The tyrosine kinase p56^{lck} is essential in coxsackievirus B3-mediated heart disease

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Infections are thought to be important in the pathogenesis of many heart diseases. Coxsackievirus B3 (CVB3) has been linked to chronic dilated cardiomyopathy, a common cause of progressive heart disease, heart failure and sudden death. We show here that the sarcoma (Src) family kinase Lck (p56^{lck}) is required for efficient CVB3 replication in T-cell lines and for viral replication and persistence *in vivo*. Whereas infection of wild-type mice with human pathogenic CVB3 caused acute and very severe myocarditis, meningitis, hepatitis, pancreatitis and dilated cardiomyopathy, mice lacking the *p56^{lck}* gene were completely protected from CVB3-induced acute pathogenicity and chronic heart disease. These data identify a previously unknown function of Src family kinases and indicate that p56^{lck} is the essential host factor that controls the replication and pathogenicity of CVB3.

Cardiovascular disease is the most frequent cause of death in the world, and bacterial and viral infections can cause the development of heart disease[1–3]. Infections with endemic picornaviruses, which are very frequent and cause common colds, diarrhea, encephalomeningitis and paralysis, may be triggers of heart disease and diabetes. Particularly notorious in this regard are coxsackieviruses, which are very contagious, positive-stranded RNA picornaviruses with a global occurrence[2–4]. The endemic coxsackievirus group B serotype 3 (CVB3), which is cytopathic for many mammalian cells, causes severe myocarditis, pancreatitis and meningitis in children, and sudden cardiac death in young adults. It has been estimated that at least 70% of the human population has come into contact with CVB3. CVB3 can be detected in the hearts of as many as 30–50% of patients with chronic dilated cardiomyopathy[5,6] (DCM), a condition that often necessitates heart transplantation. These observations indicate that CVB3 infection is a principal trigger for chronic heart disease in humans. In addition, coxsackieviruses have been linked to diabetes.

Human CVB3 infections can be mimicked in mice using a human pathogenic CVB3 isolate[7]. CVB3-infected mice develop acute and very severe myocarditis, encephalomeningitis, hepatitis and pancreatitis, and either die of the acute cytopathic effects of the virus or recover from the acute infection but develop chronic DCM. The mouse disease closely mimics histopathological findings in human DCM patients, allowing the use of this model to make observations pertinent to humans. Both acute viral replication and disease chronicity are influenced by factors in the host[8,9]. However, the results of studies attempting to identify the host genetic factors that determine viral replication, susceptibility to acute disease and progression from acute viral infection to chronic heart disease have been equivocal. Moreover, no effective prophylaxis or treatment exists to combat coxsackievirus pathogenicity *in vivo*.

Here we show that the sarcoma (Src) family kinase p56^{lck} is required for efficient CVB3 replication in T-cell lines and for viral replication and persistence *in vivo*. Infection of wild-type mice with human pathogenic CVB3 caused acute and very severe myo-carditis, meningitis, hepatitis, pancreatitis and chronic DCM, but mice lacking the *p56^{lck}* gene were completely protected from CVB3-induced acute pathogenicity and chronic heart disease. Thus, our data show that Src family kinases have another, previously unknown function, and that p56^{lck} is the essential host factor in controlling the replication and pathogenicity of CVB3.

P56^{lck} regulates CVB3 replication in human Jurkat T cells

After entering hosts through the alimentary tract, CVB3 infects and replicates in T and B cells and macrophages[10,11]. These immune cells provide a reservoir for viral RNA during acute and persistent infections[10]. Two surface receptors for CVB3 have been identified, both expressed on lymphocytes: the glycosylphosphatidyl inositol-linked surface glycoprotein CD55 (Genome Database designation, DAF; decay accelerating factor)[12] and the immunoglobulin superfamily molecule coxsackievirus and adenovirus receptor[13]. Binding of CVB3 to the short consensus region 3 domain of CD55 is required for viral attachment and internalization, whereas coxsackievirus and adenovirus receptor serves as a membrane co-receptor for productive infection[14].

