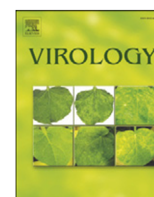
View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Virology 401 (2010) 42–48

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Melanoma differentiation-associated protein-5 (MDA-5) limits early viral replication but is not essential for the induction of type 1 interferons after Coxsackievirus infection

Michael H. Hühn [a], Stephen A. McCartney [b], Katharina Lind [a], Emma Svedin [a], Marco Colonna [b], Malin Flodström-Tullberg [a,*]

[a] Center for Infectious Medicine F59, Department of Medicine, Karolinska Institutet, Huddinge University Hospital, S-141 86 Stockholm, Sweden
[b] Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA

ARTICLE INFO

Article history:
Received 5 January 2010
Returned to author for revision 26 January 2010
Accepted 5 February 2010
Available online 5 March 2010

Keywords:
Coxsackievirus
Enterovirus
Diabetes
Innate immunity
Interferon
Hepatitis
Melanoma differentiation-associated gene-5
Pancreatitis

ABSTRACT

Coxsackievirus infections are associated with severe diseases such as myocarditis, meningitis and pancreatitis. To study the contribution of the intracellular viral sensor melanoma differentiation-associated protein-5 (MDA-5) in the host immune response to Coxsackievirus B3 (CVB3) we infected C57BL/6 and 129/SvJ mice lacking *mda-5*. Mice deficient in MDA-5 showed a dramatically increased susceptibility to CVB3 infection. The loss of MDA-5 allowed the virus to replicate faster, resulting in increased liver and pancreas damage and heightened mortality. MDA-5 was not absolutely required for the induction of type 1 interferons (IFNs), but essential for the production of maximal levels of systemic IFN-α early after infection. Taken together, our findings indicate that MDA-5 plays an important role in the host immune response to CVB3 by preventing early virus replication and limiting tissue pathology.

© 2010 Elsevier Inc. All rights reserved.

Introduction

Coxsackieviruses (CV) are positive single stranded (ss)RNA viruses belonging to the genus of enteroviruses within the *picornaviridae* family. They can be subdivided into CV group A and group B (CVB), comprising 24 and 6 serotypes, respectively. Infections with CVB can cause severe illnesses such as myocarditis, hepatitis and pancreatitis (Pallansch et al., 2007; Tracy et al., 2000). They have also been implicated in the etiopathogenesis of Type 1 Diabetes (Jaïdane and Hober, 2008; Richer and Horwitz, 2009a).

The melanoma differentiation-associated gene-5 (*mda-5*, also called *ifih1*) encodes the protein MDA-5, which belongs to a group of cellular receptors that facilitate the detection of infection by recognizing pathogen associated molecular patterns (PAMPS). When viral RNA is detected by MDA-5 a signal is relayed via the common adaptor protein interferon promoter stimulator-1 (IPS-1, also called VISA or CARDIF). This results in the activation of the transcription factors interferon regulatory transcription factor-3 (IRF-3) and nuclear factor κB (NFκB) (Takeuchi and Akira, 2009) and production of interferon beta (IFN-β). In a second wave, IFN alpha (IFN-α) and IFN-stimulated genes, such as the chemokine CXCL10 and genes involved in antiviral defense, are induced in an autocrine/paracrine manner (Smith et al., 2005).

The host's early innate immune response to CVB infections relies in particular on the functions of type I IFNs. For example, mice unresponsive to type 1 IFNs or lacking IFN-β have an increased and early mortality after infection with CVB (Deonarain et al., 2004; Wessely et al., 2001). Toll like receptors (TLRs) 3, 7 and 8 have all been suggested to recognize CVB and to initiate IFN production (Negishi et al., 2008; Richer et al., 2009; Triantafilou et al., 2005). A role for MDA-5 in recognizing enteroviruses has been suggested (Oikarinen et al., 2008; Richer and Horwitz, 2009b). This hypothesis is mainly based on the findings that encephalomyocarditis virus and Theiler's virus, also belonging to the picornavirus family, are detected by MDA-5 (Gitlin et al., 2006; Kato et al., 2006). However, the realization that Dengue virus but not hepatitis C virus, both belonging to different genera of the flaviviridea family, is recognized by MDA-5 (Loo et al., 2008; Saito et al., 2007) shows that assumptions based solely on taxonomy can sometimes be misleading. A recent study using *mda-5* deficient mice on a 29/SvJ background demonstrated that *mda-5* is important for host survival after challenge with a high dose of CVB serotype 3 (CVB3) (Wang et al., 2010). In the present study we made use of *mda-*

* Corresponding author. Center for Infectious Medicine, Department of Medicine HS, Karolinska Institutet, Karolinska University Hospital Huddinge, F59, S-141 86 Stockholm, Sweden. Fax: +46 8 746 76 37.
E-mail address: malin.flodstrom-tullberg@ki.se (M. Flodström-Tullberg).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.02.010

5 deficient mice on two different genetic backgrounds in order to evaluate the role of MDA-5 in host survival and regulation of early virus replication after infection with CVB3. We also measured the production of type I IFNs, and evaluated viral pathogenicity and the long-term consequences of infection in the *mda-5* deficient host.

