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The Role of Serpinb9/Serine Protease Inhibitor 6 in Preventing Granzyme B—Dependent Hepatotoxicity

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Abstract

Virally infected hepatocytes are resistant to cytotoxic lymphocyte killing by perforin-dependent and granzyme-dependent effector mechanisms. The present studies were designed to examine the role of serine protease inhibitor 6 (SPI-6) in limiting granzyme B—dependent cytotoxic effector mechanisms in the liver. SPI-6—specific small interfering RNA (siRNA) administration to C57Bl/6J (B6) mice elicited transient alanine aminotransferase (ALT) elevations that were not observed in either granzyme B—deficient B6 (B6.*gzmb*$^{-/-}$) or natural killer (NK) cell—depleted B6 mice. When SPI-6 expression was abolished by siRNA administration at the time of infection with a recombinant, replication-deficient adenovirus [E1-deleted adenovirus encoding *β*-galactosidase (AdCMV-*LacZ*)], earlier and dramatically increased, and earlier ALT elevations were observed in wild-type B6 but not in B6.*gzmb*$^{-/-}$ or NK cell—depleted mice. When a 3-fold higher dose of AdCMV-*LacZ* was administered to B6 mice, the coadministration of SPI-6 siRNA resulted in the early onset of lethal, acute liver failure. Of note, the accelerated clearance of AdCMV-*LacZ* was observed in recipients of SPI-6 siRNA.

Conclusion—These results indicate that the regulated expression of SPI-6 in hepatocytes during viral infection or following noninfectious causes of liver injury protects hepatocytes against excessively vigorous granzyme B—dependent killing but may also delay immune clearance of virally infected hepatocytes.

Natural killer (NK) and natural killer T (NKT) cells are prominent components of the resident liver lymphocyte population.[1] In addition, increased numbers of activated NK cells and cytotoxic T lymphocytes (CTLs) are present in the liver during systemic[2] and localized immune responses.[3] These cytotoxic effector cells play an essential role in the clearance of noncytopathic viruses from infected tissues.[4,5] In addition, NK and NKT cells amplify hepatocellular injury initiated by noninfectious causes of liver injury.[6] Mice with defects in perforin/granzyme effector mechanisms have been found to have delayed clearance of a number of viral infections from extrahepatic sites but exhibit no delay in the clearance of recombinant adenoviral vectors from the liver.[7] In contrast, mice with defects in the Fas ligand (FasL)/Fas or other death receptor pathways exhibit delayed clearance of adenoviral vectors from the liver but not from the lungs.[8] This appears to be related to the resistance of virally infected hepatocytes to killing by perforin-dependent and granzyme-dependent cytotoxic

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effector pathways, which results in a more prominent role for FasL and tumor necrosis factor (TNF) receptor—mediated killing mechanisms in the clearance of viral infection in the liver.[7,9,10]

Human proteinase inhibitor 9 (PI-9) and the murine orthologue, serine protease inhibitor 6 (SPI-6), are members of a family of ovalbumin serpin inhibitors[11-13] present in immune-privileged cells, antigen-presenting cells, and cytotoxic T cells that afford protection against the actions of granzyme B.[14-20] The absence of SPI-6 during viral infection results in a breakdown in the cytotoxic granule integrity and increased levels of cytoplasmic granzyme B, which result in higher levels of T cell apoptosis.[21] When expressed by tumor cell lines, PI-9[12,17,22,23] and SPI-6[12,22] confer resistance to perforin/granzyme B—mediated killing by CTL and NK cells. PI-9 and SPI-6 selectively inhibit granzyme B but do not affect Fas-mediated and TNF-mediated apoptosis.[24] Thus, one potential explanation for hepatocyte resistance to the perforin and granzyme cytotoxicity pathway is the expression of PI-9/SPI-6 in the liver.

Initial evaluations of human or mouse tissues for PI-9 or SPI-6 expression revealed high levels of expression in the spleen, the lungs, and a variety of immune-privileged sites, but they did not reveal high levels of PI-9 or SPI-6 expression in normal liver.[11,12] Subsequent studies conducted in our laboratory, however, noted the up-regulation of PI-9 and SPI-6 expression in human and mouse hepatocytes during viral infection and in mouse liver after an *in vitro* treatment with interferon alpha (IFN-α).[25] Therefore, the induction of PI-9/SPI-6 during viral hepatitis may serve as a mechanism to protect infected hepatocytes from excessively vigorous perforin/granzyme B—mediated killing by NK cells and CTLs.[25,26] To evaluate this hypothesis, the current studies were performed to assess the effects of inhibiting SPI-6 expression in hepatocytes in the presence or absence of viral infection of the liver.

Materials and Methods

Mice

C57BL/6J (B6), B6Smn.C3H-FasL$^{(gld)}$ (B6.*gld*), and FVB/NJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Granzyme B—knockout mice backcrossed on the B6 background for 20+ generations [granzyme B—deficient B6 (B6.*gzmb*$^{-/-}$)] were generously provided by Dr. Timothy Ley (Washington University). Mice used in individual experiments were age-matched and sex-matched and used before 12 weeks of age. No gender differences in the levels or regulation of SPI-6 expression were noted in these experiments. All animal studies were carried out in compliance with accepted standards of humane animal care and were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee.

Adenovirus Vector

An E1-deleted, replication-deficient, β-galactosidase—encoding recombinant adenovirus (AdCMV-*LacZ*) was propagated in 293 cultures and purified on cesium chloride gradients, and viral titers in optical particle units (OPU) were determined by A260 absorbance as described.[9] Mice were injected via the tail vein with 10^{11}-10^{12} OPU of AdCMV-*LacZ* to produce hepatic adenoviral infection as described.[27]

Cells and Culture Conditions

Mouse AML-12 cells (American Type Culture Collection, Manassas, VA) from a nontransformed, H-2K^{b},D^{q}—expressing hepatocyte line[10,28] were cultured in 25-cm^2 tissue culture flasks (Corning Inc., Corning, NY) at 37°C in a humidified, 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium and F12 medium (Invitrogen, Carlsbad, CA)

supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), 40 ng/mL dexamethasone (Sigma-Aldrich, St. Louis, MO), and insulin (0.005 mg/mL), transferrin (0.005 mg/mL), and selenium (5 ng/mL; Invitrogen). AML-12 cells were transfected with SilentFect reagent (Bio-Rad Laboratories, Hercules, CA) as described.[29]

IFN-*α* Administration

Recombinant human IFN-*α*2b (Intron A) was obtained from Schering Corp. (Kenilworth, NJ). Mice were injected intraperitoneally with 400 units/g of body weight of IFN-*α*, and selected tissues were harvested 24-48 hours after the treatment as described.[10] Previous work has indicated that the *in vivo* administration of human IFN-*α* A/D[30] or IFN-*α*2b[31] mice leads to hepatic accumulation of NK cells, whereas *in vitro* exposure to murine IFN-*α* leads to the induction of SPI-6 expression in hepatocytes.[25]

Lymphokine Activated Killer (LAK) Cell Generation

Red blood cell—depleted, single-cell splenocyte preparations, suspended in Roswell Park Memorial Institute medium containing 10% fetal bovine serum, were incubated on nylon wool columns at 37°C in a 5% CO_2 atmosphere for 1 hour. Nonadherent cells were eluted from each column and cultured with recombinant human interleukin-2 (Biological Resources Branch, National Cancer Institute, Frederick Cancer Research Development Center, Frederick, MD) in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum. Adherent cells were collected 24-48 hours after plating and used as effector cells in 4-6—hour chromium release assays.

Generation of Allospecific CTLs

In vitro activated allospecific CTLs were generated in a 5-day mixed lymphocyte culture containing 10-12 million responder spleen cells from B6 mice and an equal number of irradiated FVB spleen cells to generate anti—H-2^q—specific CTLs as described.[32]

Chromium Release Assay

Targets were labeled with 150 μCi of Na_2CrO_4 for 60-90 minutes at 37°C and were washed twice before incubation with the different effectors over a range of effector-to-target ratios in 200-μL cultures. After 4 (LAK assay) or 12 (CTL assay) hours, 100μL of the supernatant was harvested from experimental and control wells, and specific lysis was calculated with the following formula: percentage of specific lysis = {[experimental release (cpm) — spontaneous release (cpm)]/[maximal release (cpm) — spontaneous release (cpm)]} × 100. All assays were performed in triplicate, and the results are presented as the means ± the standard deviation (SD).

Alanine Aminotransferase (ALT) Assay

Serum ALT levels were assayed in a reduced nicotinamide adenine dinucleotide coupled reaction with ThermoTrace ALT reagent (ThermoElectron Corp., Melbourne, Australia). The decrease in the absorbance per minute at 340 nm in mixtures incubated at 37°C was measured, and the results were reported as international units per liter. The results are presented as the means ± SD for values from 3 mice per group.