As cross-linking of the short consensus region 3 of CD55 acti-

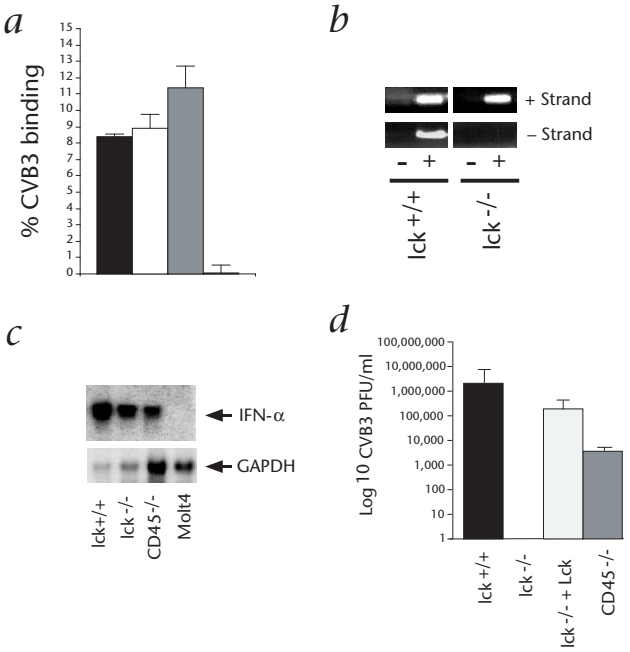

Fig. 1 p56^{lck} regulates replication of CVB3 in human Jurkat T cells. ***a***, Binding of CVB3 to specific receptors on Jurkat cells. Triplicate cultures of *p56*$^{lck+/+}$ (■) *p56*$^{lck-/-}$ (□) and *CD45*$^{-/-}$ (■) Jurkat T cells or Molt4 T cells (far right bar) were incubated with ^{35}S-labeled CVB3. Data represent mean percentages (± s.d.) of membrane-bound CVB3 (one of five different experiments). Expression of CD55 and coxsackievirus and adenovirus receptor was similar for *p56*$^{lck+/+}$, *p56*$^{lck-/-}$ and *CD45*$^{-/-}$ Jurkat cells (not shown). There is no CVB3 binding in Molt4 cells, which lack expression of the CVB3 receptor CD55. The differences in CVB3 binding between *p56*$^{lck+/+}$, *p56*$^{lck-/-}$ and *CD45*$^{-/-}$ Jurkat cells are statistically not significant ($P > 0.05$; ANOVA). ***b***, Detection of positive and negative strands of the CVB3 genome in *p56*$^{lck+/+}$ and *p56*$^{lck-/-}$ Jurkat cells. Cells (3×10^6) were left untreated (–) or were infected with 3×10^6 PFUs/ml CVB3 (+). After 24 h, positive- and negative-strand CBV3 sequences were detected in total RNA using RT–PCR. ***c***, Induction of IFN-α in infected Jurkat T cells. T-cell lines (3×10^7 cells) were inoculated with 3×10^6 PFUs/ml CVB3. After 24 h of incubation, total RNA (15 μg) was assessed by northern blot analysis with a probe detecting IFN-α mRNA expression. Bottom, GAPDH mRNA expression in the same samples (control for sample loading). ***d***, Detection of infectious CVB3. *p56*$^{lck+/+}$, *p56*$^{lck-/-}$, *CD45*$^{-/-}$ Jurkat cells and *p56*$^{lck-/-}$ Jurkat cells (3×10^6 cells/ml) reconstituted with wild-type p56^{lck} (*lck*$^{-/-}$ + *Lck*) were inoculated in triplicate with 3×10^5 PFUs/ml CVB3. After 24 h, culture supernatants were analyzed for infectious CVB3 using standard plaque-forming assays in HeLa cells. Data represent mean PFU values (± s.d.) (one of five different experiments). $P < 0.001$, *lck*$^{+/+}$ and *lck*$^{-/-}$ + *Lck* compared with *lck*$^{-/-}$ and *CD45*$^{-/-}$ (ANOVA).

vates p56^{lck} (ref. 15), we investigated whether p56^{lck} was involved in CVB3 entry into the host cell. We infected normal and p56^{lck}-deficient human Jurkat T-cell lines[16] with CVB3 and assessed viral entry. CVB3 readily bound to its surface receptors on both *p56*$^{lck-/-}$ and *p56*$^{lck+/+}$ Jurkat cells (Fig. 1*a*). This binding was specific, as CVB3 did not bind to CD55$^-$ Molt4 T cells (Fig. 1*a*) and binding of CVB3 to normal or *p56*$^{lck-/-}$ Jurkat cells could be blocked with antibodies against CD55. The presence of CVB3 in the cytoplasm of both *p56*$^{lck-/-}$ and *p56*$^{lck+/+}$ Jurkat T cells was demonstrated by PCR for the positive viral RNA strand (Fig. 1*b*, top row) and by detection of the CVB3 core protein. Furthermore, CVB3-infected *p56*$^{lck-/-}$ Jurkat cells produced alpha interferon (IFN-α), albeit in smaller amounts than did CVB3-infected *p56*$^{lck+/+}$ Jurkat cells (Fig. 1*c*); relative counts per minute (c.p.m.) for *p56*$^{lck-/-}$ Jurkat cells were 545 c.p.m. for glyceraldehyde phosphodehydrogenase (GAPDH) and 8,905 c.p.m. for IFN-α, and for *p56*$^{lck+/+}$ Jurkat cells were 320 c.p.m. for GAPDH and 7,756 c.p.m. for IFN-α. Thus, CVB3 can enter human Jurkat T cells and trigger a cellular antiviral interferon response in the absence of p56^{lck}.