Results

Mice deficient in mda-5 on a C57BL/6 background show increased mortality after CVB3 infection

Coxsackievirus Group B strain 3 (CVB3) has mainly been associated with the development of myocarditis (Tracy et al., 2000). Besides its tropism for the heart, CVB3 also infects other organs such as the liver and pancreas. Here, we determined whether MDA-5 is important for host survival following infection with CVB3. To this end, *mda-5*$^{-/-}$ and wild-type (wt) mice on the C57BL/6 (B6) and 129/SvJ (129) genetic backgrounds were challenged with a dose of CVB3 that in our hands is non-lethal for most mice on these backgrounds (10^3 PFU/mouse, Hühn, Flodström Tullberg, unpublished results). B6 mice lacking *mda-5*$^{-/-}$ demonstrated a strikingly high mortality compared to wt (B6) mice (Fig. 1A). With a higher infectious dose (10^5 PFU/mouse) the difference in survival disappeared, and few of the *mda-5*$^{-/-}$ (B6) and wt (B6) mice survived beyond day 10 post infection (p.i.) (Supplementary Fig. 1). In contrast neither *mda-5*$^{-/-}$ (129) nor wt (129) mice succumbed to an infection with either 10^3 (*mda-5*$^{-/-}$ (129), $n=8$; wt (129), $n=8$) or 10^5 (*mda-5*$^{-/-}$ (129), $n=3$; wt (129), $n=3$) PFU/mouse (Fig. 1B, and data not shown). Both *mda-5*$^{-/-}$ (129) and wt (129) mice showed a drop in weight and blood glucose levels around days 5 to 7 p.i. While the hypoglycemia was transient, the weight loss remained throughout the 28 days study period (Supplementary Figs. 2A and B). No difference was observed between *mda-5*$^{-/-}$ (129) and wt (129) mice. Hyperglycemia was not observed in any of the infected wt or *mda-5*$^{-/-}$ mice (Supplementary Fig. 2A and data not shown).

These results indicate that MDA-5 is important for host recognition of CVB3, but that the dependence on *mda5* for host survival during CVB3 infection differs between mouse strains.

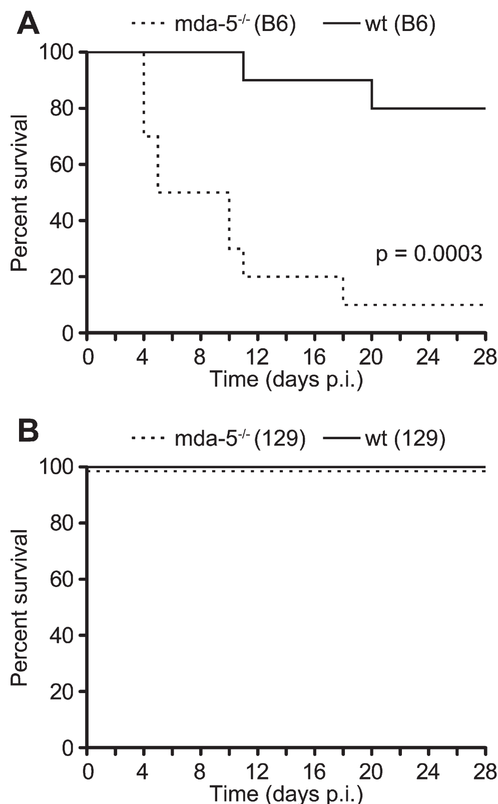


Fig. 1. C57BL/6 but not 129/SvJ mice lacking *mda-5* show increased mortality after CVB3 infection. Mice were infected with 10^3 PFU CVB3. (A) Survival curve of infected wt (B6) ($n=10$, solid line) and *mda-5*$^{-/-}$ (B6) ($n=10$, dashed line); $p=0.0003$, Log-rank (Mantel–Cox) test, and, (B) wt (129) ($n=8$ solid line) and *mda-5*$^{-/-}$ (129) ($n=8$, dashed line), n.s.

MDA-5 restricts early virus replication

Host survival during virus infections is normally dependent on an intact innate immune response, which prevents rampant virus replication and spread before activation of the adaptive immune system. The early death of the infected *mda-5*$^{-/-}$ (B6) mice indicated that MDA-5 is important during the early phase of infection. To establish whether MDA-5 contributes to limiting viral replication we measured virus titers in different organs from infected mice. As most *mda-5*$^{-/-}$ (B6) animals had succumbed within the first 4–5 days after infection, we were restricted to studies on organs harvested on day 3 or 4 p.i. On day 3, virus titers were dramatically higher in all organs from *mda-5*$^{-/-}$ (B6) animals compared to wt (B6) animals (Fig. 2A). The difference was significant for all organs except kidneys. Despite the enormous differences observed on day 3 p.i., *mda-5*$^{-/-}$ (B6) and wt (B6) mice showed comparable viral titers on day 4 p.i. (Fig. 2B).

Although the genetic background modified survival after CVB3 infection, significantly higher titers of infectious virus were also observed on day 3 p.i. in pancreata of *mda-5*$^{-/-}$ (129) mice compared to wt (129) mice (Fig. 2C, $p<0.05$). The viral titers measured in other organs showed a similar trend but failed to reach statistical significance (Fig. 2C). These results suggest that irrespective of genetic background, MDA-5 is particularly important in limiting virus replication at an early time point after infection.

MDA-5 is not essential for systemic IFN-α production

Detection of viruses by MDA-5 results in the production of type I IFNs (Gitlin et al., 2006; Kato et al., 2006; McCartney et al., 2008), cytokines known to be critical for the control of CVB replication and host survival (Deonarain et al., 2004; Flodström et al., 2002; Wessely et al., 2001). Therefore, we next investigated if the observed phenotype in *mda-5*$^{-/-}$ (B6) mice could be explained by a change in type I IFN production. Low levels of systemic IFN-α were detected in serum from mice of both genotypes 24 h after infection (Fig. 3A). A significant increase was observed after 48 h in infected wt (B6) mice (Fig. 3A). At this time point, the infected *mda-5*$^{-/-}$ (B6) mice also produced heightened levels of IFN-α. The difference compared to uninfected *mda-5*$^{-/-}$ (B6) mice was, however, not significant. Moreover, there was a non-significant trend toward lower IFN-α levels in CVB3 infected *mda-5*$^{-/-}$ (B6) mice compared to wt (B6) mice. These observations indicate that although *mda-5* may be required for maximal production of IFN-α, it is not absolutely required for the induction of this cytokine early after infection.