β-Galactosidase Assay

The *β*-galactosidase activity was quantified by the measurement of the rate of cleavage of 4-methylumbelliferyl-*β*-D-galactoside to yield the fluorescent product 4-methylumbelliferone as described.[7,9]

Real-time-PCR

Real-time-PCR primer sets were designed with Primer Express software (Applied Biosystems, Foster City, CA). Real-time-PCR was performed in a final volume of 10μL containing complementary DNA from 20 ng of reverse-transcribed total RNA, 150 nM forward and reverse primers, and SybrGreen universal polymerase chain reaction (PCR) master mix (Applied Biosystems). PCR was conducted in 384-well plates with the ABI-Prism 7900HT sequence detection system (Applied Biosystems). All reactions were performed in triplicate. A melting curve analysis and dilution curve standards were performed to identify primer sets and conditions yielding specific products with 100% amplification efficiency. Primers validated by this technique and used in the present studies were Serpinb9, Serpinb9b, Serpinb9c, Serpinb9e, Serpinb9f, Serpinb9g, cyclophilin, and 18sRNA. The primer sequences are listed in Table 1. Relative levels of messenger RNA (mRNA) were calculated by the comparative cycle threshold method (User Bulletin No. 2, Applied Biosystems). Cyclophilin mRNA and/or 18sRNA levels were used as the invariant control for each sample.

Western Immunoblotting

Cells were washed, suspended in a lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.2, 10 mM KCL, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 250 mM sucrose, and protease inhibitors (Sigma-Aldrich)], lysed by repeated freezing and thawing, and centrifuged for 10 minutes at 10,000*g* to remove debris as described.[25] Protein concentrations in tissue homogenates were assayed by the bicinchoninic acid method with reagents purchased from Pierce (Pierce Biotechnologies, Rockford, IL) and with bovine serum albumin as a standard. Indicated amounts of the total protein in the cleared lysates (10, 20, or 40 μg per lane) were separated on 15% bisacrylamide gels by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose in a pH 9.9 carbonate buffer. Immunodetection was performed with primary anti—SPI-6 and secondary horseradish peroxidase—conjugated antirabbit immunoglobulin and with the ECL western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ).

Hydrodynamic Transfection

Synthetic small interfering RNAs (siRNAs) were delivered *in vivo* with a modified hydrodynamic transfection technique,[33-35] by which 2.5, 5, or 10 μg of siRNA dissolved in 1.5-mL of lactated Ringer's solution (Baxter Healthcare, Deerfield, IL) was rapidly injected into the tail vein. Control mice were injected with an equal volume of lactated Ringer's solution. In additional experiments, mice were hydrodynamically transfected simultaneously with AdCMV-*LacZ* and siRNA.

siRNA Design and Production

siRNAs were synthesized with the Silencer siRNA construction kit (Ambion, Inc., Austin, TX). The sense and antisense strands of siRNAs were as follows: *SPI-6* (sequence 511), beginning at nucleotide (nt) 511, 5′-AATGATGAGAACCAGCCTGGTCCTGTCTC-3′ (sense) and 5′-AAACCAGGCTGGTTCTCATCACCTGTCTC-3′ (antisense); *SPI-6* (sequence 1122), beginning at nt 1122, 5′-AACAGGATGCTGTTTGCTTTGCCTGTCTC-3′ (sense) and 5′-AACAAAGCAAACAGCATCCTGCCTGTCTC-3′ (antisense); *Missense*, 5′-AAAAAGTTATCAGGATGCGCACCTGTCTC-3′ (sense) and 5′-AATGCGCATCCTGATAACTTTCCTGTCTC-3′ (antisense); and *Destabilized Missense*, 5′-AACAAAATACTGTTTGCTTTGCCTGTCTC-3′ (sense) and 5′-AACAAAGCAAACAGTATTTTGCCTGTCTC-3′ (antisense).

NK Cell Depletion

Mice were treated with 15 μg of anti—asialo-GM1 antibodies (Wako Chemicals, Richmond, VA); the treatment started 1 day before transfection and on day 4 of the experiment. The control mice were administered equivalent amounts of rabbit immunoglobulin G isotype control antibody (Sigma-Aldrich, St. Louis, MO). As determined by flow cytometry using fluorochrome-labeled anti-CD3 and anti-NK1.1 antibodies as described,[7,10] the anti—asialo-GM1 treatment led to a 96% ± 1% (mean ± standard error of the mean, n = 3) reduction in NK1.1(+), CD3(-) T cells but only a 21% ± 9% reduction in NK1.1(+), CD3(+) NKT cells in the livers of mice previously treated with human recombinant IFN-α.

Histology

Formalin-fixed liver sections were embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin. Slides were graded in a blinded manner by a pathologist, and images were captured at X10, X20, and X40 magnifications.

Results

SPI-6 Expression Protects Hepatocytes from NK Cell—Mediated and CTL-Mediated Apoptosis

AML-12 hepatocytes,[28] a mouse hepatocyte cell line that constitutively expresses SPI-6, were observed to be resistant to perforin-dependent, granzyme-dependent, and TNF-mediated cytotoxicity mechanisms in previous studies.[10,25] To analyze the role of SPI-6 in protecting AML-12 hepatocytes from these NK cell and CTL effector mechanisms, several siRNA templates were designed to selectively target SPI-6 mRNA, and the specificity of each template in knocking down SPI-6 mRNA and protein expression was examined in AML-12 hepatocytes. As illustrated in Fig. 1, the SPI-6 (511) siRNA sequence was found to effectively reduce SPI-6 mRNA and protein expression by >95% *in vitro* without altering the expression of mRNA encoding highly homologous serpins, such as serpinb9b, serpinb9c, or serpinb9f (Fig. 1A). mRNA levels of serpinb9g and serpinb9e also were unchanged in AML-12 hepatocytes after transfection with SPI-6 (511) (data not shown). Another SPI-6 siRNA sequence, SPI-6 (1122), exhibited similar specificity in decreasing SPI-6 expression but not that of other serpinb9 family members, but it was somewhat less effective than SPI-6 (511) siRNA in the knockdown of SPI-6 mRNA and protein expression in AML-12 hepatocytes (data not shown). The scrambled missense siRNA used in experiments depicted in Fig. 1 elicited a minor increase in SPI-6 mRNA and protein. This may have resulted from an unknown off-target effect because, in separate experiments, a specifically destabilized mutant of the SPI-6 (511) sequence with 3 nt differences neither increased nor decreased SPI-6 mRNA (data not shown).

To assess the role of SPI-6 expression in protecting AML-12 hepatocytes from NK cell perforin/granzyme B—mediated apoptosis, splenic lymphocytes were isolated from B6.*gld* mice and cultured with interleukin-2 to stimulate LAK cell activity. Lymphocytes from B6.*gld* mice are deficient in FasL expression and thus kill target cells predominately by perforin-dependent and granzyme-dependent mechanisms. The generation of LAK cells in the effector cell population used in the experiment detailed in Fig. 2A was confirmed by the assessment of the killing of the NK-sensitive YAC-1 cell line; 52% specific cytotoxicity was obtained at a 5:1 effector-to-target ratio (data not shown). Control, missense siRNA—transfected, and SPI-6 siRNA—transfected AML-12 hepatocytes were used as LAK cell targets in 5-hour chromium release assays. In the representative experiments presented in Fig. 2A, control and missense siRNA—transfected AML-12 hepatocytes remained resistant to killing by B6.*gld* LAK cells, whereas SPI-6 siRNA—transfected AML-12 hepatocytes became susceptible to killing. The specific cytotoxicity mediated by these granzyme-expressing and

perforin-expressing but FasL-defective LAK cells increased from 8% in untransfected AML-12 cells to approximately 38% in SPI-6 siRNA—transfected AML-12 cells.

To examine the importance of SPI-6 in protecting AML-12 hepatocytes from CTL-mediated granule effector mechanisms, splenocytes from FasL-defective B6.*gld* mice were stimulated *in vitro* for 5 days with irradiated splenocytes from FVB (H-2^q) mice to generate allospecific CTLs. These H-2^q—specific CTLs were assessed for their capacity to kill control and siRNA-transfected AML-12 hepatocytes (H-$2D^q$). As illustrated by the results presented in Fig. 2B, control and missense siRNA—transfected AML-12 hepatocytes remained resistant to killing by FasL-defective B6.*gld* effector cells, whereas AML-12 hepatocytes transfected with either of 2 independent SPI-6 siRNA sequences were susceptible to granzyme-mediated and perforin-mediated CTL killing.