CVB3 replicated in wild-type Jurkat cells, as demonstrated by the detection of the negative viral RNA strand (indicating the presence of viral replication intermediates; Fig. 1*b*, bottom row) and the recovery of high titers of infectious CVB3 from these cells (Fig. 1*d*). In contrast, we detected no negative viral RNA strand (Fig. 1*b*) and recovered no infectious CVB3 (Fig. 1*d*) in the absence of p56^{lck} expression. The introduction of wild-type p56^{lck} into *p56*$^{lck-/-}$ Jurkat cells restored viral replication and recovery of high titers of infectious CVB3 (Fig. 1*d*). To further explore the role of p56^{lck} in CVB3 replication, we infected Jurkat cells defi-

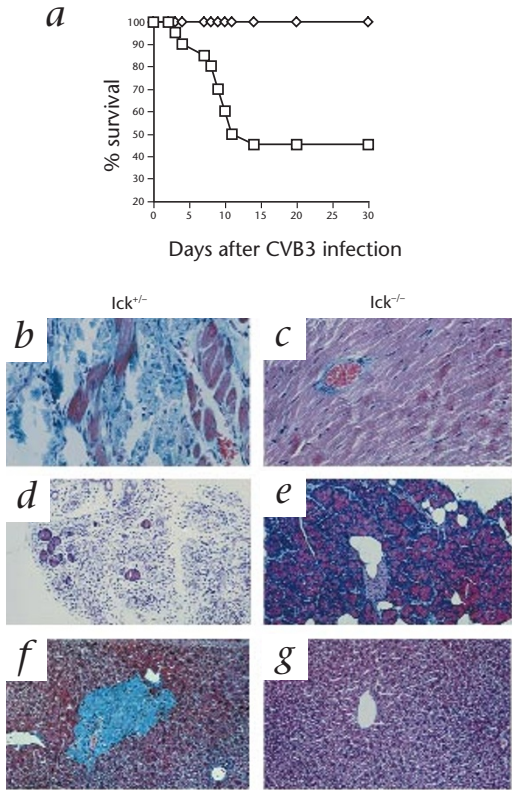

Fig. 2 P56^{lck} is essential for the pathogenesis of CVB3-mediated disease *in vivo*. ***a***, Survival of CVB3-infected *p56*$^{lck-/-}$ mice. *p56*$^{lck-/-}$ mice (◇; $n = 25$) and *p56*$^{lck+/-}$ littermates (□; $n = 22$) were inoculated with CVB3 and monitored for 30 d. *p56*$^{lck+/-}$ mice showed signs of severe systemic illness, including lethargy, ruffled coats and anorexia on days 3–15 after infection. *p56*$^{lck-/-}$ mice failed to show any signs of disease and had a 100% survival rate even at later times (more than 60 d after infection). Difference between survival curves at day 30: $P < 0.0001$ (χ^2 test). ***b***–***g***, Histopathology of hearts (*b* and *c*), pancreata (*d* and *e*) and livers (*f* and *g*) of *p56*$^{lck+/-}$ mice (*b*, *d* and *f*) and *p56*$^{lck-/-}$ littermates (*c*, *e* and *g*) infected with CVB3; organ histology was evaluated with masson staining 7 d after infection. Data represent eight mice per group. There is extensive destruction and inflammation of heart tissue, exocrine and endocrine pancreas and liver parenchyma in *p56*$^{lck+/-}$ mice. Original magnifications, ×80 (*b*, *c*, *f* and *g*) and ×8 (*d* and *e*).

