Plasmacytoid dendritic cells and Toll-like receptor 7-dependent signalling promote efficient protection of mice against highly virulent influenza A virus

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Types I and III interferons (IFNs) elicit protective antiviral immune responses during influenza virus infection. Although many cell types can synthesize IFN in response to virus infection, it remains unclear which IFN sources contribute to antiviral protection in vivo. We found that mice carrying functional alleles of the Mx1 influenza virus resistance gene partially lost resistance to infection with a highly pathogenic H7N7 influenza A virus strain if Toll-like receptor 7 (TLR7) signalling was compromised. This effect was achieved by deleting either the TLR7 gene or the gene encoding the TLR7 adaptor molecule MyD88. A similar decrease of influenza virus resistance was observed when animals were deprived of plasmacytoid dendritic cells (pDCs) at day 1 post-infection. Our results provide in vivo proof that pDCs contribute to the protection of the lung against influenza A virus infections, presumably via signals from TLR7.

Conflicting reports are available regarding the role of pDCs and the TLR7/MyD88 signalling pathway during infection with influenza viruses. Ex vivo studies demonstrated that pDCs use the TLR7 complex for sensing influenza viruses (Diebold et al., 2004). However, MyD88-deficient mice were either found (Seo et al., 2010) or not found (Koyama et al., 2007; Le Goffic et al., 2006) to exhibit enhanced influenza susceptibility compared with wild-type mice. Further, pDC depletion experiments failed to reveal a substantial contribution of these cells to influenza virus resistance (GeurtsvanKessel et al., 2008; Wolf et al., 2009). These studies were all carried out with standard Mx1-negative laboratory mice that do not develop robust resistance to influenza virus infection. We reasoned that a clearer picture might emerge from similar studies in Mx1-positive mice.

To test this hypothesis, we first introduced the defective MyD88 allele of Mx1-negative C57BL/6 mice (Adachi et al., 1998) into our Mx1-positive B6.A2G-Mx1 mouse strains. Previous work with mice lacking functional receptors for type I (α/β) or type III (λ) interferon (IFN) illustrated the importance of these virus-induced cytokines in host resistance against influenza viruses (Ank et al., 2008; Mordstein et al., 2008; Müller et al., 1994). IFN-mediated protection against influenza virus is particularly pronounced in mice carrying functional alleles of the Mx1 resistance gene. The Mx1 gene is defective in most standard laboratory mouse strains which, as a consequence, fail to mount a complete antiviral immune response and develop a high degree of resistance against influenza viruses (Staeheli et al., 1988). The Mx1 gene codes for a nuclear 72 kDa protein that interferes with the influenza virus multiplication cycle at a very early step (Haller et al., 2009; Staeheli et al., 1993). Its expression is strongly dependent on activating signals either from type I or type III IFN receptor complex (Holzinger et al., 2007; Mordstein et al., 2008).

Although most nucleated cells can synthesize IFN in response to viral infection, the various cell types differ dramatically with regard to the magnitude by which viral infections can trigger the synthesis of IFN. For example, plasmacytoid dendritic cells (pDCs) are professional IFN-producing cells that represent the major source of circulating IFN during infection with viruses such as murine cytomegalovirus (Asselin-Paturel et al., 2001; Cervantes-Barragan et al., 2007; Tabetta et al., 2004), mouse hepatitis virus (Cervantes-Barragan et al., 2007), respiratory syncytial virus (Smit et al., 2006) and vesicular stomatitis virus (Barchet et al., 2002; Diebold et al., 2004; Lund et al., 2004). During some other viral infections, including infections with influenza A virus, the contribution of pDCs to IFN synthesis in the infected host appears to be small (Jewell et al., 2007) or even negligible (GeurtsvanKessel et al., 2008; Wolf et al., 2009). pDCs strongly express Toll-like receptors (TLR) 7, 8 and 9 through which these cells can efficiently sense virus-derived nucleic acids. TLR7 recognizes ssRNA in the extracellular space. With the help of the adaptor molecule MyD88, ligand-activated TLR7 can trigger a signalling cascade in pDCs that ultimately results in vigorous activation of type I and type III IFN genes (Akira & Takeda, 2004).
line (Horisberger et al., 1983) by classical breeding, and compared the influenza virus susceptibility of $Mx1^{+/+}$ $MyD88^{+/0}$ and $Mx1^{+/+}$ $MyD88^{0/0}$ litters. As expected from earlier work (Koerner et al., 2007), the vast majority of Mx1-positive mice carrying a functional MyD88 allele survived intranasal infection with $5 \times 10^4$ p.f.u. of mouse-adapted influenza A virus SC35M, whereas all littermates lacking a functional MyD88 allele developed severe disease (Fig. 1a). SC35M is derived from A/Seal/Massachussetts/1/80 (H7N7), and it was previously shown to be highly pathogenic for mice and to disseminate into the mouse brain (Gabriel et al., 2005, 2009; Scheiblauer et al., 1995).

