are capable of directly interfering with the viral HA to block binding to cellular receptors. Interestingly, a lower concentration of CYSTUS052 was sufficient to prevent binding of FPV compared to the H5N1 isolate, which is consistent with the previous observation that higher concentrations of CYSTUS052 are required to inhibit propagation of A/Thailand/1(KAN-1)/2004 (H5N1) (Fig. 1C).

3.5. CYSTUS052 inhibits infection of cells with human rhinoviruses

Direct interaction of CYSTUS052 components with proteins at the surface of the virus particle would suggest a rather unspecific physical mode of antiviral action. If this would be the case, CYSTUS052 should not only block infectivity of influenza A viruses but also other viruses by interference with surface proteins. This hypothesis was tested using a different respiratory virus, namely human rhinovirus (HRV14). Since rhinoviruses, in contrast to influenza viruses, are non-enveloped viruses, this experiment further allowed to discriminate whether presence of a glycoprotein containing viral envelope is required for CYSTUS052 action, HRV14 was used in a concentration sufficient for full lysis of infected HeLa cells up to a dilution of 10−7. If viruses or cells were preincubated with CYSTUS052, cell lysis that indicates infectivity ofHRV14 is completely prevented even at the highest virus concentration used (Fig. 4B). This indicates that CYSTUS052 also efficiently blocks propagation of the non-enveloped HRV14, presumably by direct interaction with its viral protein surface.

3.6. CYSTUS052 does not show any tendency to induce viral resistance

A most crucial issue regarding an anti-influenza virus agent is the emergence of resistant variants to the drug. According to the data presented so far, components of the CYSTUS052 extract appear to act in a rather unspecific physical manner to interfere with the surface of viruses. In this case, the virus should not easily overcome the inhibitory effect by mutations of single amino-acid residues at its surface proteins. This was indeed the case as assessed in an established multi-passaging experiment to detect emergence of resistant viruses in cell culture (Ludwig et al., 2004). As shown in Fig. 5, in this assay virus titers from cells treated with amantadine, a blocker of the viral M2 ion channel that rapidly results in the generation of resistant variants, were up to levels of untreated cells after the fifth passage, indicating that this pool of viruses had become fully resistant to the drug. This was different for infected cells treated with CYSTUS052. While the initial inhibition of virus propagation was less efficient than in amantadine-treated cells, titers did not raise with increasing passage numbers but even

appeared to slowly decrease. This indicates that in contrast to amantadine, influenza virus does not easily become resistant to CYSTUS052 treatment.

4. Discussion

Infections with influenza A viruses are still a major health burden and the options for control and treatment of the disease are limited. Here we show that an extract derived from a special variant of the Mediterranean plant *Cistus incanus*, CYSTUS052, exerts a potent antiviral activity in cell culture. On a molecular basis the extract appears to interfere with the virussurface proteins and inhibits binding of the virus particle to cellular receptors. We cannot completely rule out an additional contribution of binding of CYSTUS052 components directly to the cell surface, e.g. to proteins, oligosaccharides or sialic acids (SA), that are receptors for influenza virus. However, since SA are widely distributed on cellular proteins on the surface, a pronounced binding to these components should harm some of the cellular functions examined (which was not the case) or binding of ligands to the cell surface, as analyzed in Fig. 2C.

CYSTUS052 has a very high polyphenol content. It is well known that polyphenols exhibit protein-binding capacity, suggesting that ingredients of CYSTUS052 may interact with pathogens via such a physical and unspecific interaction. This is not only supported by our own finding that CYSTUS052 also blocks propagation of rhinoviruses but also by data from the literature showing that the extracts from *Cistus* plants exert antibacterial and antifungal activities (Bouamama et al., 1999). The advantage of such an unspecific action may be that resistant variants cannot easily emerge and that the compound may also act against bacterial co-infections that represent a major complication in severe influenza virus infections. An unspecific interaction with the viral HA has been reported for the polyphenolic compound epigallocatechin-gallate (Nakayama et al., 1993). However, polyphenols are a very large group of chemical compounds and to date the active moiety of CYSTUS052 is not known. Nevertheless, our experimental evidence suggests that the high content of polymeric polyphenols in CYSTUS052 is required for its antiviral activity. Extracts of other polyphenol rich plants but with a lesser content of polymeric forms, e.g. from the sage plant did not exert any anti-influenza virus activity (data not shown). In fact CYSTUS052 was chosen as the most potent antiviral acting extract from an array of extracts that not only differed in plant species but also in the biochemical variety of the highly polymorphic genus *Cistus* (data not shown). Another indication of the polymeric nature of the active component comes from first attempts to fractionate the extract. None of five different soluble fractions representing around

60% of the original extract showed an antiviral or hemagglutinationinhibition activity. A final indirect indication that the active components of CYSTUS052 are of higher molecular weight and may not enter the cell comes from experiments shown in Fig. 2.

Although polyphenols are known as protein binders and antioxidants, no negative effect on proliferation, metabolism, transcriptional/translational activity or responsiveness of the cell to ligands could be attributed to CYSTUS052, which may be due to the simple reason that the active component is not taken up into the cell. This would of course imply, that CYSTUS052 as an antiviral agent has to be applied locally, e.g. as an aerosol into the lung of the patients. Consistent with that view, experiments by Planz and coworkers (Droebner et al., 2007) have recently demonstrated the potent antiviral activity of CYSTUS052 by this application route in a mouse infection model. Although the efficacy of CYSTUS052 action in infected individuals has still to be proven, the plant extract may have several advantages with regard to its use as an antiviral agent. First, *Cistus* plant extracts have been used for centuries in traditional medicine without reports of side effects or allergic reactions. Besides a therapeutic application, thiswould even allowprophylactic use in risk groups. Furthermore, by its rather unspecific and broad action directly on the pathogen, CYSTUS052 may not easily result in the emergence of virus drug resistance and may also be active against opportunistic bacterial infections.

Given that there is an urgent need for newand amply available anti-influenza drugs, an inhalable formulation of the CYSTUS052 appears to be a promising option as a replacement or supplementary strategy to currently available anti-influenza therapeutics.

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Figure Legends

FIGURE 1: CYSTUS052 inhibits influenza virus propagation. A549 (A, left panel; B) or MDCK (A, right panel) cells were left untreated or treated with the indicated amounts of CYSTUS052 before and during infection with the influenza A virus strains FPV (MOI = 0.001) (A, left panel) or PR8 (MOI = 0.01) (A, right panel; B) which were either left untreated or pretreated with CYSTUS052 as well. Supernatants were assayed for progeny virus yields 9 and 24 h (A) or 8, 24 and 36 h (B) post infection in standard plaque titrations. Virus titers (plaque forming units/ml) of mock-treated cells in (A) were 1.3×102pfu/ml (9 h) or 9.1×106pfu/ml (24 h) (FPV, left panel) and 3.2×102pfu/ml (9 h) or 1.1×103pfu/ml (24 h) (PR8, right panel), respectively, and were arbitrarily set as 100%. In (B) the pfu are given on the axis of the graph. (C) A549 cells were treated with the indicated amounts of CYSTUS052 before and during infection with FPV or the human isolate of the avian H5N1 Asia subtype (A/Thailand/1(KAN-1)/2004 (H5N1)) (MOI = 0.001). The virus was pretreated with CYSTUS052 as well. 20 h post infection supernatants were taken and assayed for progeny virus yields in standard plaque titrations. Virus yields of mock treated cells were arbitrarily set as 100%.

FIGURE 2: CYSTUS052 is not toxic or proapoptotic and does not interfere with cellular responses to cytokines. MDCK (A) cells were left untreated or treated with the indicated amounts of CYSTUS052 for the indicated time points and were subjected to propidium iodide (PI, 1µg/ml) staining and subsequent flow cytometry analysis using a FACScan (BD FACScalibur). (B) A549 cells were left untreated or were treated with 2.5µM staurosporine for 8h or 25µg/ml and 50µg/ml CYSTUS052 for 48 h, respectively. Upon stimulation cell lysates were prepared and subjected to Western blots, and caspase mediated PARP cleavage was determined as a measure for apoptosis induction. (C) A549 cells were pretreated with CYSTUS052 for 30 min or left untreated. Subsequently cells were stimulated with TNFα for 15 min in the absence or presence of CYSTUS052. Cell lysates were subjected to SDS Page and Western-blot using an anti-IκBα antiserum to detect TNFα-induced degradationof IκBα as a measure for NF-κB activity. Equal protein loads in (B) and (C) were verified in ERK blots.