To assess hepatic NK cell killing of AML-12 hepatocytes that do or do not express SPI-6, activated NK cell—enriched intrahepatic lymphocytes were isolated as described[7,10] from B6.*gld* mice 24 hours after a treatment with 400 units/g of body weight of IFN-α. As detailed in a representative experiment displayed in Fig. 2C, these activated NK cell—enriched [mean of 62% NK1.1(+), CD3(-) NK cells and 15% NK1.1(+), CD3(+) NKT cells] intrahepatic effector cells mediated minimal killing of control or missense siRNA—transfected AML-12 hepatocytes but efficiently killed SPI-6 siRNA—transfected AML-12 hepatocytes.

Inhibition of SPI-6 Results in Increased Hepatocellular Injury

In additional experiments, the *in vivo* effects of SPI-6 siRNA templates on the hepatic expression of SPI-6 RNA and protein expression were assessed. A modified hydrodynamic transfection technique was used to infuse the various siRNAs[33,35] into mice. This technique delivered SPI-6 siRNA into hepatocytes at levels that decreased IFN-α—induced SPI-6 mRNA (Fig. 3A) and protein (Fig. 4A) expression by greater than 95% to levels comparable to those in livers of untreated mice (Fig. 4C, first 4 lanes) without disrupting SPI-6 expression in organs that constitutively express SPI-6, such as the spleen (Figs. 3C and 4B) and lungs (Fig. 3D). SPI-6 (511) siRNA specifically inhibited hepatic SPI-6 mRNA expression without inhibiting the expression of mRNAs encoding highly homologous serpins, such as serpinb9b, serpinb9c, and serpinb9f (Fig. 3B). In addition, serpinb9g and serpinb9e mRNA levels remained unaltered after hydrodynamic transfection with SPI-6 (511) siRNA (data not shown). An analysis of liver homogenates prepared from control and siRNA-transfected mice revealed a greater than 10-fold decrease in SPI-6 protein expression in mice transfected with SPI-6 (511) siRNA, whereas the levels of SPI-6 protein expression were unchanged or were modestly increased in diluent control and missense siRNA—transfected mice (Fig. 4A). These results indicate that the hydrodynamic transfection of SPI-6 (511) siRNA can efficiently and selectively inhibit SPI-6 mRNA and protein expression *in vivo* in the liver without affecting expression in other organs. Of note, we found in the course of these experiments that the transient hepatic injury previously described after the rapid infusion of large (1.5 mL) but not small (0.5 mL) volumes of the lactated Ringer's solution employed in the hydrodynamic transfection technique[36-38] was associated with subsequent up-regulation of SPI-6 protein expression if no SPI-6 siRNA was included in the infusion (Fig. 4C).

In order to investigate the functional effects of impaired *in vivo* hepatic SPI-6 expression, B6 mice were hydrodynamically transfected with SPI-6 (511) siRNA, and the ALT levels were measured to assess hepatic injury. As detailed in Fig. 5 and as previously observed by others,[36-38] on day 1 after transfection, there was a mild but reproducible elevation in serum ALT values in each of the transfected mice. This is likely due to passive congestion of the liver related to the acute intravascular volume overload associated with the hydrodynamic transfection technique.[37] This mild elevation in serum ALT was resolved in the control and missense siRNA—transfected mice by day 3. However, the ALT levels in SPI-6 siRNA—

transfected mice increased further, reaching a peak level of 212 IU/L on day 3, with gradual resolution over the next 5-9 days (Fig. 5A). As illustrated by the results presented in Fig. 5B, this apparently greater level of hepatic injury in SPI-6—suppressed mice was responsive to the dose of SPI-6 siRNA administered and was seen with 2 independent SPI-6—specific siRNAs; this makes it unlikely that off-target effects of the siRNAs were responsible. In order to determine if other toxic effects of the SPI-6—specific siRNA contributed to the increase in the ALT levels or whether this more pronounced ALT increase resulted from unopposed granzyme B activity in the SPI-6—suppressed mice, the serum ALT levels were examined in granzyme B—deficient mice following hydrodynamic transfection with the various siRNAs. As illustrated in Fig. 5C, the pattern of transient ALT elevation in granzyme B—deficient mice following hydrodynamic transfection with SPI-6—specific siRNA was indistinguishable from that of missense or no siRNA hydrodynamic transfection and was resolved within 3 days. Thus, granzyme B—dependent cytotoxic mechanisms appear to be responsible for the higher levels of hepatic injury following transfection with SPI-6—specific siRNA.

The role of NK cells in mediating granzyme B—dependent hepatocellular injury in SPI-6 siRNA—treated mice was examined by the use of anti—asialo-GM1 to deplete NK cells from mice prior to transfection with SPI-6 siRNA. As detailed in Fig. 5D, on day 1 after transfection, there were comparable levels of serum ALT elevation in NK cell—depleted mice transfected with missense siRNA, SPI-6 (511) siRNA, or vehicle alone, and this elevation in serum ALT was resolved in all mice by day 3.

Inhibition of SPI-6 During AdCMV-LacZ Infection Results in Elevated ALT Levels

In further studies, the role of SPI-6 in protecting hepatocytes from perforin-mediated and granzyme B—mediated cytotoxic lymphocyte effector mechanisms during adenoviral infection was investigated. In these studies, 4×10^{11} OPU of AdCMV-*LacZ* was infused in parallel with missense or SPI-6 (511) siRNA into B6 mice, and the serum ALT levels were examined on selected days after infection. As illustrated in Fig. 6A, the serum ALT levels were modestly elevated in all hydrodynamically transfected mice on day 1 after transfection but returned to baseline levels by day 3 in adenovirus-infected control and missense siRNA—transfected mice. Mice infused simultaneously with AdCMV-*LacZ* and missense siRNA or with AdCMV-*LacZ* alone exhibited moderate increases in ALT levels, which peaked on day 8. In contrast, mice transfected with SPI-6 (511) siRNA at the time of AdCMV-*LacZ* infection exhibited much more dramatic serum ALT elevations on day 3 and continued to exhibit higher serum ALT levels than mice in other experimental groups on day 8 after adenoviral infection. When SPI-6 (511) siRNA was hydrodynamically transfected into B6 mice concomitantly with the infusion of a higher inoculum of AdCMV-*LacZ* (1.2×10^{12} OPU), the early onset of lethal, acute liver failure was observed in all SPI-6 (511) siRNA—transfected mice but not in missense or no siRNA AdCMV-*LacZ*—infected mice (Fig. 6B). Liver tissue isolated from AdCMV-*LacZ*—infected mice exhibited mononuclear cell lobular inflammation with scattered apoptotic hepatocytes, as illustrated in Fig. 7B. In contrast, as illustrated in Fig. 7D, liver tissue isolated from mice infused with both SPI-6 siRNA and AdCMV-*LacZ* exhibited numerous apoptotic hepatocytes and severe hepatocellular injury with areas of submassive, nonzonal hepatocyte death in a pattern not observed in livers of missense siRNA—transfected, AdCMV-*LacZ*—infected mice (Fig. 7C).

To investigate the role of NK cells in hepatocellular injury in response to AdCMV-*LacZ* with and without concomitant suppression of SPI-6 expression, the serum ALT levels were measured in NK cell—depleted mice at various times after infusion with AdCMV-*LacZ* and SPI-6 siRNA (Fig. 8A). In contrast to mice with normal numbers of NK cells, only a slight increase in serum ALT levels was detected at day 3 in NK cell—depleted mice cotransfected with AdCMV-*LacZ* and SPI-6 (511) siRNA with respect to ALT levels in control or missense

siRNA—transfected mice. ALT levels at all other times were similar in SPI-6 siRNA—treated and control mice.

The role of granzyme B—dependent cytotoxic effector mechanisms was investigated in similar experiments using granzyme B—deficient mice. As illustrated in Fig. 8B, the modest elevations in the ALT levels of granzyme B—deficient mice after hydrodynamic transfection with 4×10^{11} OPU of AdCMV-*LacZ* were not significantly altered by cotransfection with SPI-6—specific siRNA.

Inhibition of SPI-6 Results in the Accelerated Clearance of AdCMV-LacZ from the Liver

To assess the effect of SPI-6 inhibition on the clearance of AdCMV-*LacZ* from the livers of virally infected, siRNA-transfected mice, the hepatic expression of the adenoviral transgene product, *β*-galactosidase, was examined. Similar levels of expression of *β*-galactosidase, approximately 800-1100 —fold above basal levels of endogenous hepatic *β*-galactosidase expression, were detected in control, missense siRNA—treated, and SPI-6 siRNA—treated mice 3 days after transfection, indicating an equal level of viral infection (data not shown). As illustrated in Fig. 9, 12 days after the initial AdCMV-*LacZ* infection, livers of mice transfected with SPI-6 siRNA exhibited 5-10 —fold lower levels of *β*-galactosidase expression than livers of vehicle only or missense siRNA—transfected mice, respectively, and this indicated more rapid clearance in mice lacking SPI-6 expression during the acute phase of AdCMV-*LacZ* infection.