Tissue expression of IFN-β and IFN-stimulated genes is unaffected in mda-5 deficient mice

Because MDA-5 may play an important role in detecting CVB3 in tissues for which the virus demonstrate tropism we next studied the mRNA expression of *ifn-β* in the pancreas and livers on day 3 p.i. The majority of infected *mda-5*$^{-/-}$ (B6) mice, but only one wt (B6) mouse, expressed increased levels of *ifn-β* compared to uninfected animals (Fig. 3B). This mouse was the only wt (B6) mouse that had high virus titers both in liver and pancreata on day 3 p.i. (Supplementary Fig. 3). In addition to IFN-β we assessed the mRNA expression of two IFN-stimulated genes, 2′5′OAS1a (OAS1a), a gene known to be of importance in the host defense to CVB (Flodström-Tullberg et al.,

Fig. 2. Increased early virus replication in *mda-5*$^{-/-}$ mice. Mice were infected with 10^3 PFU CVB3. Organs were removed on day 3 (A and C) or day 4 (B) p.i. and viral titers measured by plaque assay. (A and B) Virus titers in organs harvested from infected wt (gray bars) and *mda-5*$^{-/-}$ (white bars) C57BL/6 mice on days 3 (A) or 4 (B) p.i. (C) Virus titers in organs harvested from infected wt (gray bars) and *mda-5*$^{-/-}$ (white bars) 129/SvJ mice on day 3 p.i. Results are presented as $\log_{10}$(PFU/g wet tissue). Results from individual wt and *mda-5*$^{-/-}$ animals are depicted as squares and circles, respectively, and bars show mean + S.D. *$p<0.05$ **$p<0.01$, Two-way repeated measurement ANOVA.

Fig. 3. IFNs and the IFN-stimulated genes OAS1a and CXCL10 are expressed in serum and organs from infected *mda-5*$^{-/-}$ mice. (A) Wt (B6) and *mda-5*$^{-/-}$ (B6) mice were infected with 10^3 PFU CVB3. Serum was drawn 24 and 48 h after infection and IFN-α measured by ELISA. Wt (B6) mice and *mda-5*$^{-/-}$ (B6) mice are shown in gray and white bars, respectively. (B–D) Wt (B6) and *mda-5*$^{-/-}$ (B6) mice were infected with 10^3 PFU CVB3. On day 3 p.i. small parts of the pancreata and livers were removed. RNA was isolated and the expression of IFN-β (B) OAS1a (C), CXCL10 (D) mRNA was measured by real time PCR, as described in Materials and methods. Wt (B6) and *mda-5*$^{-/-}$ (B6) are shown as gray squares and open circles, respectively. All expression levels were normalized to HPRT. Symbols represent data from individual animals, and mean values are shown as black bars. Statistical analysis was performed using ANOVA, *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

2005), and CXCL10, a chemokine expressed during CVB infection (Christen et al., 2003; Hultcrantz et al., 2007). On day 3 p.i. OAS1a mRNA expression was significantly induced both in livers and pancreata harvested from *mda-5*$^{-/-}$ (B6) mice, but not wt (B6) mice (Fig. 3C). In livers the expression was significantly higher in *mda-5*$^{-/-}$ (B6) mice than in wt (B6) mice. CXCL10 showed a similar pattern of expression, and was significantly higher expressed in liver and pancreas from *mda-5*$^{-/-}$ (B6) mice compared to wt (B6) mice (Fig. 3D). These findings show that IFN-β and the IFN-inducible genes OAS1a and CXCL10 are expressed in tissues even in the absence of MDA-5.

MDA-5 deficiency leads to increased tissue damage and inflammation after infection

A failure to block early virus replication may result in increased tissue damage (Deonarain et al., 2004). To assess if the lack of MDA-5

leads to increased pathology, we first performed a careful histological evaluation of livers and pancreata, organs that may be affected early after CVB3 infection (Flodström et al., 2001; Mena et al., 2000). The livers harvested from wt (B6) mice on day 3 p.i. ($n=5$) demonstrated signs of mild viral hepatitis. Immune cell infiltrates, mainly localized in inflammatory foci, were seen in the livers of some mice (Fig. 4A). The liver architecture was intact and there were no clear signs of hepatocyte damage. On day 4 p.i. ($n=5$) the hepatitis was still moderate in all animals analyzed (Fig. 4B). In stark contrast, moderate to severe viral hepatitis was observed in all infected *mda-5*$^{-/-}$ (B6) animals already on day 3 p.i. ($n=4$, Fig. 4A). Immune cell infiltration was more extensive and less organized than in wt (B6) animals. Moreover, there were clear signs of hepatocyte damage. Some hepatocytes showed features of ballooning degeneration and others