FIGURE 3: CYSTUS052 appears to primarily inhibit the viral entry process. (A) MDCK cells were left untreated or treated with 50µg/ml of CYSTUS052 at the indicated time points during infection with the influenza virus strain FPV (MOI = 0.001). (B) Influenza virus FPV was left untreated or pre-incubated for 2 h. 24 h post infection supernatants were taken and assayed for progeny virus yields in standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%. (C) A549 cells were left untreated (I.) or were preincubated (II.) with 50 µg/ml CYSTUS052 for 30 min and infected with the influenza virus strain FPV (MOI = 200) for 1 h. In (III.) FPV was pre-incubated with 50 µg/ml CYSTUS052 overnight and subsequently used for infection. (D) A549 cells were left untreated (I.) or treated with CYSTUS052 (100 µg/ml) (II., III.) before and during infection with FPV (MOI = 50). FPV was left untreated or was preincubated (30 min) with CYSTUS052 (100µg/ml) as well. 1h (C) or 1.5h (D) after infection RNP localization was determined by indirect immunofluorescence using a NP-specific mouse monoclonal antibody and an Alexa Fluor 488 chicken anti-mouse IgG (H + L). Picture (III.) represents an enlargement of the inlay of the middle picture. The white arrowhead shows virus particles lining on the cell borders.

FIGURE 4: CYSTUS052 inhibits hemagglutinating activity of influenza virus and blocks propagation of human rhinovirus HRV14 (A) CYSTUS052 was diluted as indicated. Virus stocks (H5N1 or H7N7) (4×104 pfu/well) were dissolved in PBS/BA and 50µl was added per well of a 96-well plate. After preincubation of 45 min chicken erythrocytes (1/20 in PBS)

were mixed with the solution. In the samples where viruses were preincubated with CYSTUS052, up to a certain dilution the viral particles are no longer capable of agglutinating erythrocytes, indicating an interaction of CYSTUS052 with the viral HA. (B) 5×104 HeLa cells in D-MEM medium were seeded into a 96-well plate to achieve 70% confluency 1 day later. The medium was then removed and replaced by 90µl of the infection medium (D-MEM, 2% FCS, 10–20mM $MgCl_2$) per well. The HRV14 virus titer was determined to 10−7/ml CCID (I.). 10µl of the virus suspension (treated (II.) or not treated (I., III., IV.) with 100µg/ml CYSTUS052 extract as indicated) was mixed with 90µl of the infection medium (supplemented (II., III., IV.) or not supplemented (I.) with 100µg/µl CYSTUS052 extract, as indicated) in the first well (triplicates) and then was serially diluted in 1:10 steps in the subsequent wells. The 96-well plate was then incubated for 5 days at 33 ◦C in a cell culture incubator. At day 5 the plate was washed twice with PBS before staining cells in each well with 100µl a crystal-violet staining solution (0.07% in EtOH) for 5 h at room temperature. The plate was subsequently washed several times with water and dried. The blue staining indicates non-lysed cells, while a white stain indicates that cells were destroyed by the lytic virus infection.

FIGURE 5: CYSTUS052 shows no tendency to induce virus drug resistance.MDCK cells were infected with the influenza A virus strain FPV (MOI = 0.01) and were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine. At 24 h post infection supernatants were taken and employed for infection in the second round of investigation. After infection cells were left untreated or treated with the indicated amounts of CYSTUS052 or amantadine again. This procedure was repeated six times. Supernatants were assayed for progeny virus yields in standard plaque titrations. Virus yields of mock-treated cells were arbitrarily set as 100%.