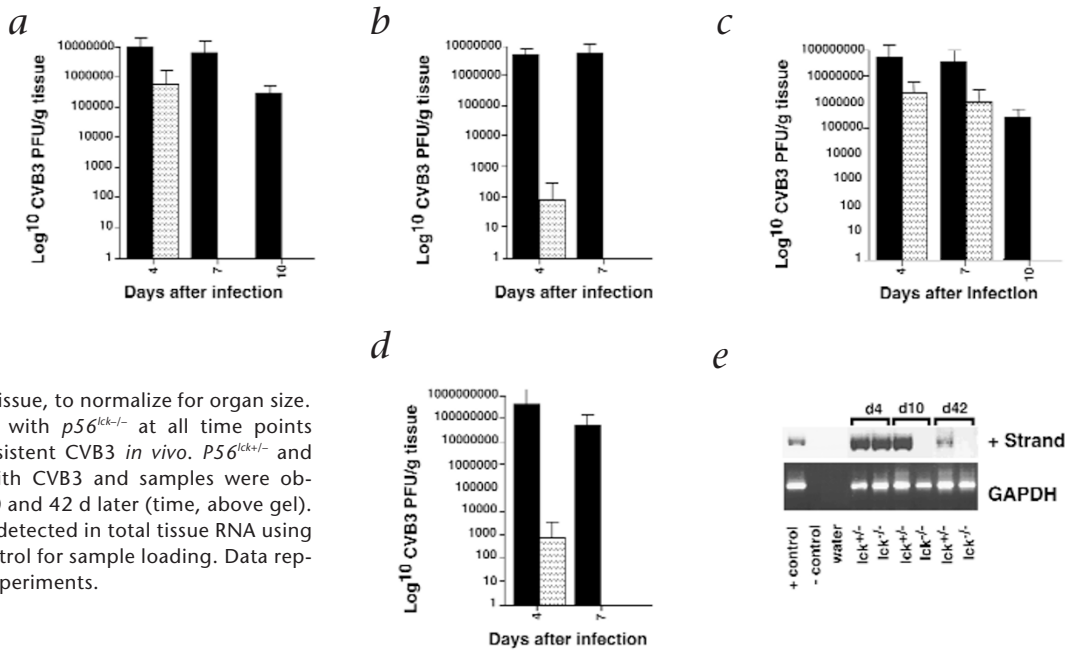

Fig. 3 Viral titers in $p56^{lck-/-}$ mice. ***a–d***, Recovery of infectious CVB3 from spleens (*a*), brains (*b*), hearts (*c*) and pancreata (*d*) of $p56^{lck+/-}$ (■) and $p56^{lck-/-}$ (⚄) littermate mice, infected with CVB3. Viral titers were determined in triplicate from total organ homogenates using plaque assays on HeLa monolayers; $n = 5–6$ mice per group analyzed for each time point. Data represent mean values of CBV3 PFUs per gram of tissue, to normalize for organ size. $P < 0.005$, $p56^{lck+/-}$ compared with $p56^{lck-/-}$ at all time points (ANOVA). ***e***, Detection of persistent CVB3 *in vivo*. $P56^{lck+/-}$ and $p56^{lck-/-}$ mice were infected with CVB3 and samples were obtained from mouse hearts 4, 10 and 42 d later (time, above gel). Positive-strand CBV3 RNA was detected in total tissue RNA using RT–PCR. GAPDH (bottom), control for sample loading. Data represent one of three different experiments.

cient in CD45 (Genome Database designation, *Ptprc*), a tyrosine phosphatase required for normal $p56^{lck}$ kinase activity[17]. CVB3 was able to bind to cell surface receptors (Fig. 1*a*) and penetrate into the cytoplasm of $CD45^{-/-}$ Jurkat cells but CVB3 replication was decreased in the absence of CD45 (Fig. 1*d*). Thus, efficient CVB3 replication in Jurkat cells depends at least partially on the $p56^{lck}$ tyrosine kinase activity. These results show that the Src family kinase $p56^{lck}$ is required for the replication of CVB3 in human T cells. The exact molecular mechanism by which $p56^{lck}$ regulates CVB3 replication needs to be determined.

$P56^{lck}$ is essential for CVB3-mediated pathogenicity

To determine whether $p56^{lck}$ expression is important in CVB3 infections *in vivo*, we inoculated $p56^{lck-/-}$ mice[18] with a human pathogenic CVB3 isolate[19]. In contrast to its effect on $p56^{lck+/-}$ mice, CVB3 infection of $p56^{lck-/-}$ littermates did not result in any disease or lethality (Fig. 2*a*). CVB3 infections in $p56^{lck+/-}$ mice led to acute and very severe myocarditis (Fig. 2*b*), pancreatitis (Fig. 2*d*), hepatitis (Fig. 2*f*) and meningoencephalitis. The histology of the hearts, livers, brains and pancreata of CVB3-infected $p56^{lck-/-}$ mice seemed normal at all times (Fig. 2*c*, *e* and *g*). We monitored the course of CVB3-mediated disease and viral replication *in vivo* in $p56^{lck+/-}$ and $p56^{lck-/-}$ littermate mice. In $p56^{lck+/-}$ mice, inoculation of CVB3 led to rapid infection of cells in spleen, brain, heart, pancreas and liver. We were able to isolate high titers of infectious CVB3 from these organs 4–10 days after the initial inoculation (Fig. 3*a–d*). Moreover, CVB3 persisted in the hearts of infected $p56^{lck+/-}$ mice for up to 42 days (Fig. 3*e*). Although we were able to recover small amounts of infectious CVB3 from hearts, brains, pancreata and spleens of $p56^{lck-/-}$ mice 4 and 7 days after infection, infectious CVB3 was no longer detectable by 10 days after viral inoculation (Fig. 3*a–e*). Thus, $p56^{lck}$ is essential for CVB3 replication, CVB3 persistence and the pathogenesis of CVB3-mediated disease *in vivo*.

$p56^{lck}$ expression in T cells is sufficient for CVB3 pathogenicity

Various host factors contribute to protection from viral pathogenicity *in vivo* (neutralizing antibodies, natural killer cells, interferons, T cells and others)[2]. Serum titers of neutralizing IgM antibodies against CVB3 and levels of IFN-α and IFN-β were similar in CVB3-infected $p56^{lck-/-}$ and $p56^{lck+/-}$ mice. *In vivo* depletion of natural killer cells in CVB3-infected $p56^{lck-/-}$ mice using antibodies against gangliotetraosylceramide did not result in exacerbation of disease, and there were no substantial changes in CVB3 replication or CVB3 titers in heart or spleen. Cytotoxic T-lymphocyte responses against CVB3 need to be determined in $p56^{lck-/-}$ mice. $p56^{lck-/-}$ mice have a defect in T-cell activation and decreased numbers of T cells in the blood and peripheral lymphoid organs[18]. To further elucidate the involvement of $p56^{lck}$-expressing T cells in CVB3 disease pathogenesis, we generated chimeras in which wild-type T cells were transferred into $p56^{lck-/-}$ mice. After chimeric re-introduction of $p56^{lck+/+}$ T cells into $p56^{lck-/-}$ mice, CVB3-mediated pathogenicity and the recovery of infectious CVB3 (Fig. 4) were restored to levels found in control littermate mice. These results indicate that expression of $p56^{lck}$ in T cells is an essential factor controlling the pathogenicity and replication of CVB3 *in vivo*.