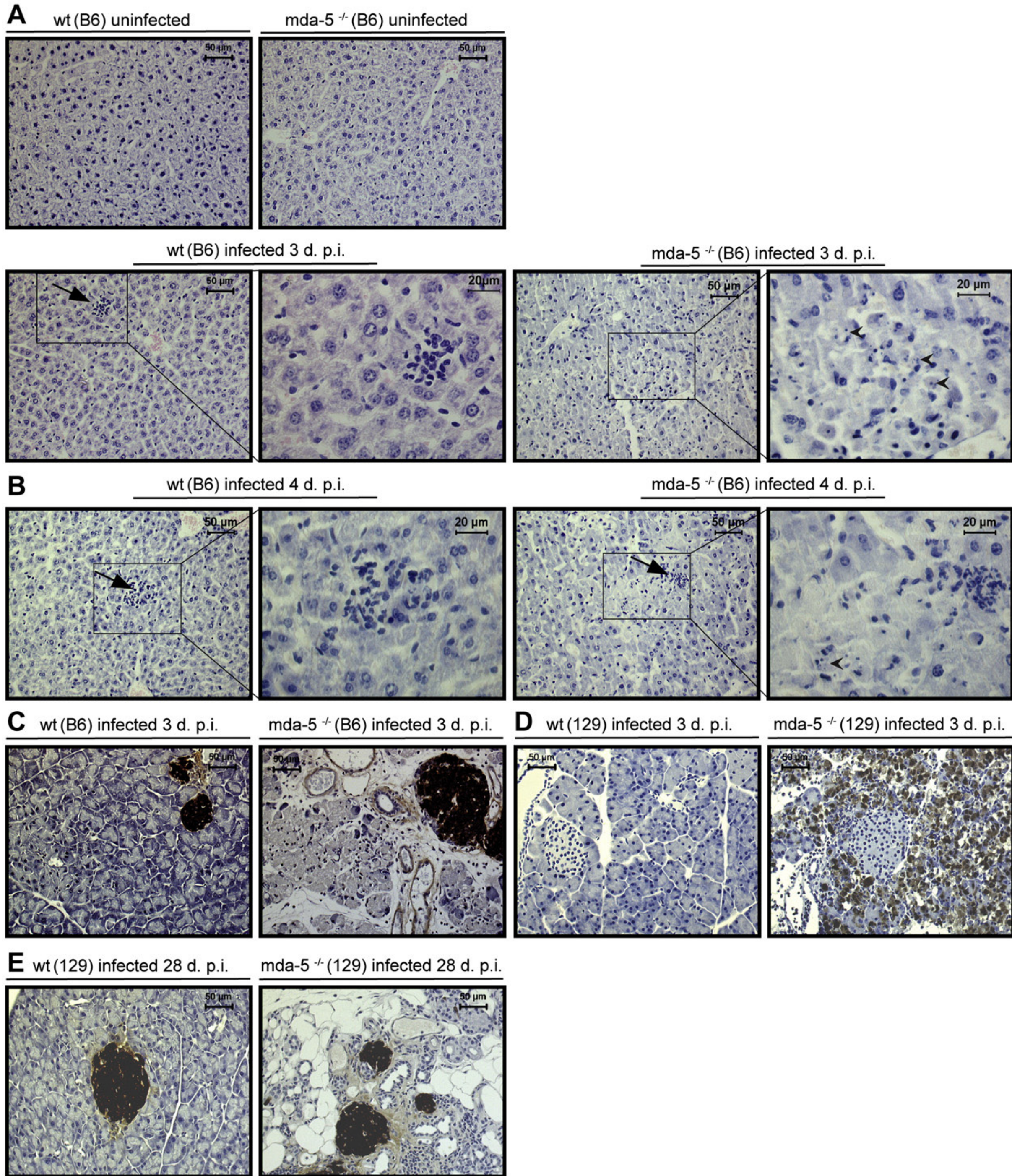


Fig. 4. Increased tissue damage and inflammation in livers and pancreata of CVB3-infected *mda-5*$^{-/-}$ mice. C57BL/6 (B6, A–C) and 129/SvJ (129, D and E) wt and *mda-5*$^{-/-}$ mice were infected with 10^3 PFU CVB3. After 3 (A, C and D), 4 (B) or 28 (E) days organs were removed and analyzed by histology. (A) Liver sections of uninfected (upper row) and infected (lower row) wt (B6) and *mda-5*$^{-/-}$ (B6) mice stained with H&E. (B) H&E staining of liver sections from infected wt (B6) and *mda-5*$^{-/-}$ (B6) 4 days after infection. Arrowheads indicate cells with apoptotic nuclei and an arrow indicates an inflammatory foci. Two different magnifications for each section are shown. The boxes in the lower magnification indicate the approximate location of the parts shown at a higher magnification. (C) Pancreas sections from infected wt (B6) (left) and *mda-5*$^{-/-}$ (B6) (right) mice (day 3 p.i.) were stained with an antibody against insulin. (D) Pancreas sections of infected wt (129) (left) and *mda-5*$^{-/-}$ (129) (right) mice (day 3 p.i.) were stained using an antibody to the enterovirus capsid protein VP1. (E) Pancreas sections of infected wt (129) (left) and *mda-5*$^{-/-}$ (129) (right) mice (day 28 p.i.) were stained using an antibody to insulin. Shown are tissue sections representative for the indicated groups (A and C, $n=3$–5, and D and E, $n=8$ mice/group). Scale bars are shown in every picture as a point of reference.

of apoptosis (Fig. 4A, and data not shown). Livers harvested on day 4 p.i. ($n=3$) showed similar pathology with the exception that the infiltrating immune cells were now present both as inflammatory foci and as single cells spread throughout the parenchyma (Fig. 4B). The liver inflammation observed in 129/SvJ mice was milder than in C57/BL6 animals. The majority of *mda-5*$^{-/-}$ (129) (7/8) but none of the wt (129) (0/8) mice showed clear signs of inflammation (data not shown).

Three days after infection all infected *mda-5*$^{-/-}$ (B6) mice ($n=4$) demonstrated widespread pancreatic exocrine tissue destruction (Fig. 4C). At this time point only one out of five infected wt (B6) mice (Fig. 4C) had signs of acinar cell damage. Four days after infection the majority of both *mda-5*$^{-/-}$ (B6) (2/3) and wt (B6) mice (4/5) suffered from exocrine pancreas damage (data not shown). No abnormalities were found in uninfected control animals (*mda-5*$^{-/-}$ (B6), $n=3$, and wt (B6), $n=3$, data not shown). Lymphocyte infiltration (pancreatitis) and severe damage to the exocrine pancreas were present in several of the CVB3 infected *mda-5*$^{-/-}$ (129) mice (2/8 and 3/8, respectively), but not in wt (129) (0/8) mice on day 3 p.i. (data not shown). CVB virus, as visualized with the VP-1 antibody, was present in the exocrine tissue of the majority of *mda-5*$^{-/-}$ (129) mice, but not in wt (129) mice. The islets of both *mda-5*$^{-/-}$ (129) and wt (129) mice remained free from VP-1 staining (Fig. 4D).

Collectively, these observations show that *mda-5*$^{-/-}$ mice suffer from increased tissue damage after CVB3 infection. The difference was particularly prominent in *mda-5*$^{-/-}$ mice on a C57BL/6 background.