Figures

Figure 1

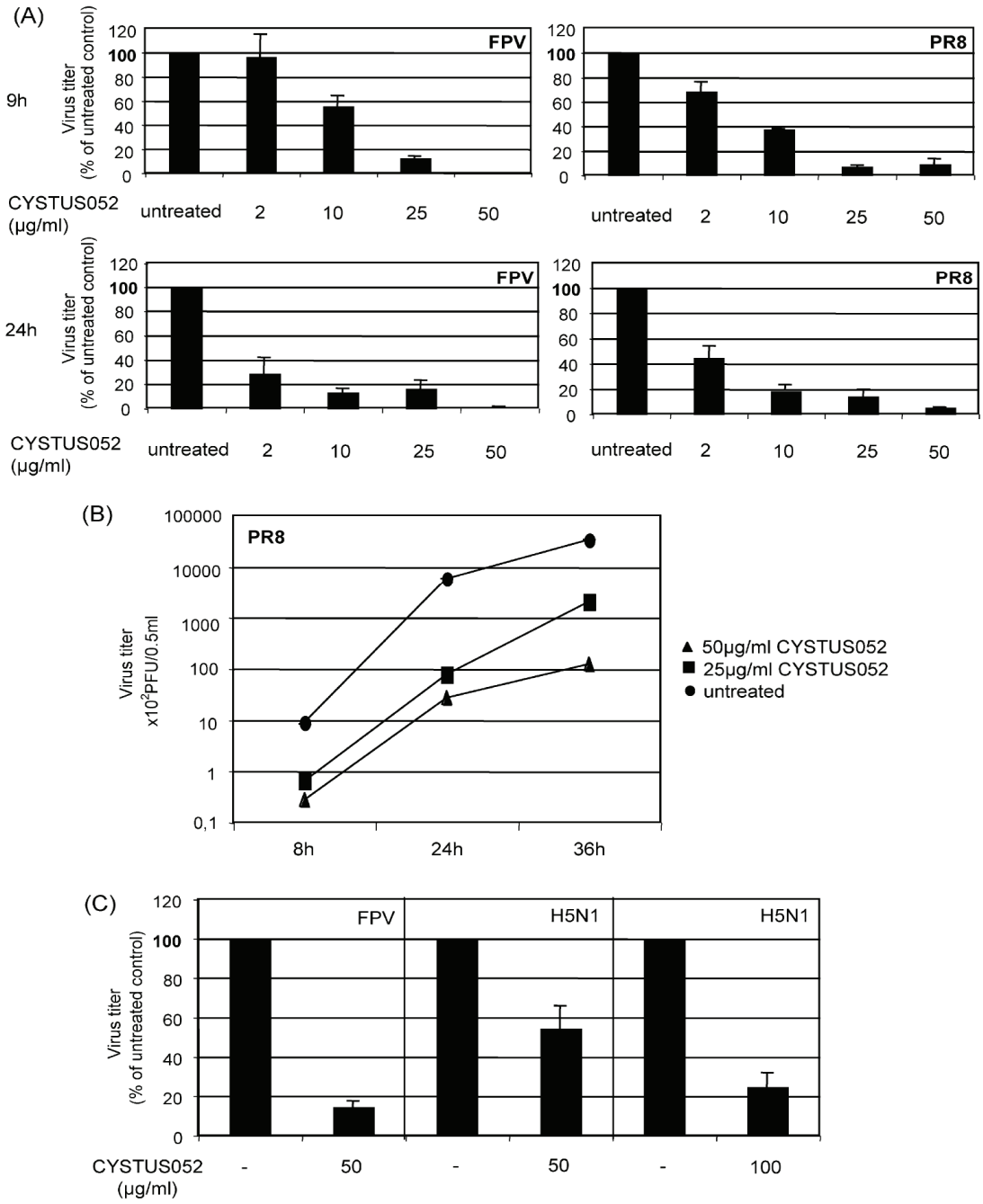


Figure 2

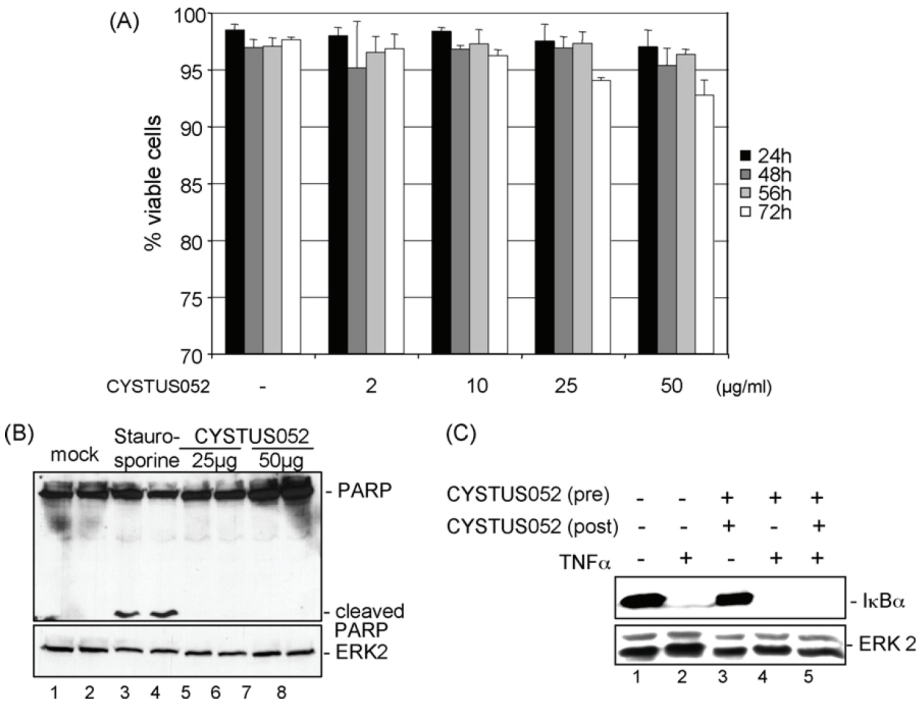


Figure 3

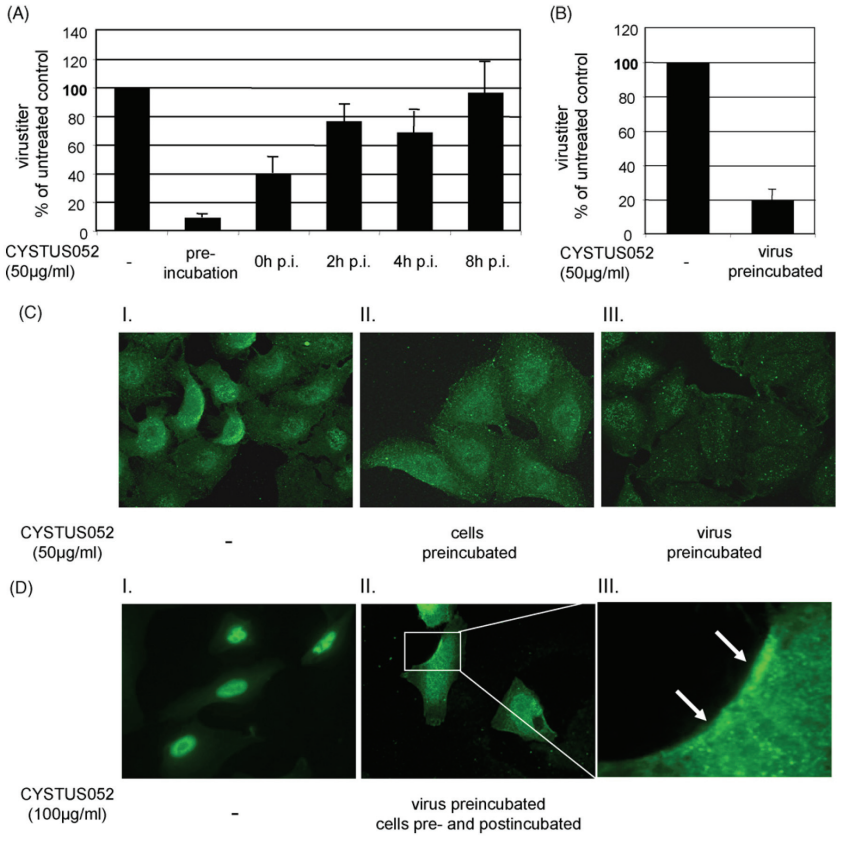


Figure 4

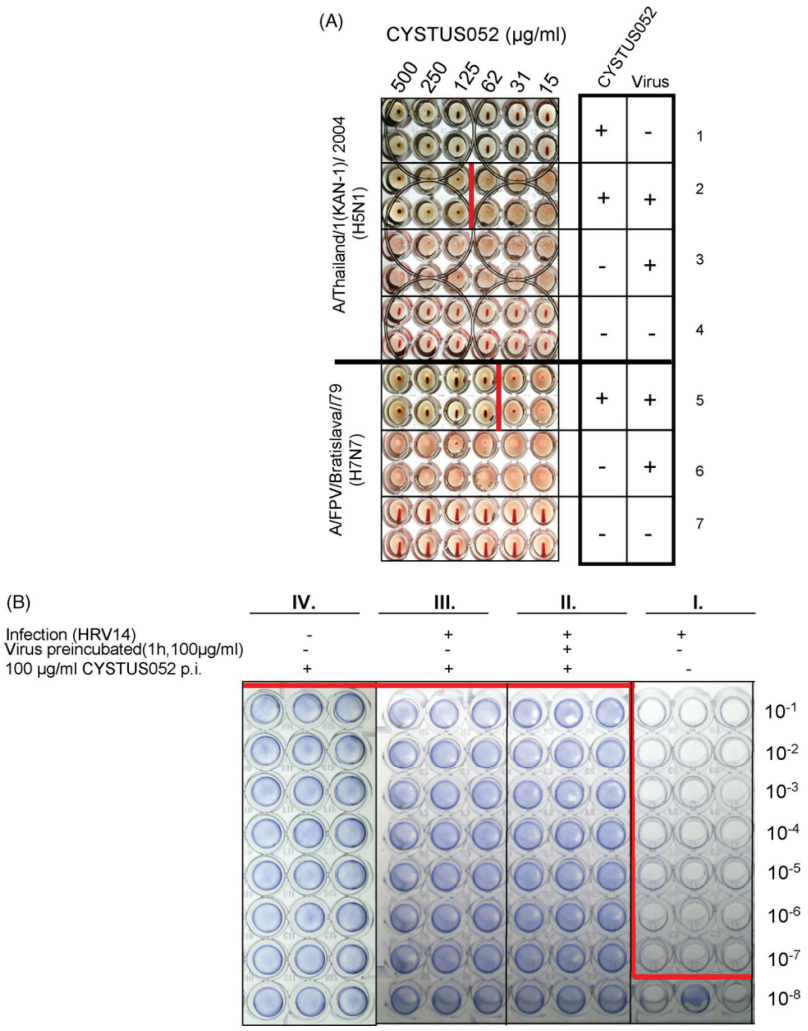
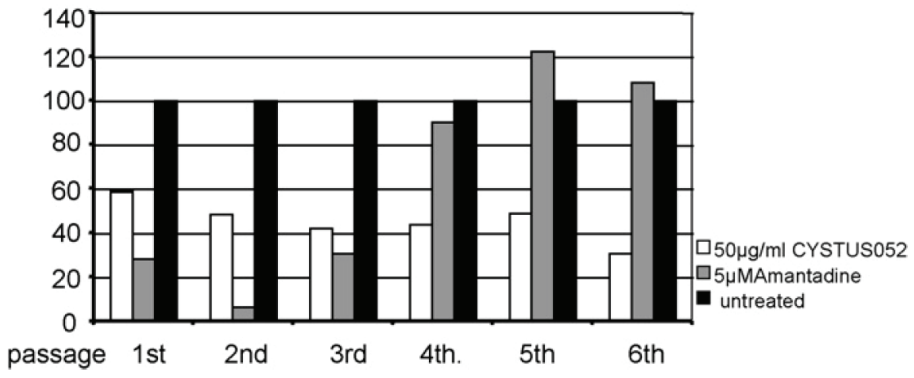


Figure 5



PUBLICATION #6:

CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice

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Keywords: Influenza A virus; *Cystus incanus*; Polyphenols

Abstract

Influenza, a respiratory disease caused by influenza viruses, is still a worldwide threat with a high potential to cause a pandemic. Beside vaccination, only two classes of drugs are available for antiviral treatment against the pathogen. Here we show that CYSTUS052, a plant extract from a special variety of *Cistus incanus* that is rich in polymeric polyphenols, exhibits antiviral activity against a highly pathogenic avian influenza A virus (H7N7) in cell culture and in a mouse infection model. *In vitro* and *in vivo* treatment was performed with an aerosol formulation, because the bioavailability of high molecular weight polyphenols is poor. In MDCK cells, a 90% reduction of plaque numbers on cells pre-incubated with the plant extract was achieved. For *in vivo* experiments we used a novel monitoring system for influenza A virus-infected mice that allows measurement of body temperature and gross motor-activity of the animals. Mice treated with CYSTUS052 did not develop disease, showed neither differences in their body temperature nor differences in their gross motor-activity and exhibited no histological alterations of the bronchiolus epithelial cells.