Discussion

The results of the present studies demonstrate that the inhibition of serpinb9/SPI-6 expression *in vitro* and *in vivo* renders hepatocytes more sensitive to killing by granzyme B—expressing NK cells. These results indicate that the regulated expression of this specific inhibitor of granzyme B[11-13] in the liver accounts, at least in part, for the previously observed resistance of virally infected hepatocytes to perforin-dependent and granzyme-dependent effector mechanisms[7] and, in turn, the greater importance of Fas and related death receptor—dependent cytotoxic effector mechanisms in immune-mediated injury and the clearance of viral infections from the liver.[9,10,39] Of note, when serpinb9/SPI-6 expression was inhibited during adenoviral infection, the immune clearance of adenovirally encoded transgenes was accelerated. These results are in agreement with a large body of clinical literature indicating that the presence of a vigorous cytopathic immune response during acute viral infection is associated with a high likelihood of viral clearance and that inhibition of the cell-mediated component of the immune response significantly delays this process.[40,41] However, the risk to the host of an unregulated antiviral cytopathic immune response in the liver is illustrated by the observation during the studies reported here that a lack of SPI-6 expression can lead to fatal acute liver failure as the result of excessively rapid and extensive NK cell killing of virally infected hepatocytes.

When hepatic SPI-6 expression was knocked down *in vivo* by SPI-6—specific siRNA, enhanced hepatocellular injury mediated by granzyme B—expressing NK cells was observed not only in response to adenoviral infection but also in response to the modest level of hepatocyte injury associated with the hydrodynamic transfection technique used to deliver siRNA to hepatocytes. Previous analyses of siRNA and/or recombinant DNA delivery to hepatocytes *in vivo* have noted that the efficacy of the hydrodynamic transfection technique is closely tied to the volume and rate of infusion and associated transient disruption of the hepatocyte membrane.[36-38] NK cells kill target cells in response to a variety of stimuli that elicit the expression of stress proteins,[42] and thus, in addition to their role in the clearance of malignant or virally infected cells, NK cells play a role in killing cells injured by drugs or other agents.[6] As fully differentiated granzyme B—expressing NK cells reside in the normal liver

and are poised to promptly eliminate injured hepatocytes expressing appropriate stress protein ligands for NK cell—activating receptors, the regulation of granzyme B—mediated apoptosis by serpinb9/SPI-6 expression during the course of ischemic or toxin-induced liver injury is also likely of importance in determining the magnitude of liver injury. Of note, significant fractions of NKT cells also reside in livers of normal mice.[1] However, these effector cells were not implicated in the models of SPI-6 regulated hepatocellular injury observed in the present studies as anti-asialo-GM1 treatments effective only in the depletion of CD3(-), NK1.1(+) NK cells were sufficient to abolish enhanced hepatic injury in SPI-6 siRNA—treated mice. In part, this may be related to the fact that following adenoviral infection, NKT cells constitute only a minor fraction of the lymphocytic immune response in the liver.[7,10]

Under the experimental conditions employed in the present studies, the inhibition of SPI-6 expression during adenoviral infection in the liver induced an accelerated course of NK cell—mediated hepatocellular injury that evolved prior to the development of adaptive CD8+ T cell responses, which peak later in the course of this infection.[5,7,39] Of note, the significant recovery of SPI-6 expression is observed *in vivo* 6-7 days after SPI-6 siRNA administration, a time preceding the time of peak CTL responses in the adenoviral infection models employed in the present studies.[7,10] Thus, any separate role for serpinb9/SPI-6 expression in the protection of hepatocytes from CTL-mediated immune injury *in vivo* was not apparent in the present studies. However, hepatic SPI-6 mRNA levels remain more than 10-fold above basal levels throughout the first 3 weeks of *in vivo* adenoviral infection when granzyme B expression is also increased in the liver (unpublished observation). In addition, *in vitro* SPI-6 siRNA—treated hepatocytes were observed to become more sensitive to perforin-dependent and granzyme-dependent CTL killing. Therefore, it remains likely that the regulated expression of SPI-6 plays an equally important role in preventing excessive CTL-mediated hepatic injury. During adenoviral infection, hepatic NK cells and CTLs also express high levels of granzyme A,[7] a serine protease capable of inducing perforin-dependent apoptosis in the absence of granzyme B.[4] In previous studies,[25] we noted that human and murine hepatocytes appear to constitutively express proteinase inhibitor 8, a putative granzyme A inhibitor.[12] Thus, the hepatic expression of this serpin and/or additional cytoprotective factors also may contribute to the resistance of virally infected hepatocytes to the multiple effector molecules that are components of the granule exocytosis pathway of cell-mediated immune injury.

The results of the present study demonstrate that the serpinb9-mediated regulation of the cytotoxic lymphocyte function in the liver plays a central role both in the determination of the rate of immune clearance of viral pathogens from the liver and in protection from immune-mediated hepatocellular injury. These findings suggest that the severity of illness and the potential for development of fatal acute liver failure both during acute viral hepatitis and following noninfectious forms of injury capable of triggering NK cell responses are likely to be related not only to the vigor of the host cytotoxic lymphocyte response but also to the efficiency with which hepatocytes up-regulate the expression of cytoprotective molecules such as murine SPI-6 and human PI-9.

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Abbreviations

AdCMV-LacZ, E1-deleted adenovirus encoding β-galactosidase
ALT, alanine aminotransferase

B6, C57Bl/6J
B6.gld, B6.Smm.C3H-FasL$^{(gld)}$
B6.gzmb$^{-/-}$, granzyme B—deficient B6
CTL, cytotoxic T lymphocyte
FasL, Fas ligand
IFN-α, interferon alpha
LAK, lymphokine activated killer
mRNA, messenger RNA
NK, natural killer
NKT, natural killer T
nt, nucleotide
OPU, optical particle unit
PCR, polymerase chain reaction
PI-9, proteinase inhibitor 9
SD, standard deviation
SDS-PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis
siRNA, small interfering RNA
SPI-6, serine protease inhibitor 6
TNF, tumor necrosis factor

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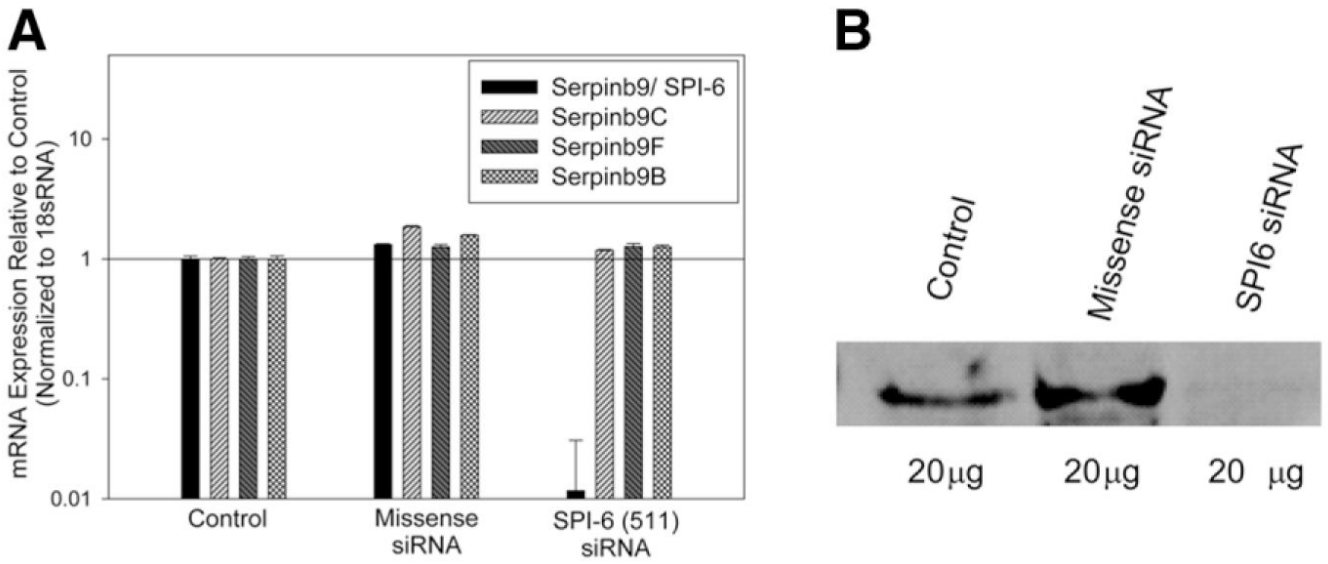

Fig. 1.

Evaluation of SPI-6 mRNA and protein expression after the transfection of AML-12 hepatocytes with SPI-6 (511) siRNA. (A) Serpin mRNA levels were measured in control, missense siRNA—transfected, and SPI-6 siRNA—transfected AML-12 hepatocytes with real-time PCR. Each bar represents the mean ± SD of triplicate determinations of mRNA expression with respect to control AML-12 hepatocytes and normalized to 18S RNA. (B) SPI-6 protein expression was measured in lysates prepared from transfected AML-12 hepatocytes. Protein levels were assayed, and 20 μg of protein was loaded per lane prior to the SDS-PAGE separation of proteins and western blotting with SPI-6—specific antibodies. The data presented in parts A and B are representative of 4 experiments with similar results.