Enhanced susceptibility of MyD88-deficient mice correlated with inefficient control of virus multiplication in the lung. This difference reached statistical significance at day 5 post-infection (Fig. 1b). MyD88-deficient animals that survived the viral challenge for more than 10 days showed neurological symptoms of variable severity. Infectious virus was found in the brain of most MyD88-deficient, but not MyD88-competent mice (Fig. 1c). Immunohistochemical analysis further revealed that viral antigen was abundantly present in the cerebral cortex of animals with neurological disease (Fig. 1d), confirming enhanced influenza virus susceptibility of MyD88-deficient mice.

**Fig. 1.** MyD88-dependent signalling is crucial for survival and influenza A virus clearance. (a) $Mx1^{+/+}$ $MyD88^{+/0}$ ($n=25$) and $Mx1^{+/+}$ $MyD88^{0/0}$ ($n=10$) mice were infected intranasally with $5 \times 10^4$ p.f.u. of influenza A virus strain SC35M (H7N7) and monitored daily for survival. Mice that lost more than 25% of the original body weight were sacrificed and scored dead. (b) Viral titres in lung homogenates were determined at days 2 and 5 post-infection by plaque assay. Each symbol represents one animal. (c) Viral titres of brain homogenates were determined at days 5 and 11 post-infection by plaque assay. The virus detection limit in this experiment was 10 p.f.u. ml$^{-1}$. Each symbol represents one animal. (d) Brain tissue from a diseased $Mx1^{+/+}$ $MyD88^{0/0}$ mouse displaying neurological symptoms at day 11 post-infection was analysed for the presence of virus nucleoprotein by immunohistochemistry (brown staining).
MyD88 is an adaptor molecule that plays a key role in many signalling pathways, including the TLR7 pathway that can be triggered by influenza virus-derived RNA (Diebold et al., 2004). To determine if the enhanced influenza virus susceptibility observed in our MyD88-deficient mice can be attributed to the lack of TLR7 signalling, we next analysed influenza virus resistance of TLR7-deficient mice. Since TLR7 maps to the X chromosome, we used the F1 offspring from crosses between Mx1-negative TLR7-deficient females and Mx1-positive wild-type males for our experiments. Females from such crosses carry one functional TLR7 allele, whereas males do not. All animals from such crosses carry one functional allele of the autosomal Mx1 gene which, even in the heterozygous state, can provide a high degree of influenza virus resistance. Challenge experiments with SC35M demonstrated that male offspring developed substantially more severe disease than the female littermates (Fig. 2a). Enhanced susceptibility correlated with inefficient control of influenza virus multiplication in the lung, which was most pronounced at day 5 post-infection (Fig. 2b and c). To control for the possibility that the sex difference rather than the defective TLR7 allele was responsible for the observed enhanced susceptibility of our TLR7-deficient males, we performed reciprocal breeding experiments in which Mx1-negative TLR7-deficient males were crossed with Mx1-positive wild-type females. All F1 offspring from such crosses carry one functional Mx1 allele as well as one functional TLR7 allele. Challenge experiments showed no statistically significant differences in viral lung titres at day 5 post-infection between males and females from these particular crosses (Fig. 2d), confirming the decisive role of TLR7.