1. Introduction

Influenza is still one of the major plagues worldwide. This respiratory disease is caused by influenza A viruses that are highly contagious pathogens for humans and several animal species. Beside annual epidemics, these pathogens are also capable to cause pandemic outbreaks. Three of these pandemics occurred in the last century, in 1918, 1957, and 1968 (Wright and Webster, 2001; Palese, 2004). The most severe pandemic outbreak known as the "Spanish flu" occurred in 1918 and caused at least 20–40 million deaths worldwide (Reid et al., 2001; Taubenberger et al., 2001). The statistical likeliness of a new pandemic outbreak and the emergence of highly pathogenic avian influenza viruses (HPAIV) of the H5 and H7 subtype that infected and killed humans highlight the urgent need for new and amply available antiviral drugs. At present, even though H5N1 influenza A viruses occasionally infect humans, they are not freely transmissible between humans. Nevertheless, due to mutations and/or reassortment of genes with circulating human influenza A viruses H5 and/or H7 strains might acquire such transmissibility between humans. Vaccination is the first option for controlling influenza and reducing the impact of pandemics. Changes of the viral proteins require annual adaptation of the influenza vaccine formulation. In addition, antiviral drugs provide a fundamental complementary line of defense, particularly for fast spreading pandemic influenza A virus strains where vaccines might be not available in time (Ferguson et al., 2005; Longini et al., 2005). Only two classes of anti-influenza virus drugs are currently

accessible. These are viral neuraminidase inhibitors (oselatamivir and zanamivir) and viral M2 ion channel protein inhibitors (amantadine, rimantadine) (Wright and Webster, 2001; De Clercq, 2004). The emergence of influenza A viruses resistant to the M2 inhibitors occurs at high frequency in treated patients (Cox and Subbarao, 1999; Suzuki et al., 2003). Many of the human isolates of H5N1 viruses are already resistant to these inhibitors (Puthavathana et al., 2005). In addition, a recent study has shown that influenza A viruses resistant to the neuraminidase inhibitor oseltamivir occurred in 20% of the children treated with this drug (Kiso et al., 2004). In fact, H5N1 viruses that are partially resistant to oseltamivir have recently been reported (Le et al., 2005). The emergence of influenza A viruses resistant to these two classes of antiviral drugs highlights the need for additional antiviral drugs against these pathogens. Even though several antiviral compounds have been developed against influenza virus, their long-term efficacy is often limited due to toxicity or the emergence of drug-resistant virus mutants (Hayden, 2006). Phenolic compounds, or polyphenols, constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known. Polyphenols are products of the secondary metabolism of plants. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds (Harborne, 1980). Polyphenols exhibit a wide range of biological effects. Bioavailability differs greatly from one polyphenol to another (Manach et al., 2005). The knowledge of absorption, bio-distribution and metabolism of polyphenols is partial and incomplete. Some polyphenols like epigallocatechin (EGC) are bioactive compounds that are absorbed from the gut, while most polymeric polyphenols like flavan-3-ols and proanthocyanidins are not absorbed (Urquiaga and Leighton, 2000; Manach et al., 2004). Due to the overall poor absorption and low plasma concentrations of polymeric polyphenols, it was suggested that phenols might exert direct effects (Halliwell et al., 2005). Plant-derived polyphenols have been shown to be strong antioxidants with potential health benefits as they are able to protect the heart, support the biological activity of vitamin C, protect from premature skin ageing, and bind viruses and bacteria (Urquiaga and Leighton, 2000; Arts and Hollman, 2005; Halliwell et al., 2005). Various reports exist on antiviral and antibacterial potential and the mode of action of polyphenols (reviewed in Cos et al., 2004), including several reports describing the antiviral activity of polyphenols against influenza virus. Epigallocatechin gallate (EGCG) and theaflavin digallate, two polyphenols present in tea are able to bind the hemagglutinin of influenza virus and thus block its infectivity (Nakayama et al., 1993). In another investigation it was shown that the antiviral effect of epigallocatechin gallate, epicatechin gallate (ECG)

and epigallocatechin on influenza virus is mediated not only by specific interaction with the hemagglutinin, but also by altering the physical properties of viral membrane (Song et al., 2005). Other reports demonstrate an antiviral activity of a polyphenol-rich extract from the medicinal plant *Geranium sanguineum* L. against influenza virus *in vitro* and *in vivo* (Ivanova et al., 2005; Sokmen et al., 2005). Resveratrol, another polyphenol found in grapes strongly inhibited the replication of influenza virus in cell culture by blockade of the nuclear-cytoplasmic translocation of the viral ribonucleoprotein complex, by reducing expression of late viral proteins and by inhibition of protein kinase C (PKC) activity and PKC dependent pathways (Palamara et al., 2005). CYSTUS052 is a plant extract that is very rich in highly polymeric polyphenols. The extract is a preparation of a selected variety of the biochemical polymorphic species *Cistus incanus*. *Cistus incanus* (Pink Rockrose) is one species of the genus *Cistus* L. The genus *Cistus* comprises a group of about 20 shrub species found in wide areas throughout the whole Mediterranean region to the Caucasus. *Cistus* species are used as anti-diarrheic, as general remedies in folk medicine for treatment of various skin diseases, and as anti-inflammatory agents (Danne et al., 1993; Petereit et al., 1991). Being one of the main constituents of the Mediterranean-type maquis, this plant genus is peculiar in that it has developed a range of specific adaptations to resist summer drought and frequent disturbance events, such as fire and grazing (Comandini et al., 2006). CYSTUS052 extract was defined by the producer as having a content of polymeric polyphenols in particular flavan-3-ols and proanthocyanidins of around 26% (determined after Singleton and Rossi, 1965; Singleton et al., 1999). The total content of monomeric components like gallic acid, gallocatechin, catechin, epicatechin was lower than 2% (Danne et al., 1993; Petereit et al., 1991). In the present communication we raised the question if the polyphenol-rich plant extract CYSTUS052 exert antiviral activity against influenza virus. The bioavailability of flavan-3-ols and proanthocyanidins are poor. Therefore, with regard to an antiviral action against influenza virus a local administration in an aerosol formulation would be more favorable. Here, we demonstrate that CYSTUS052 inhibits influenza A virus replication *in vitro*. Furthermore, CYSTUS052 aerosol treatment of mice infected with a mouse-adapted highly pathogenic avian influenza virus (FPV, H7N7) protected the animals against clinical disease symptoms. From our data we propose a mechanism, where polyphenolic ingredients of CYSTUS052 extract block virus infection by a direct (physical) interaction with the virus particles.

2. Experimental/materials and methods

2.1. Mice

Inbred female Balb/c and C57Bl/6 mice at the age of 6–8 weeks were obtained from the animal breeding facilities at the Friedrich-Loeffler-Institute, Federal Research Institute for Animal

Health, Tübingen, Germany and were used throughout all the experiments.

2.2. Virus and infection

Mouse-adapted avian influenza A/FPV/Bratislava/79 (H7N7) (FPV) virus grown on Madin-Darby canine kidney (MDCK) cells was used throughout this study. The LD_{50} of the virus stock used in the present study was $1x10^2$ pfu (plaque forming units). The Bratislava strain of the H7N7 avian influenza A virus (FPV) was originally obtained from the Institute of Virology, Justus-Liebig University, Giessen, Germany and further propagated at the Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Tübingen, Germany.