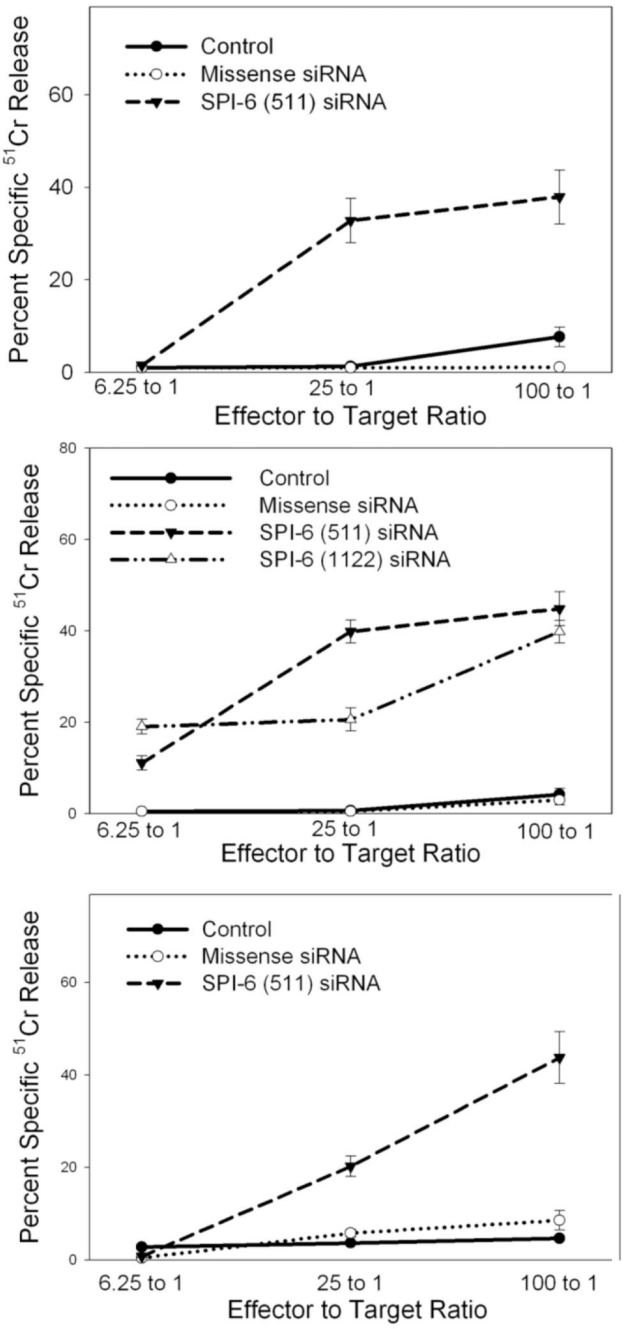

Fig. 2.

LAK, CTL, and intrahepatic NK cell killing of SPI-6 siRNA—transfected AML-12 hepatocytes. (A) AML-12 hepatocytes previously transfected for 48 hours with SPI-6—specific siRNA, missense siRNA, or the transfection reagent alone were used as targets in 5-hour chromium release assays of killing by FasL-defective (*gld*) LAK cells. (B) Anti—H-2^{q}—specific, FasL-defective (*gld*) CTLs were generated in a 5-day mixed lymphocyte culture and assessed in a 12-hour chromium release assay for the killing of AML-12 hepatocytes previously transfected with SPI-6—specific or missense siRNAs. (C) AML-12 hepatocytes previously transfected for 48 hours with SPI-6—specific siRNA, missense siRNA, or the transfection reagent alone were used as targets in 5-hour chromium release assays of killing by IHL isolated

48 hours after the IFN-α treatment of FasL-defective (*gld*) mice. The data shown are representative of 3 experiments with similar results.

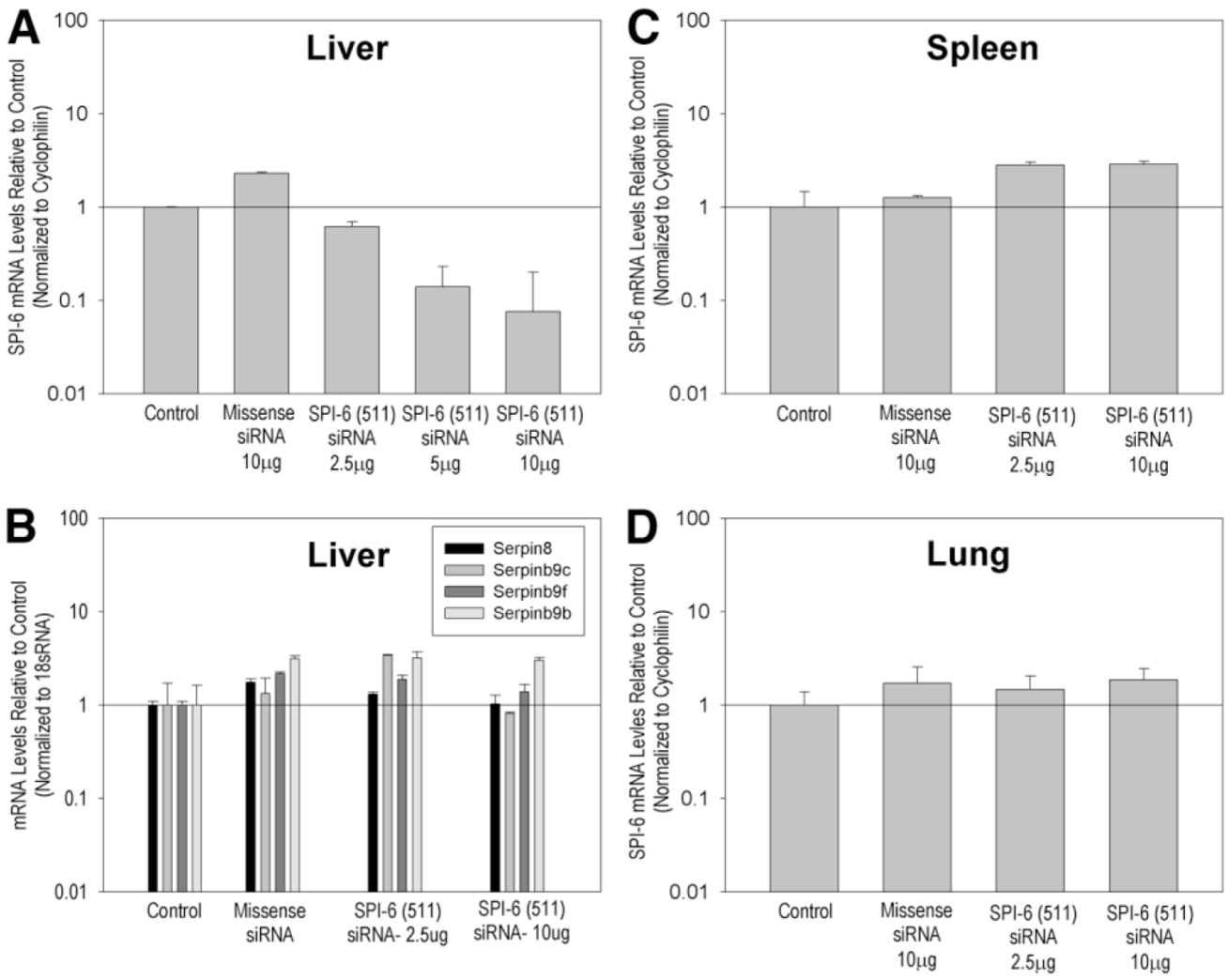

Fig. 3.

Evaluation of SPI-6 (511) siRNA specificity *in vivo*. RNA was isolated from the livers of hydrodynamically transfected B6 mice stimulated with IFN-α 24 hours after transfection. Gene expression was evaluated by real-time PCR. Equal quantities of RNA were reverse-transcribed and assessed for mRNA levels of (A) SPI-6 or (B) homologous serpins by real-time PCR, as described in the Materials and Methods section. The SPI-6 mRNA levels in (C) spleens and (D) lungs of siRNA-transfected mice also were determined. The presented values are the mean SDs from triplicate determinations in a single experiment and are representative of 3 independent experiments with similar results.