**Fig. 2.** TLR7-deficient mice show enhanced lethality and elevated viral lung titres after infection with influenza A virus. (a) Male TLR7<sup>0</sup> (n=10) and female TLR7<sup>+/0</sup> (n=8) mice carrying one functional Mx1 allele from the B6.A2G-Mx1 strain were infected intranasally with 5×10<sup>4</sup> p.f.u. of influenza A virus SC35M and monitored daily. Animals that lost more than 25% of the original body weight were sacrificed and scored dead. (b) Viral titres in lung homogenates of other groups of animals were determined at days 2 and 5 post-infection by plaque assay. Each symbol represents one animal. (c and d) TLR7 deficiency rather than sex determines influenza virus susceptibility. The offspring from crosses of (c) wild-type B6.A2G-Mx1 males with B6-TLR7<sup>0/0</sup> females or (d) B6-TLR7<sup>0</sup> males with wild-type B6.A2G-Mx1 females were infected intranasally with 5×10<sup>4</sup> p.f.u. of influenza A virus. Lung virus titres were determined at day 5 post-infection by plaque assay. Each symbol represents one animal.
TLR7 is prominently expressed by pDCs, but other cell types such as B-cells and macrophages were also reported to express TLR7 (Rubtsov et al., 2011; Wang et al., 2011). To determine if pDCs confer virus protection in our system, we transiently depleted this cell population from Mx1+/+ wild-type mice by a single intraperitoneal injection of 0.5 mg of a mAb against PDC1 at day 1 post-infection with SC35M. Control animals received the same amount of rat IgG2b. At day 5 post-infection, the animals were killed and virus titres in the lung were determined. Animals in which the pDCs were depleted contained about 100-fold more virus in the lung than controls (Fig. 3).

Our data with MyD88-deficient mice are in agreement with recent studies which suggested that MyD88 is important for influenza virus defence (Koyama et al., 2007; Seo et al., 2010). However, our data are not in agreement with an older publication (Le Goffic et al., 2006) which reported similar survival rates of MyD88-deficient and wild-type mice after challenge with an H3N2 influenza A virus strain. In contrast to our present finding, earlier depletion experiments further failed to reveal a substantial contribution of pDCs to influenza virus resistance (GeurtsvanKessel et al., 2008; Wolf et al., 2009). We believe that these differences are due to the fact that we worked with mice that carry functional alleles of the IFN-regulated influenza virus resistance gene Mx1. Since influenza resistance in Mx1-positive mice is highly dependent on IFN, it is likely that genetic alterations which only slightly compromise the host’s ability to synthesize IFN have a more pronounced phenotype in Mx1-positive mice than in standard Mx1-negative mice. The results reported here seem to support this view and suggest that Mx1-positive mice are indeed particularly well-suited tools for evaluating the in vivo potency of IFN.

Taken together, our results strongly suggest that TLR7-mediated sensing of influenza viruses by pDCs substantially contributes to antiviral protection, presumably by enhancing the synthesis of type I and type III IFN in the infected host. This conclusion is compatible with the recently introduced concept that all the various virus sensor systems of mammalian hosts contribute to varying degrees to IFN synthesis (Wang et al., 2011). As the healthy lung does not contain large numbers of pDCs, it is likely that other cells in this organ, including alveolar macrophages (Kumagai et al., 2007), are primarily responsible for the very early synthesis of IFN. In contrast, pDCs contribute to IFN synthesis at a later stage when immune cells start to enter the infected lung. Such a scenario is compatible with our observation that viral lung titres of MyD88-deficient, TLR7-deficient and wild-type mice were similar at day 2 post-infection, whereas titre differences increased during the following days during which MyD88- or TLR7-deficient mice cleared the virus less efficiently than the wild-type controls.

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References


Fig. 3. pDCs promote virus clearance from influenza A virus-infected lungs. Wild-type B6.A2G-Mx1+/+ mice were infected intranasally with 5×104 p.f.u. of influenza A virus SC35M. At day 1 post-infection pDCs were depleted by intraperitoneal injection of 0.5 mg anti-PDC1 per mouse (Milenyi Biotec). Control mice were treated with 0.5 mg irrelevant rat IgG2b per animal (clone RMG2b1; Biolegend). Lung virus titres were determined at day 5 post-infection by plaque assay. Each symbol represents one animal.