The *in vitro* infections were performed in an aerosol-chamber ($5.0x10^{-2}$ m^3); 46 cm x 33 cm x 33 cm l/ [w h]). Two PARI® aerosol nebulizers (PARI, Starnberg, Germany, Art. No. 73-1963) were connected to either the left or right side of the chamber (Fig. 1A). Ten 3.5 cm Petri dishes with MDCK cells were placed without lid into the chamber as demonstrated in Fig. 1A. Nebulized virus preparation was introduced to the chamber from the left side with 1.5 bar for 10 min (total volume of 2 ml), while either CYSTUS052 extract (1 mg/ml; Charge-Number: 40121T01B/04) or in a different experiment buffer solution with a pressure of 1.5 bar was given for 20 min (roughly 4 ml) from the right side to the chamber starting 10 min prior to virus infection. After infection Petri dishes were placed in a 37 °C incubator with 5% CO_2 for 48 h. Thereafter, the cells were stained for plaque formation as described previously (Ölschläger et al., 2004). For infection of mice, the animals were anaesthetized by intraperitoneal injection of 150 µl of a ketamine (Sanofi, Germany) rompun (Bayer-Leverkusen, Germany)-solution (equal amounts of a 2%-rompun solution and a 10%-ketamine solution were mixed at the rate of 1:10 with PBS) and infected intranasally (i.n.) with $1x10^2$ pfu/50 µl of FPV (H7N7). For infection of mice with FPV pre-incubated with CYSTUS052, either 10^2/25 µl, 10^3/25 or 10^4/25 µl FPV was incubated either with 25 µl CYSTUS052 (1 mg/ml) or with 25 µl PBS for 30 min at room temperature. Thereafter, mice were infected i.n. with 50 µl of FPV/CYSTUS052 or FPV/PBS.

2.3. CYSTUS052 extract

CYSTUS052 extract was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (Charge- Number: 40121T01B/04, 8-2004; Glandorf). The extract is a special preparation from a distinct variety of *Cistus incanus* (*Cistus incanus* PANDALIS). CYSTUS052 extract was defined by the producer as having a polyphenolic content of more than 26% (determined after Singleton and Rossi, 1965; Singleton et al., 1999). The total content of monomeric components (gallic acid, gallocatechin, catechin, epicatechin) was lower than 2%. CYSTUS052 granulate was solved either in sterile PBS (for *in vitro* experiments) or in sterile H_2O (for *in vivo* experiments) for 10 min at 60 ◦C or up to 1h at 100◦C until the granulate was completely solved. The extract was stored at RT for 1 week.

2.4. Treatment of Balb/c mice with CYSTUS052

Treatment of mice was performed in inhalation chambers. Either five mice were treated at the same time in an inhalation chamber ($2.1x10^{-2}$ m^3) or single mice were treated in an inhalation tube. Five tubes were connected to a central cylinder with an overall volume of $8.1x10^{-4}$ m^3. A PARI® nebulizer (Aerosol Nebulizer; Art. No. 73-1963) was connected to either the inhalation chamber or the central tube cylinder. CYSTUS052 extract (10 mg/ml; Charge-Number: 40121T01B/04) or buffer solution with a pressure of 1.5 bar was given for 10 min (roughly 2 ml) to the chambers. The CYSTUS052 extract was dissolved in sterile ddH_2O (stock solution: 10 mg CYSTUS052/ml distillate water) and incubated for 1 h at 100 °C in a water bath. Balb/c mice were placed in the tube-cylinder and exposed to 2 ml of aerosolized CYSTUS052 extract for 10 min three times a day at 9:00 a.m., 12:00 a.m. and 3:00 p.m. The treatment was performed for 5 days. Ten minutes after the first treatment mice were infected with FPV. Balb/c controls were treated with the same amount of sterile ddH_2O. After FPV infection, the general health status of the animals was controlled twice a day. Furthermore, the animals were weighted every day. According to the German animal-protection law, the mice were sacrificed after they had lost 25% of their initial weight. All animals were monitored for 15 days after infection.

2.5. Mouse monitoring

The monitoring of body temperature and gross motor activity of the animals was performed with the VitalView® software and hardware system (Mini Mitter U.S.A.). This system allows data acquisition of physiological parameters. The hardware includes a transmitter (E-Mitter)/receiver system. The E-Mitter collects data on temperature and gross motor activity

for the lifetime of the animal. The temperature sensitive devices alter their pulse rate in response to temperature changes. The Vital View system records the average rate and converts this to temperature using temperature calibration values specific to each unit. The vitality or gross motor activity measurement provides a basic index of the movement of mice with implanted E-Mitter. As the mouse moves, movement of the implanted E-Mitter results in subtle changes in the transmitted signal. These small changes are detected by a receiver *via* telemetry and registered by a connected computer as activity counts. In our experiments the Vital View software recorded an index of movement every 5 min to produce a longitudinal record of the activity. For implantation of the E-Mitters the mice were anesthetized with intraperitoneal injection of 150 µl ketamine/rompun. The ventral surface of the abdomen was shaved and a midline abdominal skin incision was made 0.5–1 cm below the diaphragm with no more then 2 cm in length. The abdomen was opened with a 2 cm incision along the *linea alba* and the E-Mitter was positioned in the abdominal cavity. After the incision was closed with two to three wound clips (autoclip 9 mm; Becton & Dickinson, Germany). The animals were placed into the cage and successful implantation of the E-Mitter was controlled by Vital View software. Health status was controlled for 7 days before infection.

2.6. LCMV infection and footpad swelling

Lymphocytic choriomeningitis virus strain WE was propagated in L-cells. Infection was performed with 10^4 ffu (focusforming unit) in 50 µl buffer solution into the left hind footpad. Delayed-type hypersensitivity reaction (DTH) was determined as local swelling after subcutaneous inoculation of virus into the left hind footpad of C57Bl/6 mice (Moskophidis and Lehmann-Grube, 1989). At daily intervals, the dorsoventral thicknesses of both hind feet were measured with a dial calliper (Oditest; Kroplin,Germany), and swelling was expressed as percent of the factor with which the thickness of the inoculated foot exceeded the thickness of the contra lateral (right) un-inoculated control foot.

2.7. Histology and immunohistology

Mice were treated with nebulized CYSTUS052 (10 mg/ml) three times at 9:00 a.m., 12:00 a.m. and 3:00 p.m. for 10 minor H_2O (as control). Immediately after the treatment the mice were killed and lungs were obtained and fixed in buffered 4% paraformaldehyde. The lungs were stained with hematoxylin and eosin as described before (Ölschläger et al., 2004). Lectinstaining of lung sections was performed with *Sambucus nigra*agglutinin (SNA; Vector Laboratories) for sialic acid α-2.6 linked to galactose and *Maackia amurensis* agglutinin

(MALII; Vector Laboratories) for sialic acid α-2.3 linked to galactose.Secondary staining was performed with an ABC-kit (Vector) for 30 min at RT. Substrate reaction was performed with DAB kit (Vector Laboratories).

2.8. Hemagglutination assay

Hemagglutination assays were carried out in V-bottomed microtiter plates using 50 µl of 2.5% suspensions of chicken red blood cells in PBS. Fresh chicken blood was supplemented with 1.6% sodium-citrate in sterile H_2O and was centrifuged at 800× *g* for 10 min at room temperature to separate red blood cells. Thereafter the cells were washed for three times with PBS. Fifty microliters of a 10% lung homogenate was added and serially diluted in PBS. Assays were read following a 1 h-incubation on ice. Untreated red blood cells would precipitate to the bottom of the plate, while red blood cells incubated with influenza virus showed a diffuse distribution on the microtiter plates.

3. Results

3.1. Antiviral effect of CYSTUS052 extract in vitro

To investigate whether the CYSTUS052 extract exerts an antiviral action after being delivered as an aerosol, we performed infectivity studies in aerosol chambers. Both, the virus and theCYSTUS052 extract were applied to MDCK cells as an aerosol as described in detail in Section 2. Cells were cultured in Petri dishes and were placed at different positions in the chamber (Fig. 1A). As a control, MDCK cells were treated with nebulized PBS in a second set of experiment at the same way. When CYSTUS052 extract was nebulized in parallel or after virus infection, no differences regarding plaque formation on MDCK monolayers compared to control cells were detected (data not shown). In contrast, when MDCK cells were treated with CYSTUS052 extract 10 min prior to influenza virus infection with 10^5 pfu/ml, a reduction of plaque numbers on the MDCK cells was found. Depending on the position of the plate in the aerosol chamber the plaque reduction was up to 90% in MDCK monolayers that were pre-treated with CYSTUS052 extract (Fig. 1B). The absolute number of plaques in control cell cultures was 2–7 x10^2 pfu. When an infectious dose of 10^6 pfu/ml was used for aerosol infection, again a reduction of plaque numbers between 70 and 90% was observed in cells that were treated with CYSTUS052extract starting prior to infection (data not shown).