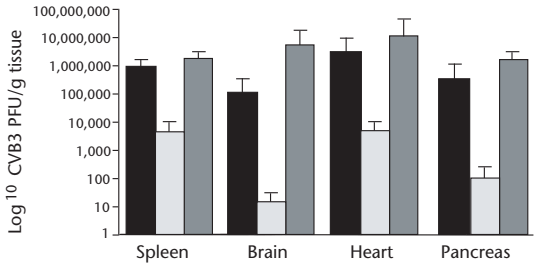

Fig. 4 $p56^{lck}$ expression in T cells is sufficient for CVB3 pathogenicity. Recovery of infectious CVB3 from spleens, brains, hearts and pancreata of $p56^{lck+/+}$ (■) and $p56^{lck-/-}$ (▩) mice and $p56^{lck-/-}$ mice reconstituted with $p56^{lck+/+}$ T cells (■). Mice (6 weeks old; $n = 5$ per group) were infected with CVB3. Data represent mean values of CBV3 PFUs per gram of tissue on day 4 after infection. $P < 0.01$, $p56^{lck+/+}$ and $p56^{lck-/-}$ mice reconstituted with wild-type T cells compared with $p56^{lck-/-}$ mice (ANOVA).

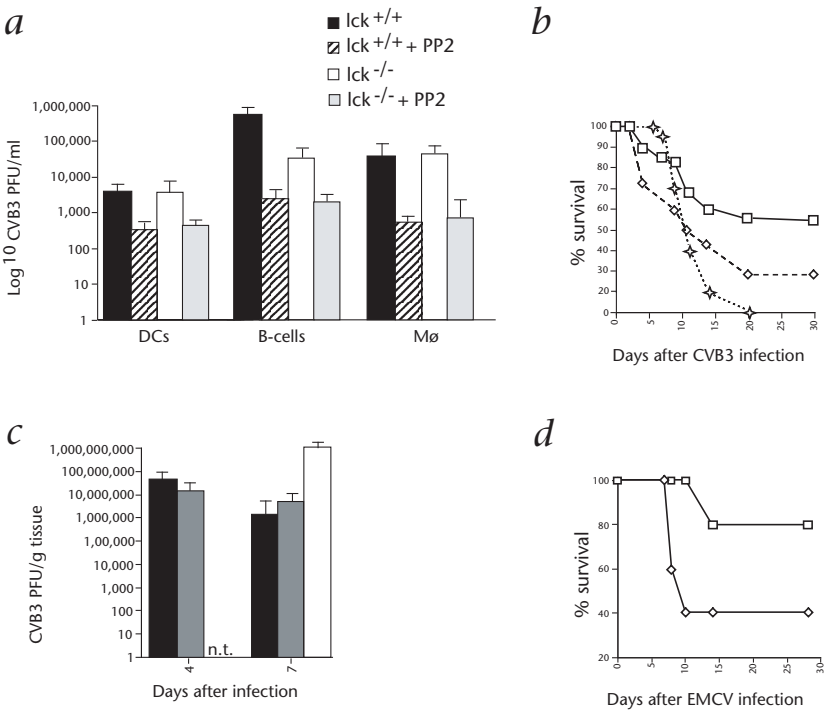

Fig. 5 CVB3 infections in different cell types and $p56^{lck-/-}$, $Src^{-/-}$ and $Rag1^{-/-}$ mice. ***a***, CVB3 replication in $p56^{lck+/+}$ and $p56^{lck-/-}$ dendritic cells (DCs; 1×10^5), B cells (5×10^5) and macrophages (Mø; 1×10^6). Purified cells were infected in triplicate with 5×10^5 PFUs CVB3 in the presence or absence of 2 µM PP2, and viral titers were determined 24 h after infection. $P < 0.05$, $p56^{lck+/+}$ B cells compared with $p56^{lck-/-}$ B cells (ANOVA). ***b***, Survival curves of $Rag1^{+/+}$ (□; $n = 21$), $Src^{-/-}$ (◇; $n = 15$) and $Rag1^{-/-}$ (☆; $n = 20$) mice inoculated with CVB3 and monitored for 30 d. Day 30 survival: $P < 0.001$, $Rag1^{+/+}$ compared with $Src^{-/-}$; $P < 0.0001$, $Rag1^{+/+}$ compared with $Rag1^{-/-}$ (χ^2 test). ***c***, Recovery of infectious CVB3 in the hearts of $Rag1^{+/+}$ (■; $n = 3$), $Src^{-/-}$ (■; $n = 4$) and $Rag1^{-/-}$ (□; $n = 4$) mice infected with CVB3. Viral titers were determined from total heart tissue in triplicate using plaque assays. n.t., not tested. Data represent mean values of CBV3 PFUs per gram of tissue, to normalize for organ size. $P < 0.005$ at day 7, $Rag1^{+/+}$ or $Src^{-/-}$ mice compared with $Rag1^{-/-}$ mice, (ANOVA). ***d***, Survival curves of $p56^{lck+/-}$ (□; $n = 12$) and $p56^{lck-/-}$ (◆; $n = 15$) mice inoculated with EMCV and monitored for 30 d after infection. Day 30 survival: $P < 0.005$ (χ^2 test).