Irreversible pancreas damage in mda-5 deficient mice on a 129/SvJ background

The low mortality in *mda-5*$^{-/-}$ mice on a 129/SvJ background allowed us assess if the absence of MDA-5 had long-term consequences for the infected host. On day 28 p.i., a time point when infectious CVB is usually cleared from all organs (Flodström et al., 2001; Mena et al., 2000), no VP-1 staining could be seen in the pancreata of infected wt (129) and *mda-5*$^{-/-}$ (129) mice (data not shown). Around 75% (6/8) of the *mda-5*$^{-/-}$ (129) mice showed severe damage to the exocrine pancreas, as well as lymphocyte infiltration (Fig. 4E). In contrast, only 25% (2/8) of the infected wt (129) mice demonstrated exocrine tissue damage, and infiltrating lymphocytes were only seen in 37.5% (3/8) of the mice at this time point.

We also examined liver and heart tissues harvested from *mda-5*$^{-/-}$ (129) ($n=8$) and wt (129) ($n=8$) mice on day 28 p.i. We did not observe any signs of inflammation or cell death in the livers of either genotype (data not shown). Mild fibrosis could be detected in the hearts by Masson's Trichrome staining, with no difference between wt (129) and *mda-5*$^{-/-}$ animals (data not shown).

Discussion

A detailed understanding on how the host immune system recognizes viral infections may help in the development of new treatments preventing virus-induced pathology. In this study, we investigated the role of MDA-5 in the host immune response to CVB3, a virus linked to severe diseases such as myocarditis, pancreatitis and meningitis. We found that *mda-5*$^{-/-}$ mice show a strikingly increased susceptibility to infections with CVB3.

Our findings support a critical role for MDA-5 in regulating early CVB3 replication. By day 3 p.i. the virus was replicating vigorously in organs of most animals lacking *mda-5*, but only in a minority of the infected wt mice. Thus, the loss of MDA-5 appeared to allow rapid viral replication and spread. The difference in virus titers disappeared on day 4 p.i., suggesting that MDA-5 temporarily hinders virus replication. The momentary block in virus replication appeared to provide essential protection from tissue damage. Indeed, mice lacking MDA-5 demonstrated more pancreatitis and severe hepatitis, as well as increased acinar cell and hepatocyte damage, compared to infected wt mice. In *mda-5*$^{-/-}$ mice on a C57BL/6 background this had a devastating outcome, as the infection was lethal.

A CVB infection is lytic in many cells (Chehadeh et al., 2000; Schmidtke et al., 2000). Hence, direct cytolytic damage caused by the replicating virus is a likely explanation to the observed cellular damage to hepatocytes and pancreatic acinar cells. The large influx of immune cells and augmented inflammation observed in livers and pancreata of *mda-5*$^{-/-}$ mice may, at least in part, have been an effect of the increased expression of the T- and natural killer cell attracting chemokine CXCL10 (Fig. 3). Notably this expression was low in organs of wt mice with no or barely detectable virus titers (Supplementary Fig. 3).

We measured increased serum levels of IFN-α in both wt (B6) and *mda-5*$^{-/-}$ (B6) mice 48 h after CVB3 infection (Fig. 3A). Keeping in mind the important role for type I IFNs in the host immune response to CVB infections (Deonarain et al., 2004; Flodström et al., 2002; Wessely et al., 2001), it is of interest that 48 h after infection the infected wt (B6) mice produced two-fold more IFN-α than *mda-5*$^{-/-}$ (B6) mice. Although this difference failed to reach statistical significance, a weak IFN response during early infection may have contributed to increased virus titers and pathology. The increased production of IFN-α in *mda-5*$^{-/-}$ (B6) mice also indicates that MDA-5 is not absolutely required for the induction of this cytokine during early CVB3 infection.

We also found that the expression of *ifn-β* mRNA was increased in livers and pancreata from both *mda-5*$^{-/-}$ (B6) and wt (B6) mice having high levels of replicating CVB3 (Fig. 3B, and Supplementary Fig. 3). Besides MDA-5 other pattern recognition receptors (PRR) have been shown to be important in the recognition of CVBs (Flodström-Tullberg et al., 2005; Negishi et al., 2008; Richer et al., 2009; Triantafilou et al., 2005). The fact that we could detect a robust induction of *ifn-β* and the IFN-stimulated genes OAS1a and CXCL10 in *mda-5*$^{-/-}$ mice suggests that MDA-5 is dispensable for the induction of type 1 IFN in these tissues. This indicates that other PRR are more important in detecting CVB infections in liver and pancreas. Both CXCL10 and OAS1a can be induced also by type II interferons (Flodström-Tullberg et al., 2005; Hultcrantz et al., 2007). However, we did not find an increased mRNA expression of IFN-γ in livers and pancreata of infected mice (data not shown), indicating that IFN-γ is not a valid explanation. Collectively, our results suggest that for example one of the TLRs also contributes to the recognition of CVB3, a hypothesis that will be addressed in future studies.

The severity of CVB3 infection in *mda-5*$^{-/-}$ mice seems to, at least in part, be dependent on the genetic background. The majority of C57BL/6 mice lacking *mda-5* succumbed to 10^3 PFU CVB3 within 3 to 9 days, while all infected *mda-5*$^{-/-}$ (129) mice survived this and a higher dose (10^5 PFU/mouse) of virus. It has previously been described that mice on the 129 background are less susceptible than C57BL/6 mice to CVB3 (Wang et al., 2010; Zaragoza et al., 1999). The present study and the work by Wang et al. (2010) showed that the virus replicates less efficiently in organs from 129/SvJ mice compared to those from C57BL/6 mice. Moreover, our histopathological analyses revealed that both wt and *mda-5*$^{-/-}$ mice on the C57BL/6 background suffer from more tissue damage than 129/SvJ mice. Others have reported a correlation between the degree of hepatitis/liver damage and mortality after CVB infections (Wang et al., 2010; Wessely et al., 2001). Thus, the severe hepatitis observed in both wt and *mda-5*$^{-/-}$ mice on a C57BL/6 background could explain the high mortality in this strain. It is also of interest that an important role for *mda-5* in host survival was uncovered only on the C57BL/6 background. The explanation to this is at present not known. Genes other than *mda-5* influence the outcome of CVB infections (Flodström-Tullberg et al., 2005; Negishi et al., 2008; Richer et al., 2009) and it is possible that differences in the function or expression of these or other genes between the two strains of mice could explain this somewhat unexpected observation.