3.2. Evaluation of toxic effects in Balb/c mice and interference with the immune response after aerosol treatment with CYSTUS052 extract

In a first set of experiments the effects of CYSTUS052 extract on the health and immune status of uninfected mice was determined. Five female Balb/c mice were treated in an aerosol chamber, three times a day for five consecutive days with 2 ml CYSTUS052 extract (10 mg/ml, 1.5 bar) to investigate whether CYSTUS052 extract treatment induces any apparent side effects in the animals. In addition, five female Balb/c mice (littermates) were kept untreated and served as controls. Since mice treated with CYSTUS052 extract exhibited no differences in their bodyweight during the treatment (Fig. 2A) and showed no differences in the overall health status compared to untreated control animals, it can be concluded that the treatment with CYSTUS052 extract (10 mg/ml aerosol) is non-toxic for the animals. Furthermore, the results also show that the treatment procedure itself (three times a day for five consecutive days in an aerosol chamber) was not harmful to the animals. This indicates that the treatment-protocol is well suitable for experiments to investigate the influence of CYSTUS052 extract in mice after influenza A virus infection. It is of common knowledge that certain polyphenols including some polyphenols found in CYSTUS052 extract have an anti-inflammatory function. To elucidate if local treatment of CYSTUS052 extract to the lungs would interfere with the systemic cellular immune response, we performed a classical and well-established footpad-swelling assay after lymphocytic choriomeningitis virus (LCMV) infection (Moskophidis andLehmann-Grube, 1989). LCMV infection into the footpad induces a local delayed-type hypersensitivity (DTH) reaction that consists of phases that are sequentially mediated by class I restricted cytotoxic $CD8^+$ T cells and class II-restricted $CD4^+$ T cells, cytokines/lymphokines and inflammatory cells. The infiltration of these immune cells and the immune mediators into the footpad results in a footpad-swelling that can be measured with a dial calliper. Interference with the immune system or immunesuppression leads to reduced footpad swelling.

LCMV was injected into the left hind foot of C57Bl/6 mice, while the right foot served as a control. Aerosol-treatment with CYSTUS052 extract or H_2O was identically performed as described for the experiments above (see also Section 2).The treatment started 5 days after infection and 1 day prior to the first sign of footpad swelling that was first detectable at day 6after LCMV infection. Until day 10 p.i., there was a strong increase in footpad swelling in mice that were either treated with CYSTUS052 extract or with H_2O. Thereafter, the swelling declined in both groups and after day 16 p.i. swelling was no longer detectable (Fig. 2B). In comparison of CYSTUS052extract-treated mice with control animals, there was no difference

in footpad-swelling. This experiment clearly demonstrates that CYSTUS052 extract aerosol treatment had neither a positive nor a negative effect on the systemic immune response against a viral pathogen.

3.3. Aerosol-treatment of influenza A virus-infected Balb/c mice with CYSTUS052 extract

To investigate whether CYSTUS052 extract has an antiviral effect *in vivo*, Balb/c mice were treated with CYSTUS052 extract as described above (Fig. 2A; open squares). To allow monitoring of body temperature and the activity of the mice during infection, transponders were implanted into the mice 7 days prior to infection as described in Section 2. To assure that all conditions including physiological stress were equal between the control group and mice that received CYSTUS052 extract, control mice were treated with H_2O, the solvent of CYSTUS052 extract. Treatment and infection were described in detail in Section 2. As expected 1 day prior to infection there was no difference between the two groups of animals regarding body temperature (Fig. 3A left panel) and activity status (Fig. 3Bleft panel). In general, Balb/c mice showed a circadian rhythm with respect to these two parameters. During daytime a decrease of body temperature and activity was observed. In the evening body temperature and activity increased again. Eight days after infection all control animals showed clinical symptoms, whereas none of the CYSTUS052 extract-treated animals developed disease. While CYSTUS052 extract-treated mice still showed a circadian rhythm of their body temperature (Fig. 3A right panel, black squares) and activity status (Fig. 3B right panel, black squares), a strong decrease of body temperature (Fig. 3A right panel, red squares) and vitality (Fig. 3B right panel, red squares)was found in control animals without any signs of a circadian rhythm. These symptoms correlated with the bodyweight of the animals. During the first 5 days after infection a slight weight loss was found in both groups that was similar to the loss of weight found in the experiment without infection. After 6 days post-infection the H_2O-treated mice developed first signs of clinical symptoms, like ruffled fur, reduced activity and disturbances of the circadian changes in body temperature. Furthermore, the animals of the control group started to loose weight (Fig. 3C). Weight loss increased until day 10 p.i. Thereafter the animals of the control group regained weight. In the described experiment 60% (n = 3) of the control mice died. In contrast, CYSTUS052 extract-treated mice did not develop clinical disease symptoms, showed no weight reduction and no differences in the body temperature or activity status (Table 1). From this data one might speculate that CYSTUS052-treated mice were completely protected from the infection. Therefore, we looked for the presence of FPV 6 days after infection in the lung of CYSTUS052-treated and

control mice. While virus was present in the lung of control mice (240 ± 60HA units/ml), the amount of virus found in the plant extract-treated mice was drastically reduced (40 HA units/ml), indicating that CYSTUS052-treated mice were partially protected against the infection (Fig. 4A). Taken together, CYSTUS052 extract given three times daily for 5 days *via* an aerosol route resulted in effective reduction of influenza A virus infectivity in mice. After successful treatment of FPV-infected mice (10^2 pfu/50 µl i.n.) with CYSTUS052 extract as an aerosol, we now raised the question whether oral application of CYSTUS052 extract would also suppress disease in influenza A virus-infected mice. Therefore, mice were treated with CYSTUS052 extract *via* the oral route three times a day for 5 days. Control mice were treated with H_20. In one experiment, already at day 6 p.i., both control mice and CYSTUS052-treated animals developed first signs of disease symptoms and lost weight. The severity of disease symptoms increased until day 8 p.i. By day 10 p.i. mice from both groups showed reduced disease symptoms and gained weight (Fig. 4B). In a second experiment, control mice and CYSTUS052-treated animals developed first signs of disease symptoms by day 6 p.i. and all mice died between days 8 and 10 after infection (Table 1).Taken together, CYSTUS052 extract given three times daily for five days *via* an aerosol route resulted in effective reduction of influenza A virus infectivity in mice, while no antiviral effect of CYSTUS052 extract was found after oral treatment of infected mice. We raised the question whether pre-incubation of the virus with the compound would result in an antiviral effect. Therefore, 10^2 pfu/25 µlwas either incubated with 25 µl CYSTUS052 (1 mg/ml) or with 25 µl PBS for 30 min at room temperature. Thereafter mice were infected i.n. with 50 µl of either FPV/CYSTUS052 or FPV/PBS. Mice that were infected with FPV pre-treated with PBS developed disease and two out of five mice died. In contrast, none of the mice that were infected with FPV pre-treated with the plant extract developed disease or died. Since a 90% reduction of infectivity was found *in vitro* after pre-incubation of virus with CYSTUS052, we then used 10^3 pfu/25 µl for pre-incubation. All mice that were infected with FPV pre-treated with PBS died. Mice that were infected with FPV pre-treated with CYSTUS052 developed disease or died similarly to mice infected with the LD_{50} of 10^2 pfu (Table 2).

3.4. Histology and immunohistology of lungs sections fromCYSTUS052-treated mice

To answer the question if aerosol treatment of CYSTUS052 would lead to alterations on epithelial bronchiolus cells we performed hematoxylin and eosin staining of mice that were either treated with an aerosol formulation of the plant extract (+CYSTUS052) or with H_2O (−CYSTUS052), the dissolvent of the extract as described above. Immediately after three

treatments the mice were killed and lungs were fixed in 4% paraformaldehyde. No alterations of epithelial cells were found after CYSTUS052 treatment (Fig. 5; H & E). Moreover we also investigated the expression of sialic acid α-2.6 and sialic acid α-2.3 on epithelial bronchiolus cells after CYSTUS052 staining. Again no alterations of the expression of these two viral receptors were found in CYSTUS052-treated mice (Fig. 5; 2.6 SA, 2.3 SA).