Involvement of other Src family kinases and non-T cells

$p56^{lck-/-}$ mice have a defect in T-cell activation[18]. To determine if the resistance of $p56^{lck-/-}$ mice to CVB3-induced disease was due to a deficiency in activated T cells, we inoculate CBV3 into $Cd4^{-/-}$ mice, which lack $Cd4^+$ T helper cells[20], $Cd8^{-/-}$ mice, which lack $Cd8^+$ cytotoxic lymphocytes[21] and mice lacking the T-cell costimulatory receptor Cd28, which have a defect in T-cell activation[22]. CVB3-induced disease as well as replication and persistence of CVB3 *in vivo* were mostly independent of $Cd4^+$ helper and $Cd8^+$ cytotoxic T-cell subsets and independent of T-cell activation itself (as shown in $Cd28^{-/-}$ mice). These results show that not functional T cells themselves but $p56^{lck}$ is the essential factor that controls pathogenicity and replication of CVB3 *in vivo*. Thus, we analyzed CVB3 replication in other hematopoietic cell lines isolated from $p56^{lck-/-}$ mice. Whereas T and B cells expressed $p56^{lck}$, macrophages and dendritic cells did not express $p56^{lck}$, as determined by RT–PCR and western blot analysis. CVB3 replication was similar for $p56^{lck+/+}$ and $p56^{lck-/-}$ dendritic cells and macrophages (Fig. 5*a*). However, CVB3 replication was partially reduced in $p56^{lck-/-}$ B cells (Fig. 5*a*). These results show that $p56^{lck}$ is important for CVB3 replication in B cells. However, CVB3 replication can occur in cells that normally do not express $p56^{lck}$.

To further elucidate cellular requirements for CVB3 replication, we infected cells with CVB3 in the presence of a specific inhibitor of Src family kinase, PP2. The addition of PP2 decreased CVB3 replication in dendritic cells, B cells and macrophages (Fig. 5*a*), indicating that other Src family kinases can regulate CVB3 replication in these cells. The nature of this Src family kinase needs to be determined in genetic experiments. We excluded the possibility of essential involvement of the prototypical kinase Src, as $Src^{-/-}$ mice are very susceptible to CVB3 infections *in vivo* (Fig. 5*b* and *c*). From our data it is evident that $p56^{lck}$ is the limiting and essential host factor that controls *in vivo* replication and pathogenicity of CVB3.

As $p56^{lck}$ is mainly expressed in T and B cells, one explanation for the protective effect of the $p56^{lck}$ mutation against CVB3 infection *in vivo* could be that CVB3 requires T and/or B cells for infection and a normal life cycle. To test this, we inoculated CVB3 into mice deficient in the recombination activation gene ($Rag\text{-}1^{-/-}$ mice), which completely lack T and B cells due to a defect in T- and B-cell receptor gene rearrangement[23]. $Rag\text{-}1^{-/-}$ mice remained very susceptible to CVB3 infection. All $Rag\text{-}1^{-/-}$ mice died after CVB3 inoculation (Fig. 5*b*), because of uncontrolled viral replication in the heart (Fig. 5*c*) and severe tissue damage. As-yet-unidentified immunomodulatory mechanisms in $p56^{lck-/-}$ mice could contribute to increased protection against CVB3.

$p56^{lck}$ is not a general regulator of picornavirus pathogenicity

In contrast to their resistance to the picornavirus CVB3, $p56^{lck-/-}$ mice show a profound lack of anti-viral activity against viruses of other replicative classes, including vaccinia virus, lymphocytic choriomeningitis virus and vesicular stomatitis virus[24]. These viruses belong to different replicative classes: Lymphocytic choriomeningitis virus is an RNA arenavirus; vaccinia is a DNA-containing poxvirus; and vesicular stomatitis virus is an RNA rhabdovirus. To determine whether $p56^{lck}$ is required for infection by other picornaviruses, we inoculated $p56^{lck-/-}$ mice with encephalomyocarditis virus. Like CVB3, encephalomyocarditis virus is a picornavirus that causes acute myocarditis and encephalomyocarditis in mice[25]. $p56^{lck-/-}$ mice were very susceptible to encephalomyocarditis virus infections (Fig. 5*d*) and were unable to clear the virus (data not shown). The fact that $p56^{lck}$ expression is required for CVB3 but not for encephalomyocarditis virus infection indicates that $p56^{lck}$ is not a general regulator of picornavirus pathogenicity.