The present finding that MDA-5 is an important factor in the immune response to CVB3 is in line with a recently published study (Wang et al., 2010). Wang et al. reported that MDA-5 deficient mice on a 129/SvJ background have an increased susceptibility to infection with CVB3. The present study adds further to the understanding of the function of MDA-5 in several ways. For example, Wang et al. reported that *mda-5*$^{-/-}$ 129/SvJ mice suffered from increased tissue damage early after infection. Here we confirmed and extended this finding by using MDA-5 deficient mice on both the 129/SvJ and C57BL/6 backgrounds. Importantly, by expanding the studies to a time point after which the virus was cleared, the present study also demonstrated that the increased early tissue damage observed in mice lacking MDA-5 had long-term consequences resulting in chronic damage to the pancreatic acinar tissue. Furthermore, one of the most important and novel findings in the present study was that MDA-5 limited early viral replication. Finally, by using a non-lethal infection with CVB3 our study also clearly highlighted the role of other viral sensors in the induction of type 1 IFNs during CVB3 infection.

Enterovirus infections (e.g. Coxsackieviruses) have been associated with the development of the autoimmune disease type 1 diabetes (Dotta et al., 2007; Jaïdane and Hober, 2008; Richardson et al., 2009; Richer and Horwitz, 2009a). Recent findings link certain polymorphisms in the human *mda-5* gene to an increased risk for development of type 1 diabetes (Grant et al., 2009; Nejentsev et al., 2009; Smyth et al., 2006). The present study together with the recent study by Wang et al. (2010) now firmly establish that MDA-5 plays an important role in the host response to a type of virus that has been associated with the development of this disease. These findings encourage further studies on the link between *mda-5* and type 1 diabetes.

In summary, we show that MDA-5 is involved in the recognition of CVB3. A lack of MDA-5 allows unbridled virus replication early after infection. This results in enhanced inflammation and increased tissue damage in organs such as the pancreas and liver. In the pancreas, the damage persists even after the virus is cleared. Our findings also suggest that MDA-5 is not solely responsible for the systemic increase in IFN-α observed early after infection. Furthermore, they indicate that MDA-5 is dispensable for the induction of type 1 IFN and IFN-stimulated genes on the tissue level. Future studies will show how MDA-5 together with other PRRs orchestrates the host anti-viral response.

Materials and methods

Mice and animal husbandry

In the present study we used mice deficient in MDA-5. *Mda-5* was targeted in SSC#10 (129/SvJ) ES cells and some mice were maintained on a pure 129/SvJ background (here denoted "129"). The mutation was also backcrossed onto a C57BL/6 background (here denoted "B6"), facilitated by genome-wide screening of polymorphic microsatellite markers at 10-centimorgan intervals at each generation (performed by the Rheumatology Speed Congenics Core Laboratory at Washington University School of Medicine, St Louis, USA). The *mda5*$^{-/-}$ B6 mice used for these experiments were ≥99% C57BL/6. Wild-type (wt) C57BL/6 and 129/SvJ mice were used as controls. The mice were breed and housed under specific pathogen free conditions at a facility of the Washington University School of Medicine, St. Louis, USA. All animals were matched for genetic background, sex and age. The animal experiments were conducted in accordance with institutional guidelines for animal care and use.

Virus

CVB3 strain "Nancy" was originally obtained from G. Frisk (Uppsala University, Sweden) and propagated and titered in HeLa cells, as previously described (Hühn et al., 2008).

Infections, organ recovery and virus titration

Mice aged 8 to 10 weeks were infected with one intra-peritoneal (i.p.) injection of CVB3 (10^3 or 10^5 PFU/mouse in 200 µl PBS). In experiments measuring viral titers the mice were sacrificed by cervical dislocation at the indicated time points, and organs removed under aseptic conditions. One part of the removed organs was frozen immediately for later virus recovery and another part fixed in 4% formalin for histology. Viral titers were determined by standard plaque assay on HeLa cell cultures. Titers were quantitated as PFU/g wet tissue and presented as $\log_{10}$ (PFU/g tissue). For RNA isolation small biopsies from the pancreas and liver were immersed in RNAlater (Qiagen, Stockholm, Sweden) and stored at −20 °C.

Blood glucose determination

Venous blood glucose levels of non-fasting mice were measured using a Contour blood glucose meter (Bayer, Stockholm, Sweden).

RNA isolation

Organs were removed from RNAlater, grinded and homogenized with a gentelMACS dissociator using M-tubes and the RNA_02 program (Miltenyi Biotech, Bergisch Gladbach, Germany). Total RNA was isolated with a RNeasy Kit (Qiagen, Stockholm, Sweden) according to the manufacturer's protocol. RNA was quantified using a NanoDrop ND-1000 (Thermo Scientific, Stockholm, Sweden).