4. Discussion

The goal of this study was to determine whether the polyphenol-rich plant extract from a distinct variety of *Cistus incanus* would function as an antiviral agent against influenza A virus in mice. Aerosol treatment with CYSTUS052 extract of mice that were infected with a mouse-adapted fowl plague virus (FPV), a highly pathogenic avian influenza virus of the H7 subtype, protected these animals from disease and death. The CYSTUS052 extract-treated mice neither showed changes in their body temperature nor changes in their gross motoric activity, while drastic differences in these parameters were detectable in mice from the control groups. Our experimental setup to apply CYSTUS052 extract as an aerosol was based on the fact that the bioavailability of high molecular weight polyphenols is very poor. Furthermore, *in vitro* studies demonstrated that CYSTUS052 extract given as an aerosol results in reduced plaque formation after FPV infection of MDCK cells. Interestingly, this antiviral activity of CYSTUS052 extract was only detectable when CYSTUS052extract was nebulized prior to virus infection. In contrast, when CYSTUS052 extract was nebulized in parallel to or after virus infection, no antiviral effect was detected (data not shown).One might now speculate about the mechanism that is responsible for the antiviral properties of CYSTUS052 extract. From our findings and from studies of the active compounds in *Cistus incanus* (Petereit et al., 1991), we would like to hypothesize a rather physical mode of action where the content of polymeric polyphenols in the CYSTUS052 extract that by far exceeds other well-known sources of polyphenols, bind to the virus prior to infection and will prevent adsorption of the virus to cells. This hypothesis is based on the fact that in our *in vitro* studies we were able to demonstrate that CYSTUS052 extract showed no antiviral properties, when given after or during the infection (Ehrhardt et al., 2007). Only few polymorphic polyphenols are absorbed and metabolized (Urquiaga and Leighton, 2000; Manach et al., 2004; Halliwell et al., 2005). The active compounds of *Cistus incanus* are most probably flavan-3-ols and proanthocyanidins. This group of polyphenols comprises monomers but also dimers and polymers (Petereit et al., 1991). Proanthocyanidins are active compounds against viral and bacterial pathogens (Cos et al., 2004). It is known that proanthocyanidins down regulate the

expression of CCR2b, CCR3 and CCR5 on peripheral blood mononuclear cells. CCR2b, CCR3 and CCR5 function as co-receptors for HIV (Nair et al., 2002). This prompted us to investigate if CYSTUS052 would have an influence on the expression of sialic acid influenza virus receptors. The immunohistochemistry experiments revealed no alterations in the expression of the viral sialic acid receptors on bronchiole epithelial cells after CYSTUS052 treatment. Some polyphenols of low molecular weight are bioactive compounds that are absorbed from the gut in their native or modified form and show antiviral activity against influenza A virus. One of these bio-reactive compounds is Resveratrol, a polyphenol that is synthesized by various plant species including grapes (Fremont, 2000). Resveratrol appears to interfere with several intracellular signalling pathways and showed an antiviral activity against influenza A virus *in vitro* and *in vivo* (Palamara et al., 2005). The fact that oral treatment of CYSTUS052 extract was not effective against influenza A virus infection in mice (Fig. 4,Table 1) argues against a mode of action of CYSTUS052 extract similar to Resveratrol and further supports the mechanism of a direct physically function. The plant *Cistus incanus* is very rich in polymeric polyphenols. This allowed us to perform the experiments with an extract of the plant without purification of the active component. The great advantage of using the crude plant extract is the lower risk of toxic side effects. Indeed, aerosol treatment of CYSTUS052 extract in a concentration of 10 mg/ml did not lead to toxic effects in mice, when given three times a day for five consecutive days. Furthermore, it is well known that some polyphenols have an anti-inflammatory function. In experiments that examined LCMV-induced footpad-swelling aerosol treatment of CYSTUS052 extract did not result in alterations of the systemic immune reaction against the viral pathogen. These findings are in line with the fact that *Cistus incanus* plant extracts are used in traditional medicine in southern Europe for centuries without complications.

Our studies with the polyphenol-rich extract from the plant *Cistus incanus* demonstrated a pronounced antiviral effect against influenza A virus *in vitro* and *in vivo*. It is commonly accepted that in addition to a direct effect influenza virus alters the lungs through epithelial damage in a way that facilitates superinfections by pneumococci (McCullers, 2006). Since polyphenols are also functional against bacteria (Urquiaga and Leighton, 2000), CYSTUS052 extract may have a significant potential against influenza, where bacterial co-infections contribute to the severity of the disease.

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Figure Legends

FGURE 1: Reduction of influenza A virus plaque formation after aerosol CYSTUS052extract treatment. (A) Ten 3.5 cm Petri dishes with a monolayer of MDCK cells were placed at different positions in the aerosol chamber (for detail, see Section 2). (B) Virus plaques on MDCK monolayers after treatment with nebulized PBS were calculated as 100%

(black bars). Plaque reduction after CYSTUS052 extract treatment compared to control was given (grey bars). A mean value for positions 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10 was calculated for each experiment. The data represents the mean value of five independent experiments.

FIGURE 2: No differences in the general health status of mice after treatment with CYSTUS052 extract. (A) Five female Balb/c mice were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days or kept untreated (black triangles). For detail, see Section 2. The general health status of the mice was monitored until day 11 after the start of the treatment by measuring the weight of the animals. The graph represents the mean value of five mice (S.E.M. ≤ 5%). (B) No alteration of LCMV-induced footpad swelling after CYSTUS052 treatment. Five female C57Bl/6 mice were treated with H_2O (black triangles) and five littermates were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days. The mice were infected into the left footpad with 10^4 ffu LCMW-WE 5 days prior treatment. The graph represents the mean value of five mice (S.E.M. ≤ 5%). The experiment was performed twice with similar results.

FIGURE 3: Antiviral effect of CYSTUS052 treatment in FPV-infected mice. Five female Balb/c mice were treated with H_2O (red squares) and five littermates were treated with CYSTUS052 extract (black squares) three times a day for five consecutive days. Directly after the first treatment the animals were infected with 10^2 pfu/50 µl FPV (A, left panel). No difference in the body temperature was found 1 day prior to infection. (A, right panel) Loss of circadian rhythm in control mice 8 days p.i. (B, left panel) No difference in the gross body-activity was found 1 day prior to infection. (B, right panel) Absence of gross body-activity in control mice 8 days p.i. (C) Body weight reduction in control mice 7–11 days p.i. All CYSTUS052 extract treated animals showed no disease symptoms, while H_2O-treated mice developed disease starting at day 7 post-infection and three H_2O-treated mouse died. The H_2O-treated mice recovered between days 10 and 11 post-infection. The graph represents the mean value of five mice (S.E.M. ≤ 5%). Three independent experiments were performed with similar results, except one experiment, where two CYSTUS052 extract-treated mice developed disease and died (see also Table 1).

FIGURE 4: Hemagglutination units and weight curves. (A) Balb/c mice were infected with 10^2 pfu/50 µl FPV and were treated as three times a day for 5 days either with CYSTUS052

(10 mg/ml) or with H_2O (control) in an aerosol formulation. Six days after infection the animals were killed, lungs were taken and a 10% homogenate of the lungs was produced. Fifty microliters of the lung-homogenate was added to a hemagglutinin assay. Lung-homogenate of uninfected mice showed no reaction with chicken red blood cells. The graph represents the mean value of three mice. (B) No antiviral effect against influenza virus infection after oral treatment of mice with CYSTUS052 extract. Five female Balb/c mice were treated with CYSTUS052 extract (open squares) three times a day for five consecutive days or kept untreated (black triangles). For detail, see Section 2. The health status of the mice was monitored until day 15 after the start of the treatment by measuring the weight of the animals. The graph represents the mean value of five mice (S.E.M. ≤ 5%).