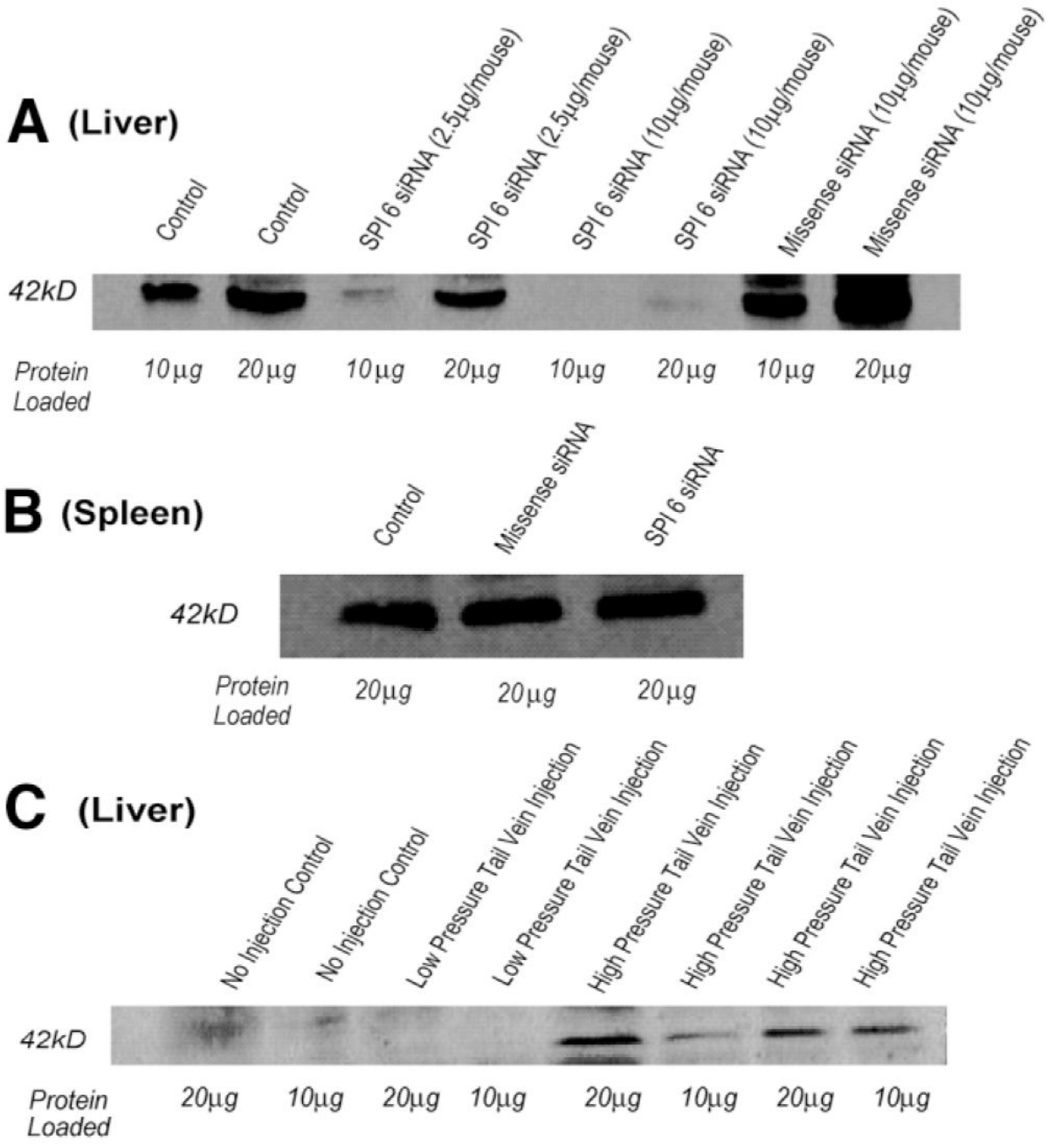

Fig. 4.

Evaluation of SPI-6 (511) siRNA efficacy *in vivo*. Lysates from (A,C) livers and (B) spleens isolated from (A,B) hydrodynamically transfected, IFN-*α*—stimulated mice or (C) mice infused with high (1.5 mL; results from 2 are mice shown) or low (0.5 mL) volumes of lactated Ringer's solution were prepared as detailed in the Materials and Methods section. The protein levels were assayed, and, as indicated, 10 or 20 *µ*g of protein was loaded per lane before the SDS-PAGE separation of proteins and immunoblotting with SPI-6—specific antibodies. The data represent the results of 1 of several experiments with similar results.

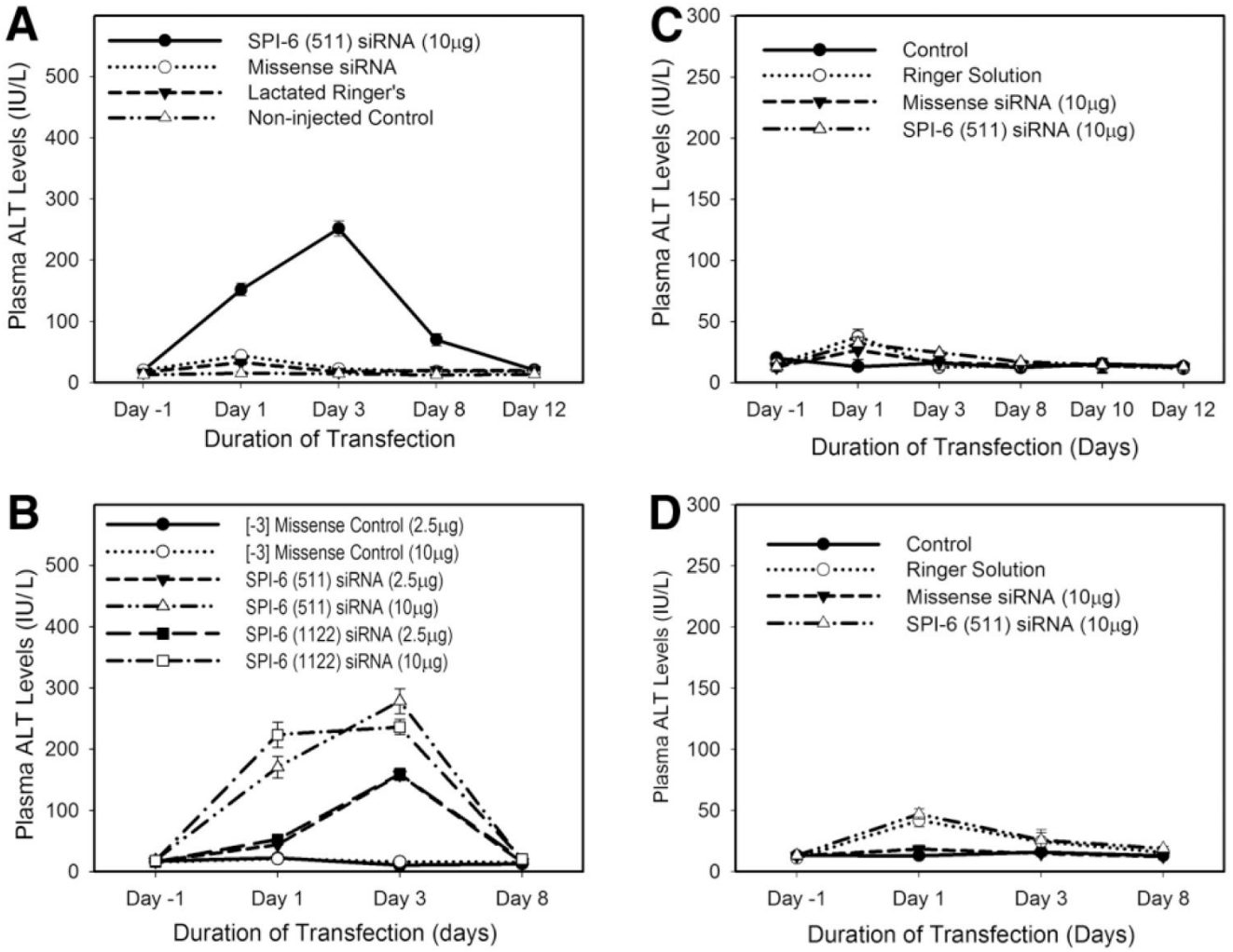

Fig. 5.

ALT levels in siRNA-transfected mice. Plasma was isolated on select days after hydrodynamic transfection from (A,B) B6 mice, (C) B6.*gzmb*$^{-/-}$ mice, and (D) mice previously treated with anti—asialo-GM1 on day -1 and day 4, and the ALT levels were determined as described in the Materials and Methods section.