$p56^{lck-/-}$ mice are protected from chronic DCM

Viral persistence, chronic low-grade infections and autoimmune attacks 'sparked' by CVB3-induced damage to cardiomyocytes may be involved in the progression of acute disease to chronic

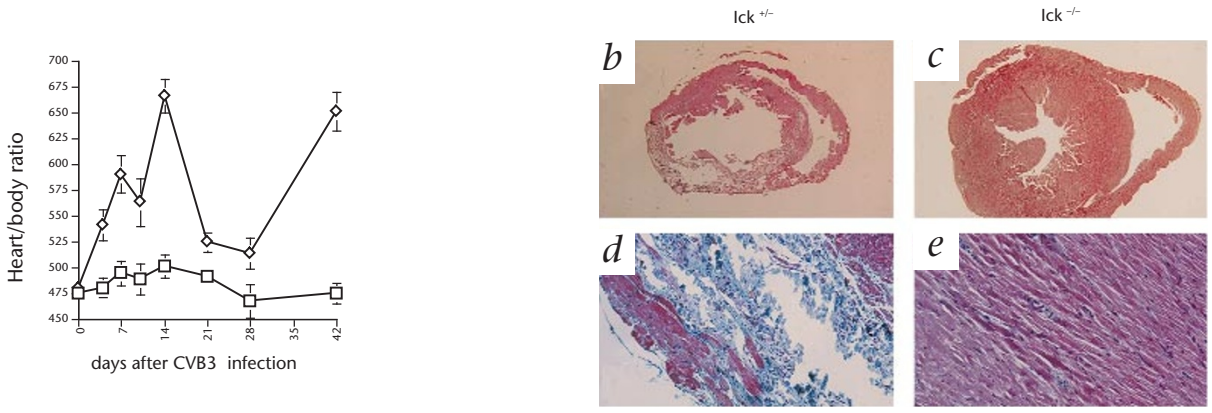

Fig. 6 *p56*$^{lck-/-}$ mice are protected from chronic DCM. ***a***, Ratios of heart weight to body weight. *p56*$^{lck+/-}$ (◇) and *p56*$^{lck-/-}$ (□) mice were infected with CVB3. Data represent mean heart weight:body weight ratios (± s.d.) for at least three mice per group for each time point. The ratios of the *p56*$^{lck+/-}$ group were significantly higher ($P < 0.05$) than those of *p56*$^{lck-/-}$ mice on days 4, 7, 10, 14 and 42 after infection (variance analysis using Neuman-Keul's statistics). There is a biphasic disease progression in *p56*$^{lck+/-}$ mice: The first phase is due to acute CBV3 cytopathicity and heart inflammation; the second phase is caused by chronic DCM (ref. 26). ***b***–***e***, Heart histopathology of *p56*$^{lck+/-}$ (*b* and *d*) and *p56*$^{lck-/-}$ (*c* and *e*) mice infected with CVB3; hearts were analyzed on day 42 after infection. There is thinning of ventricular walls and enlargement of cardiac chambers (*b*) and severe destruction of the heart muscle characterized by necrosis, fibrosis and cellular infiltration (*d*) that occurs in all infected *p56*$^{lck+/-}$ mice. *p56*$^{lck-/-}$ mice have normal heart morphologies and heart weight:body weight ratios. Original magnifications (and staining): ×4 (hematoxylin and eosin, midventricular regions; *b* and *c*) and ×40 (masson; *d* and *e*).

DCM (refs. 26,27). Wild-type (data not shown) and *p56*$^{lck+/-}$ mice rapidly developed severe cardiomyopathy characterized by increased ratios of heart weight to body weight (Fig. 6*a*), thinning of ventricular walls and dilation of the ventricles (Fig. 6*b*) and considerable destruction of heart muscle (Fig. 6*d*). In contrast, CVB3-infected *p56*$^{lck-/-}$ mice had normal heart morphology and heart weight:body weight ratios (Fig. 6*a*, *c* and *e*). Whereas CVB3 persisted in the hearts of *p56*$^{lck+/-}$ mice, we did not detect virus in *p56*$^{lck-/-}$ heart muscle 10 and 42 days after the primary infection, as determined by PCR for positive-stranded CVB3 RNA in total heart tissue (Fig. 3*e*). An increase in the number of CVB3 plaque-forming units (PFUs) to a viral load that leads to 100% lethality in wild-type mice still did not trigger acute or chronic disease in infected *p56*$^{lck-/-}$ mice. Thus, in the absence of p56^{lck}, CVB3 can start to replicate in some organs and induce a low-grade infection. However, the specific expression of p56^{lck} is necessary for efficient CVB3 replication to cause acute pathogenicity and for viral persistence *in vivo* to produce chronic dilated heart disease.

Discussion

DCM is a common cause of progressive heart disease, heart failure, and sudden death and often necessitates heart transplantation. Infections are now thought to be involved in the pathogenesis of many heart diseases, and CVB3 has been epidemiologically linked as the most prevalent virus associated with DCM in humans[2,3]. Our study here has shown that the Src family kinase p56^{lck} is the essential host factor required for the replication, persistence and pathogenicity of CVB3 *in vivo*. Mice lacking the *p56*^{lck} gene were completely protected from CVB3-induced acute pathogenicity and chronic heart disease. These data therefore define a previously unknown function for Src family kinases in the pathogenesis of heart disease. Identification of p56^{lck} as an essential regulator of CVB3 replication may allow the design of drugs that specifically interfere with the replication and persistence *in vivo* of this virus, avoiding the onset of acute myocarditis, pancreatitis and encephalitis in children, and preventing the development of CVB3-associated chronic heart diseases.