FIGURE 5: No alterations of epithelial bronchiolus cells after treatment of mice with CYSTUS052 extract. Female Balb/c mice were treated with CYSTUS052 extract (+CYSTUS052) as an aerosol formulation (10 mg/ml) or were treated with H_2O (−CYSTUS052). No damage of epithelial bronchiolus cells was found in the plant extract-treated mice after H & E staining. Lectinstaining of lungsections was performed to detect sialic acid α-2.6 (2.6 SA) and sialic acid α-2.3 (2.3 SA) receptors. Also no different receptor expression was found in CYSTUS052-treated mice (80x magnification).

Tables

Table 1

Summary of the mouse-experiments with CYSTUS052 treatment

Treatment	Route	Number of animals	Disease symptoms	Onset of disease (day p.i.)	Survival
CYSTUS052	Aerosol	5	0/5	–	5/5
CYSTUS052	Aerosol	15	2/15[a]	8	13/15[a]
H2O	Aerosol	15	13/15	7	7/15
CYSTUS052	Oral	10	10/10	7	2/10*
H2O	Oral	10	10/10	7	2/10*

[a] Two mice from a single experiment developed disease and died.

* In one experiment 5/5 mice died in both groups.

Table 2

Disease in mice after pre-incubation of virus with CYSTUS052

Infectious dose (pfu)	CYSTUS052 pre-incubation	Onset of disease[a] (day p.i.)	Death[a] (day p.i.)	Survival
10^3	−	4	9	0/5
10^3	+	6	11	3/5
10^2	−	7	10	3/5
10^2	+	–	–	5/5

[a] Mean value of five Balb/c mice (S.E.M. ≤ 16%).

Figures

Figures 1

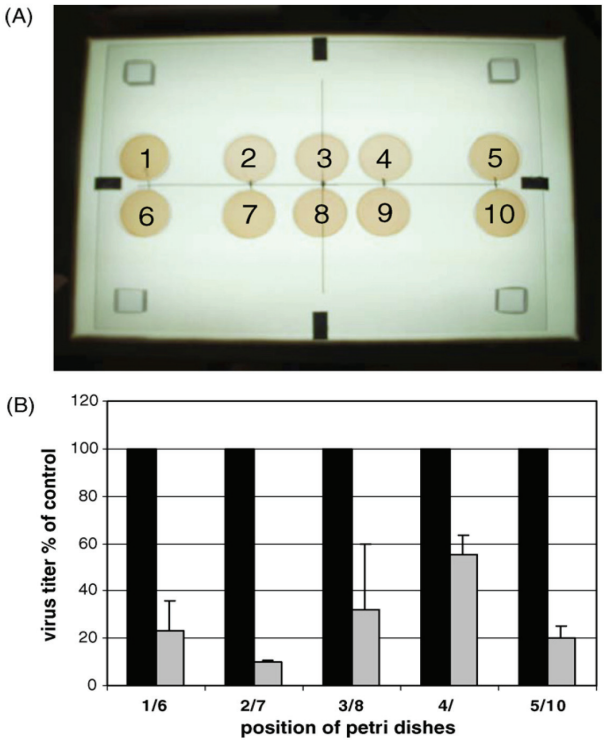

Figure 2

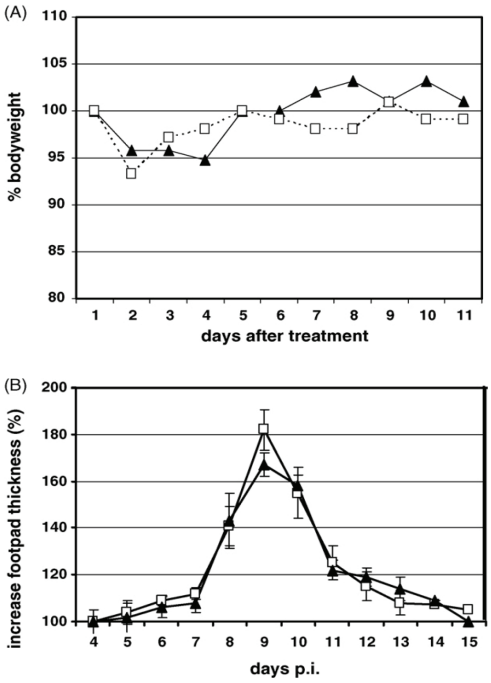

Figure 3

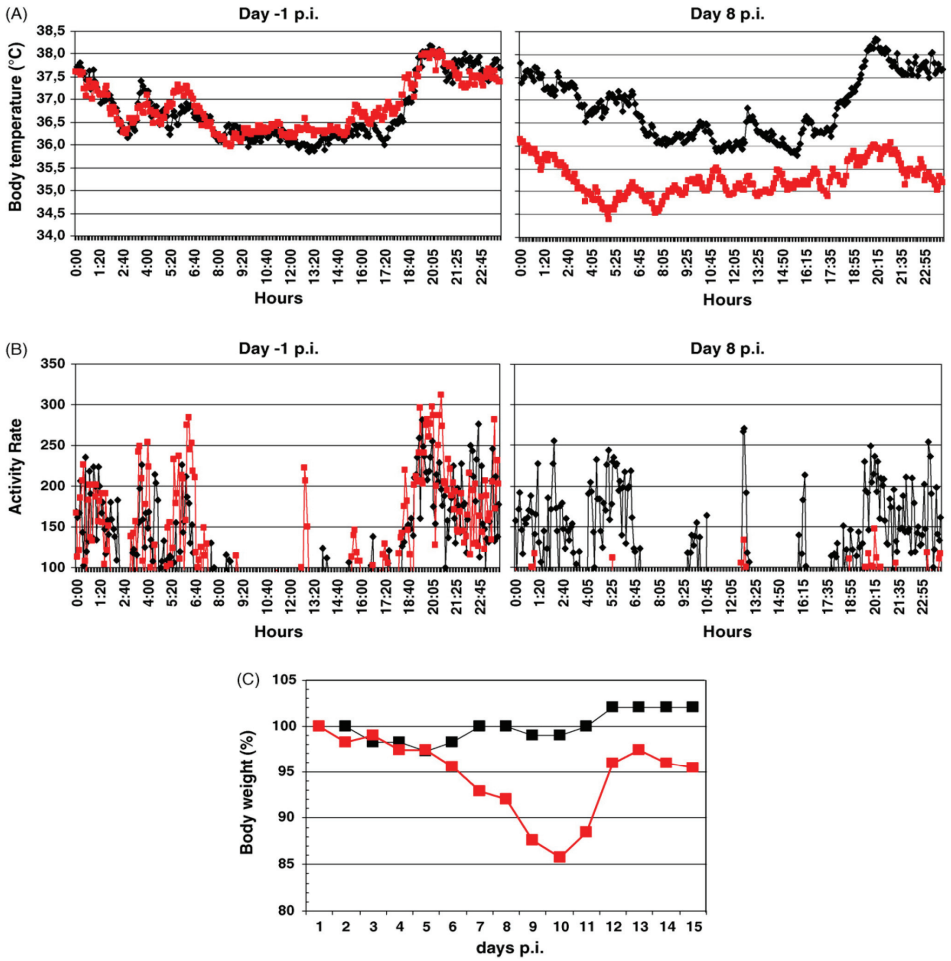

Figure 4

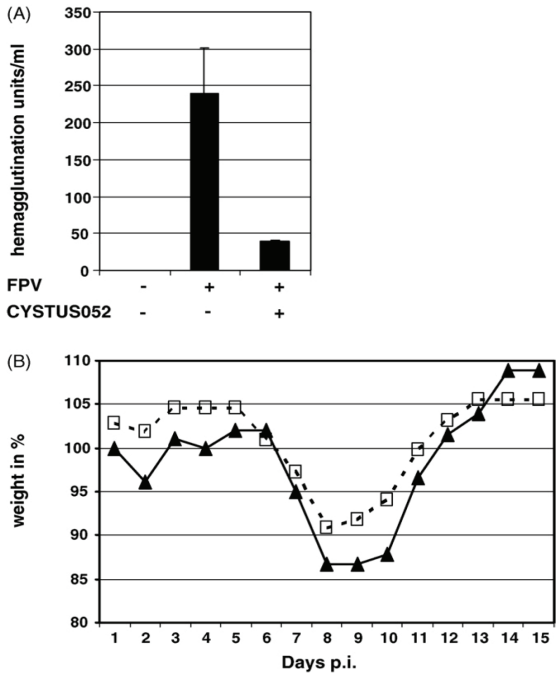

Figure 5