Reverse transcription and real-time PCR

cDNA was prepared by treating 1 µg RNA with Turbo DNase (Applied Biosystems, Stockholm, Sweden) before reverse transcription using SuperScript III First Strand Synthesis Kit and random hexamers for priming (Invitrogen, Stockholm, Sweden). Real time (RT-) PCR for CXCL10, 2–5 OAS1a and HPRT was performed with TaqMan Gene Expression Assays (Mm00445235_m1, Mm00836412_m1 and Mm00446968_m1 respectively) and MasterMix (all Applied Biosystems, Stockholm, Sweden) and for IFN-β and IFN-γ (published in (Kallewaard et al., 2009) using RT² SYBR Green/ROX (SABiosciences distributed by In vitro Sweden AB, Stockholm, Sweden). All experiments were performed on a ABI 7500 Real Time system (Applied Biosystems, Stockholm, Sweden) and expression was normalized to HPRT using the $\Delta\Delta C_t$ method. In samples that did not show any measurable gene expression Ct values were set to 41.

Measurement of IFN-α serum levels

Animals were infected with 10^3 PFU CVB3, serum collected after 24 and 48 h and IFN-α was measured using ELISA (PBL Biomedical Laboratory, New Brunswick, NJ, USA), according to the manufacturer's protocol.

Histology and immunohistochemistry

Paraffin sections (4 µm) were stained with hematoxylin and eosin (H&E) or with the following primary antibodies purchased from DAKO Cytomation, Stockholm, Sweden: guinea pig anti-insulin (1:5500), rat anti-glucagon (1:2500) or mouse anti-VP1 (biotinylated in-house; 1:50), as previously described (Flodström-Tullberg et al., 2005). Tris–EDTA Buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0) was used for heat induced epitope retrieval to improve detection of VP1. Trichrome staining was done using the Artisan staining system (DAKO Cytomation, Stockholm, Sweden) according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was done with GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). A p-value ≤ 0.05 was considered statistically significant. Survival was analyzed using Log-rank (Mantel–Cox) test, viral titers and gene expression using two-way repeated measurement ANOVA and ANOVA, respectively, followed by a Bonferroni post-test.

Acknowledgments

Dr. J. Bergelson (University of Pennsylvania School of Medicine, Philadelphia, PA, USA) and colleagues are gratefully acknowledged for assistance with the RT-PCR analyses. These studies were supported by the European Foundation for the Study of Diabetes (EFSD) (M.F-T), the Erik and Edith Fernströms stiftelse (M.H.H.), the Swedish Foundation for Strategic Research (M.F-T), the Swedish Research Council (M.F-T), The Swedish Diabetes Association Research Foundation (M.F-T). M.H.H, was also supported by an Albert Renold Fellowship from the EFSD. MC was supported by JDRF grant # 24-2007-420. SM is supported by NRSA Award Number F30HL096354 from the NHLBI. The opinions in this manuscript as those of the authors and do not reflect the positions of the JDRF or NHLBI.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.02.010.

References

Chehadeh, W., Kerr-Conte, J., Pattou, F., Alm, G., Lefebvre, J., Wattré, P., Hober, D., 2000. Persistent infection of human pancreatic islets by Coxsackievirus B is associated with alpha interferon synthesis in beta cells. J. Virol. 74 (21), 10153–10164.

Christen, U., McGavern, D.B., Luster, A.D., von Herrath, M.G., Oldstone, M.B., 2003. Among CXCR3 chemokines, IFN-gamma-inducible protein of 10 kDa (CXC chemokine ligand (CXCL) 10) but not monokine induced by IFN-gamma (CXCL9) imprints a pattern for the subsequent development of autoimmune disease. J. Immunol. 171 (12), 6838–6845.

Deonarain, R., Cerullo, D., Fuse, K., Liu, P.P., Fish, E.N., 2004. Protective role for interferon-beta in Coxsackievirus B3 infection. Circulation 110 (23), 3540–3543.

Dotta, F., Censini, S., van Halteren, A.G.S., Marselli, L., Masini, M., Dionisi, S., Mosca, F., Boggi, U., Muda, A.O., Prato, S.D., Elliott, J.F., Covacci, A., Rappuoli, R., Roep, B.O., Marchetti, P., 2007. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc. Natl. Acad. Sci. U. S. A. 104 (12), 5115–5120.

Flodström, M., Horwitz, M.S., Maday, A., Balakrishna, D., Rodriguez, E., Sarvetnick, N., 2001. A critical role for inducible nitric oxide synthase in host survival following Coxsackievirus B4 infection. Virology 281 (2), 205–215.

Flodström, M., Maday, A., Balakrishna, D., Cleary, M.M., Yoshimura, A., Sarvetnick, N., 2002. Target cell defense prevents the development of diabetes after viral infection. Nat. Immunol. 3 (4), 373–382.

Flodström-Tullberg, M., Hultcrantz, M., Stotland, A., Maday, A., Tsai, D., Fine, C., Williams, B., Silverman, R., Sarvetnick, N., 2005. RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during Coxsackievirus infection. J. Immunol. 174 (3), 1171–1177.

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R.A., Diamond, M.S., Colonna, M., 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. U. S. A. 103 (22), 8459–8464.

Grant, S.F.A., Qu, H.-Q., Bradfield, J.P., Marchand, L., Kim, C.E., Glessner, J.T., Grabs, R., Taback, S.P., Frackelton, E.C., Eckert, A.W., Annaiah, K., Lawson, M.L., Otieno, F.G., Santa, E., Shaner, J.L., Smith, R.M., Skraban, R., Imielinski, M., Chiavacci, R.M., Grundmeier, R.W., Stanley, C.A., Kirsch, S.E., Waggott, D., Paterson, A.D., Monos, D.S., Group, D.E.R., Polychronakos, C., Hakonarson, H., 2009. Follow-up analysis of genome-wide association data identifies novel loci for type 1 diabetes. Diabetes 58 (1), 290–295.

Hühn, M.H., Hultcrantz, M., Lind, K., Ljunggren, H.-G., Malmberg, K.-J., Flodström-Tullberg, M., 2008. IFN-gamma production dominates the early human natural killer cell response to Coxsackievirus infection. Cell. Microbiol. 10 (2), 426–436.

Hultcrantz, M., Hühn, M.H., Wolf, M., Olsson, A., Jacobson, S., Williams, B.R., Korsgren, O., Flodström-Tullberg, M., 2007. Interferons induce an antiviral state in human pancreatic islet cells. Virology 367 (1), 92–101.