Methods

CVB3 binding assays and replication *in vitro*. *p56*$^{lck-/-}$ and *CD45*$^{-/-}$ Jurkat T cells and Molt4 T cells have been described[16,17] and were purchased from American Type Culture Collection (Rockville, Maryland). *p56*$^{lck-/-}$ Jurkat cells reconstituted with wild-type *p56*^{lck} cDNA have been described[16]. B cells and dendritic cells from *p56*$^{lck-/-}$ and *p56*$^{lck+/+}$ mice were purified by fluorescence-activated cell sorting (purity: more than 99% B220^{+} B cells and more than 98% CD11c^{+} dendritic cells). Macrophages were purified from the peritoneal cavity 4 d after intraperitoneal injection of thioglycolate. In all experiments, the CVB3 isolate Gauntt-Chow was used[19]. CVB3 was grown in HeLa cell monolayers, and infectious CVB3 released by multiple cycles of freezing and thawing was fractionated by sucrose gradient centrifugation. For viral binding, CBV3 was radiolabeled with 100 μCi ^{35}S-methionine in methionine-free RPMI medium for 4 h. The radiolabeled virus (1×10^4 c.p.m.) was incubated with 1×10^7 T cells for 1 h at room temperature, after which cells were washed free of medium and excess labeled virus. The percentage of CVB3 bound was calculated as (c.p.m. of membrane-bound virus/c.p.m. of total CVB3 in medium before binding) × 100%. For the detection of viral RNA, IFN-α and infectious CVB3, cells were inoculated with CVB3 for 1 h at 37 °C, then washed and resuspended in RPMI medium containing 10% fetal bovine serum. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), an inhibitor specific for the Src family kinase, was used according to the manufacturer's specifications (Calbiochem, La Jolla, California). At days 1, 2 and 5 after infection, cells were collected by centrifugation and the supernatants recovered for plaque assays. Plaque assays were done in triplicate according to established protocols on HeLa cell monolayers plated in six-well plates (Nunc, Rochester, New York).

Positive and negative strands of CVB3 RNA were identified in total cellular RNA by RT-PCR as described[6]. Total RNA (1 μg) was reverse-transcribed into cDNA using Superscript II (RNase H-negative; Life Technologies). The following primers were used: positive-strand, 5'–CACCGGATGGCCAATCCA–3'; negative-strand, 5'–GCGAAGAGTCTATTGAGCTA–3'. cDNA was analyzed for positive- and negative-strand CVB3 sequences using Taq Polymerase (Boehringer) and the primers 5'–GCGAAGAGTC-

TATTGAGCTA–3′ and 5′–CTCTCAATTGTCACCATAAGCAGCCA–3′. PCR products were analyzed on a 2% agarose gel and visualized using ethidium bromide. Amplification products were analyzed for specificity by cycle sequencing of gel-purified PCR products. The CVB3 control was transcribed CVB3 cDNA. For the detection of IFN-α mRNA, total RNA was isolated from infected cells, and 15 μg were separated by formaldehyde agarose gel electrophoresis followed by transfer to a nitrocellulose membrane; blots were hybridized to probes specific for IFN-α and GAPDH.

CVB3 infections *in vivo*. Mutant mice with deletions of the *p56*^{lck}, *Src*, *Cd4*, *Cd8*, *Cd28* or *Rag1* genes have been described[18,20–22]. Mice were backcrossed to an A/J (H2$^{k/k}$) background (five generations) and a C57BL/6 background (ten generations). Littermate mice were used in all experiments. For CVB3 infection, mice 4 and 6 weeks old were inoculated intraperitoneally with 1×10^5 PFUs CVB3. For the generation of T-cell chimeras, *p56*$^{lck+/+}$ T cells were purified using Dynal beads (purity, more than 98% Cd3$^+$ T cells) and 1×10^7 T cells were transferred intraperitoneally into 4-week-old *p56*$^{lck-/-}$ hosts; these T-cell-chimeric mice were infected 10 days after T-cell transfer with CVB3 as described above. For EMCV infection, mice were inoculated intraperitoneally with 1×10^5 PFUs EMCV variant M (ref. 25). For lethality assessment, mice were monitored daily after initial infection. At different times, mice were killed and their organs were processed for histology and CVB3 detection. For histology, organs were fixed in 10% neutral buffered formalin, embedded in paraffin, and extensively sectioned at various levels. For detection of DCM, hearts were sectioned transversely at the mid-ventricular region. Sections were stained with hematoxylin and eosin and with masson blue. For detection of infectious CVB3 or EMCV, whole organs were weighed, homogenized with a polytron homogenizer and then subjected to three cycles of freezing and thawing to release the virus from the homogenates. CVB3 plaque assays were done on HeLa monolayers. EMCV titers were detected using plaque assays on BHK21 cells. CVB3 persistence was detected by RT–PCR of positive-stranded viral RNA using the primers and controls described above.

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