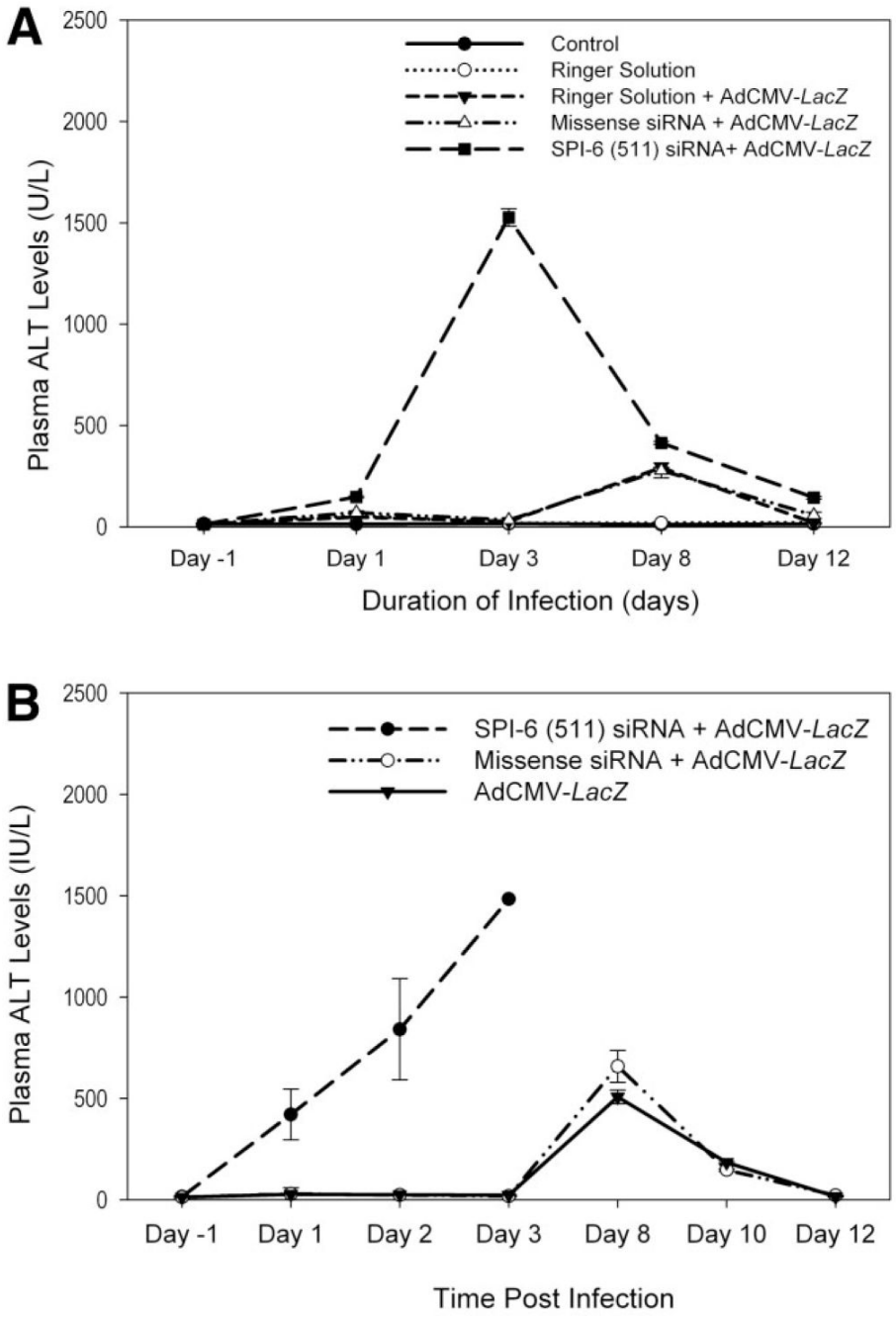

Fig. 6.

ALT levels in control, missense siRNA—transfected, and SPI-6 siRNA—transfected mice following AdCMV-*LacZ* infection. Plasma was isolated from B6 mice infected with (A) 4×10^{11} OPU of AdCMV-*LacZ* or (B) 1.2×10^{12} OPU of AdCMV-*LacZ* at select times after hydrodynamic transfection with vehicle, missense, or SPI-6 (511) siRNA, and the ALT levels were evaluated. The data for each treatment group are representative of at least 3 independent experiments with similar results.

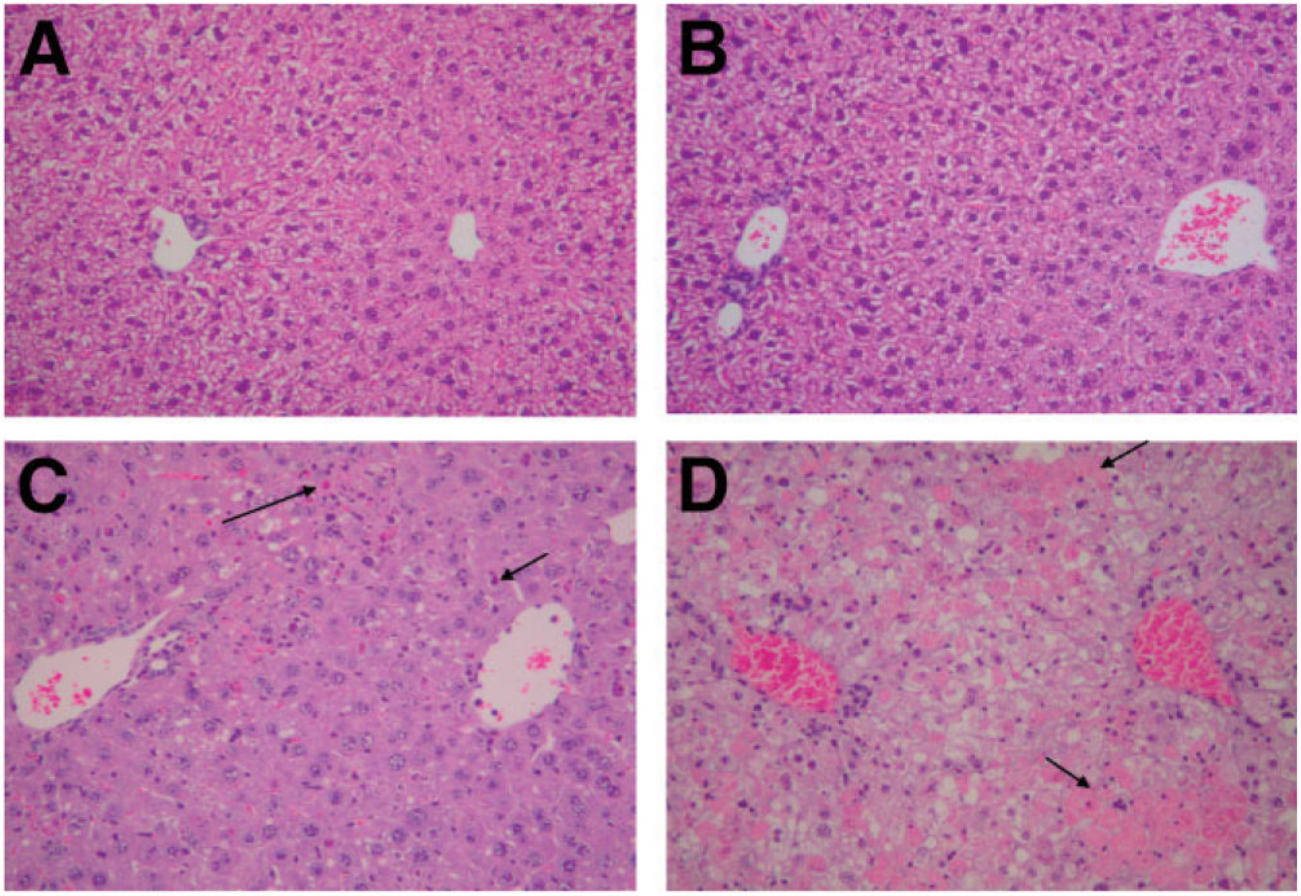

Fig. 7.

Effect of SPI-6 depletion during hepatic viral infection. Formalin-fixed liver sections were embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin. The photomicrographs are of livers obtained on day 3 from (A) control, (B) missense siRNA—treated and AdCMV-*LacZ*—infected, (C) SPI-6 siRNA—treated and AdCMV-*LacZ*—infected (day 2), and (D) SPI-6 siRNA—treated and AdCMV-*LacZ*—infected (day 3) mice.