Jaïdane, H., Hober, D., 2008. Role of Coxsackievirus B4 in the pathogenesis of type 1 diabetes. Diabetes Metab. 34 (6 Pt 1), 537–548.

Kallewaard, N.L., Zhang, L., Chen, J.-W., Guttenberg, M., Sanchez, M.D., Bergelson, J.M., 2009. Tissue-specific deletion of the Coxsackievirus and adenovirus receptor protects mice from virus-induced pancreatitis and myocarditis. Cell Host Microbe 6 (1), 91–98.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.-S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441 (7089), 101–105.

Loo, Y.-M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M.A., García-Sastre, A., Katze, M.G., Gale, M., 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J. Virol. 82 (1), 335–345.

McCartney, S.A., Thackray, L.B., Gitlin, L., Gilfillan, S., Virgin Iv, H.W., Colonna, M., 2008. MDA-5 recognition of a murine norovirus. PLoS Pathog. 4 (7), e1000108.

Mena, I., Fischer, C., Gebhard, J.R., Perry, C.M., Harkins, S., Whitton, J.L., 2000. Coxsackievirus infection of the pancreas: evaluation of receptor expression, pathogenesis, and immunopathology. Virology 271 (2), 276–288.

Negishi, H., Osawa, T., Ogami, K., Ouyang, X., Sakaguchi, S., Koshiba, R., Yanai, H., Seko, Y., Shitara, H., Bishop, K., Yonekawa, H., Tamura, T., Kaisho, T., Taya, C., Taniguchi, T., Honda, K., 2008. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc. Natl. Acad. Sci. U. S. A. 105 (51), 20446–20451.

Nejentsev, S., Walker, N., Riches, D., Egholm, M., Todd, J.A., 2009. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 324 (5925), 387–389.

Oikarinen, M., Tauriainen, S., Honkanen, T., Oikarinen, S., Vuori, K., Kaukinen, K., Rantala, I., Mäki, M., Hyöty, H., 2008. Detection of enteroviruses in the intestine of type 1 diabetic patients. Clin. Exp. Immunol. 151 (1), 71–75.

Pallansch, M., Roos, R., Knipe, D.M., 2007. Enteroviruses: polioviruses, Coxsackieviruses, echoviruses, and newer enteroviruses. “Fields Virology”, Vol. 1, pp. 839–894.

Richardson, S., Willcox, A., Bone, A., Foulis, A., Morgan, N., 2009. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia 52 (6), 1143–1151.

Richer, M.J., Horwitz, M.S., 2009a. Coxsackievirus infection as an environmental factor in the etiology of type 1 diabetes. Autoimmun. Rev. 8 (7), 611–615.

Richer, M.J., Horwitz, M.S., 2009b. The innate immune response: an important partner in shaping Coxsackievirus-mediated autoimmunity. J. Innate Immun. 1 (5), 421–434.

Richer, M.J., Lavallée, D.J., Shanina, I., Horwitz, M.S., 2009. Toll-like receptor 3 signaling on macrophages is required for survival following Coxsackievirus B4 infection. PLoS ONE 4 (1), e4127.

Saito, T., Hirai, R., Loo, Y.-M., Owen, D., Johnson, C.L., Sinha, S.C., Akira, S., Fujita, T., Gale, M., 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc. Natl. Acad. Sci. U. S. A. 104 (2), 582–587.

Schmidtke, M., Selinka, H.C., Heim, A., Jahn, B., Tonew, M., Kandolf, R., Stelzner, A., Zell, R., 2000. Attachment of Coxsackievirus B3 variants to various cell lines: mapping of phenotypic differences to capsid protein VP1. Virology 275 (1), 77–88.

Smith, P.L., Lombardi, G., Foster, G.R., 2005. Type I interferons and the innate immune response—more than just antiviral cytokines. Mol. Immunol. 42 (8), 869–877.

Smyth, D.J., Cooper, J.D., Bailey, R., Field, S., Burren, O., Smink, L.J., Guja, C., Ionescu-Tirgoviste, C., Widmer, B., Dunger, D.B., Savage, D.A., Walker, N.M., Clayton, D.G., Todd, J.A., 2006. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. Nat. Genet. 38 (6), 617–619.

Takeuchi, O., Akira, S., 2009. Innate immunity to virus infection. Immunol. Rev. 227 (1), 75–86.

Tracy, S., Höfling, K., Pirruccello, S., Lane, P.H., Reyna, S.M., Gauntt, C.J., 2000. Group B Coxsackievirus myocarditis and pancreatitis: connection between viral virulence phenotypes in mice. J. Med. Virol. 62 (1), 70–81.

Triantafilou, K., Orthopoulos, G., Vakakis, E., Ahmed, M.A.E., Golenbock, D.T., Lepper, P.M., Triantafilou, M., 2005. Human cardiac inflammatory responses triggered by Coxsackie B viruses are mainly Toll-like receptor (TLR) 8-dependent. Cell. Microbiol. 7 (8), 1117–1126.

Wang, J., Cerny, A., Asher, D., Kurt-Jones, E., Bronson, R., Finberg, R., 2010. MDA5 and MAVS mediate type I IFN responses to Coxsackie B virus. J. Virol. 84 (1), 254–260.

Wessely, R., Klingel, K., Knowlton, K.U., Kandolf, R., 2001. Cardioselective infection with Coxsackievirus B3 requires intact type I interferon signaling: implications for mortality and early viral replication. Circulation 103 (5), 756–761.

Zaragoza, C., Ocampo, C.J., Saura, M., Bao, C., Leppo, M., Lafond-Walker, A., Thiemann, D.R., Hruban, R., Lowenstein, C.J., 1999. Inducible nitric oxide synthase protection against Coxsackievirus pancreatitis. J. Immunol. 163 (10), 5497–5504.