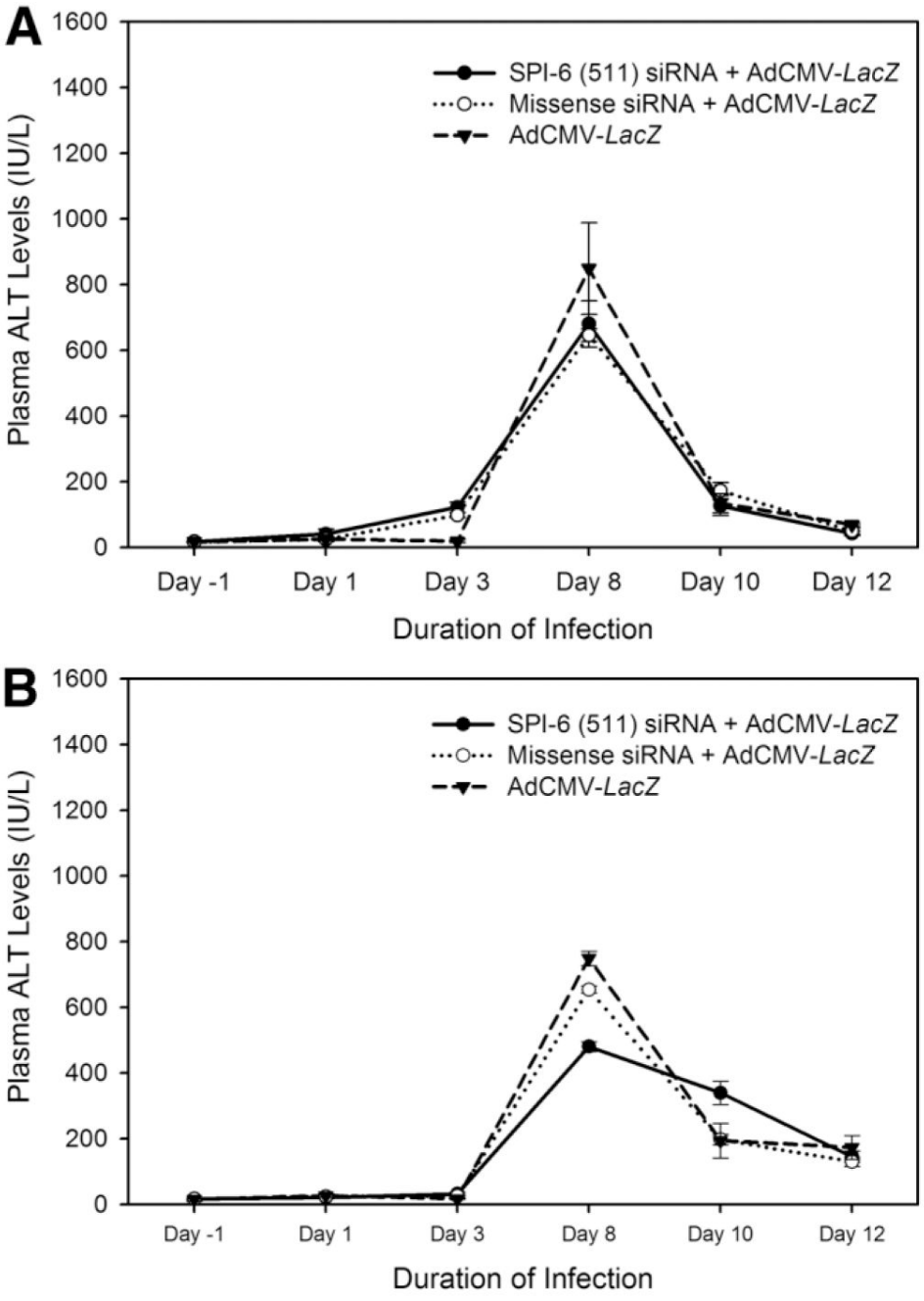

Fig. 8.

ALT levels in NK cell—depleted mice. (A) B6 mice were treated with anti—asialo-GM1 24 hours prior to transfection, or (B) B6.*gzmb*$^{-/-}$ mice were infused with vehicle, missense, or SPI-6 (511) siRNA and infected with 4×10^{11} OPU of AdCMV-*LacZ*. The plasma ALT levels were measured at selected times during infection.

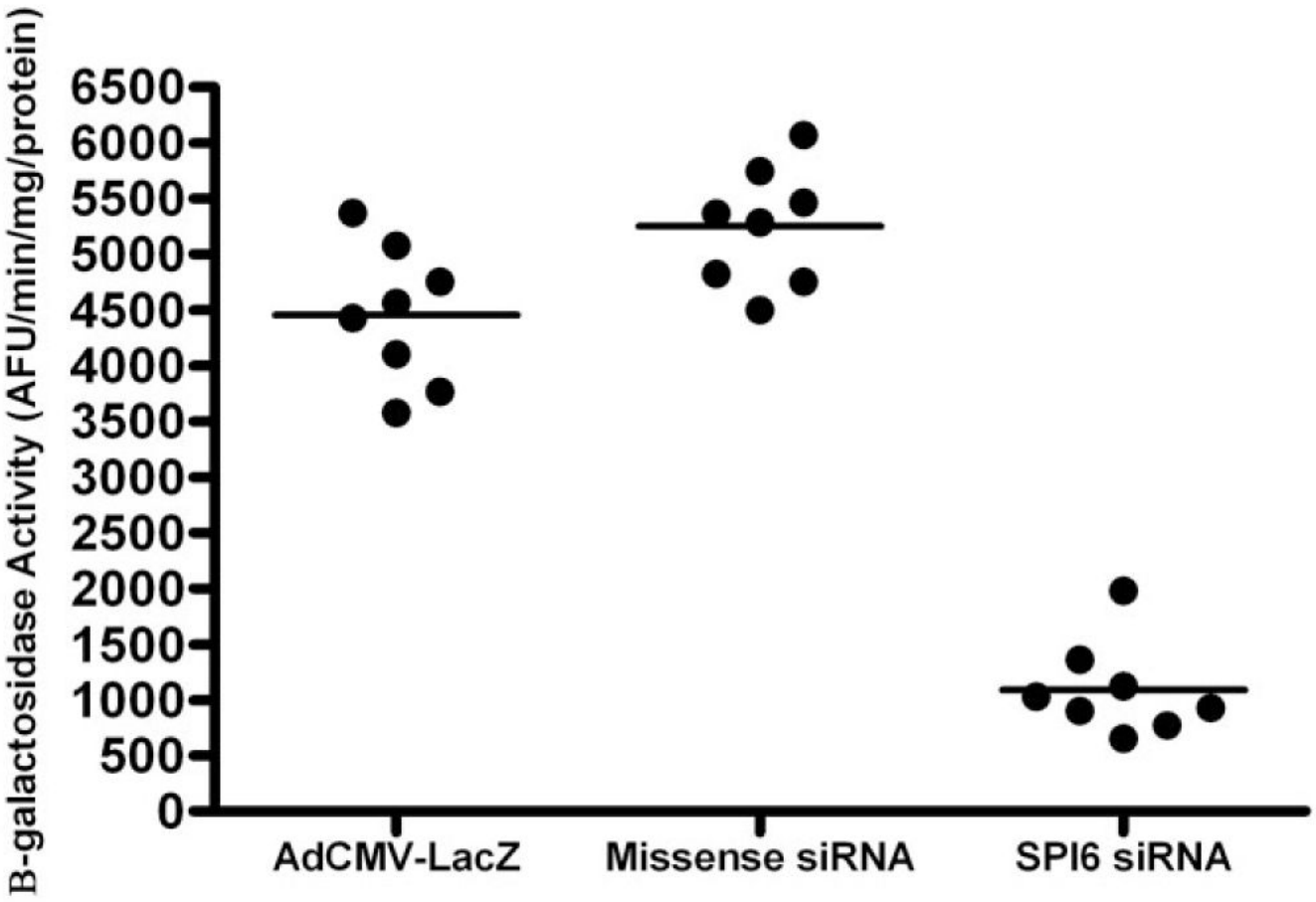

Fig. 9.

β-Galactosidase levels in SPI-6 siRNA mice following AdCMV-*LacZ* infection. The *β*-galactosidase levels were assayed in livers isolated 12 days after hydrodynamic transfection from B6 mice infused with AdCMV-*LacZ* alone, B6 mice infused with AdCMV-*LacZ* plus missense siRNA, or B6 mice infused with AdCMV-*LacZ* and treated with SPI-6 (511) siRNA.

Table 1

RT-PCR Primers

Gene	Forward Primer	Reverse Primer
Cyclophilin	5′-gcccgtagtgcttcagctt-3′	5′-ggagatggcacaggaggaa-3′
18sRNA	5′-gcctcagttccgaaaacca-3′	5′-accgcagctaggaataatgga-3′
Serpinb9	5′-tatctcaggcacttggtttgaataaa-3′	5′-tttctgtctggcttgttcagctt-3′
Serpinb9b	5′-caagcccacatccctttgaa-3′	5′-tgttggcagatgactcagttaatttc-3′
Serpinb9c	5′-ttgggaggaattcacaagtgttc-3′	5′-gaagccaaccagaaagtacacctt-3′
Serpinb9e	5′-acagcatttgggaatcttgg-3′	5′-gcaacagcctctgttccttc-3′
Serpinb9f	5′-acaacccttcaaaaatgtgtgttat-3′	5′-gtctcccttagcacccaagaga-3′
Serpinb9g	5′-gaccagtgcagatgatgttgg-3′	5′-cactagcacttgtgcctgga-3′