SUPPORTING INFORMATION

MISPAIRING C57BL/6 SUBSTRAINS OF GENETICALLY ENGINEERED MICE AND WILD-TYPE CONTROLS CAN LEAD TO CONFOUNDING RESULTS AS IT DID IN STUDIES OF JNK2 IN ACETAMINOPHEN AND CONCANAVALIN A LIVER INJURY

Mohammed Bourdi, John S. Davies, and Lance R. Pohl

Molecular and Cellular Toxicology Section, Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892-1760, USA.

Corresponding Author

Mohammed Bourdi: bourdim@nhlbi.nih.gov;, NIH, Building 10, Room 8N 110, Bethesda MD, 20892-1760. Telephone, 301-451-2599; Fax, 301-480-4852

Materials and Methods

Mice. Male C57BL/6J, C57BL/6NJ, and JNK2$^{-/-}$ mice on a C57BL/6 background all 7-8 weeks old were purchased from JAX (Bar Harbor, ME). Experiments were conducted with the approval of the National Heart Lung and Blood Institute Animal Use and Care Committee, and all animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the NIH (NIH publication 86-23 [revised 1985]).

Mice Treatment. Prior to treatment with acetaminophen (APAP, Sigma, St. Louis, MO), mice were fasted overnight (15-16 hours) as described previously to deplete hepatic glutathione levels (*1*). The following morning, food was restored after treatment of mice with APAP (300 mg/kg intraperitoneally in 20 ml/kg of warm saline). Other non-fasted mice were administered concanavalin A (Sigma, 10 mg/kg intravenously in 10 ml/kg of saline). At times thereafter, blood was collected by retro-orbital puncture and the livers were removed. Blood samples were allowed to clot in microtainer serum separator tubes (Becton Dickinson and Co., Franklin Lakes, NJ) for approximately 2 hours at room temperature and then overnight at 4^{o}C. Aliquots of sera were separated and stored at 4^{o}C. A portion of each liver was fixed in buffered formalin (Fischer Scientific, Fair Lawn, NJ) and embedded in paraffin. Sections were mounted onto glass slides, and stained with hematoxylin and eosin (American Histolabs, Gaithersburg, MD).

Assessment of liver injury. Liver injury was assessed by measuring serum alanine aminotransferase (ALT) activity with the use of a kit from Teco Diagnostics (Anaheim, CA) and by examining liver sections for histopathological changes by light microscopy.

Nicotinamide nucleotide transhydrogenase (*Nnt*) allele genotyping. Based on the finding that C57BL/6J mice can be differentiated from other C57BL/6 substrains by the presence of the mutant allele of the *Nnt* gene (*2*), the following DNA samples derived from GEM and WT mice from JAX were genotyped for WT and mutant *Nnt* alleles by Charles River Genetic Testing Services (Troy, NY) following a published method (*3*): 80 stock samples of stored DNA from GEM and tail DNA from C57BL/6J and C57BL/6NJ WT mice.

Statistical analyses. Statistical analyze was performed using one-way analysis of variance (ANOVA) with Newman-Keuls Multiple Comparison Post Test. Differences were considered significant when $P < 0.05$.

Table S1. Genetically engineered C57BL/6 mice found carrying a *Nnt*$^{-/-}$ genotype after PCR analysis using DNA stock samples from The Jackson Laboratory

JAX Stock #	Strain	*Nnt* Genotype
001913	*Pkrdc* $^{-/-}$	-/-
002087	*B2m* $^{-/-}$	-/-
002099	*Fos* $^{+/-}$	-/-
002127	*Icam1* $^{-/-}$	-/-
002128	*Itgb2* $^{-/-}$	-/-
002253	*Il4* $^{-/-}$	-/-
002289	*Selp* $^{-/-}$	-/-
002609	*Nos2* $^{-/-}$	-/-
002629	*hSOD1 Tg*	-/-
002650	*Il6* $^{-/-}$	-/-

002665	*Cd8a* $^{-/-}$	-/-
002666	*Cd28* $^{-/-}$	-/-
002682	*Agtr1a* $^{+/-}$	-/-
002684	*Nos3* $^{-/-}$	-/-
002687	*Ccl3* $^{-/-}$	-/-
002692	*Il12a* $^{-/-}$	-/-
002693	*Il12b* $^{-/-}$	-/-
002767	*Nfe2* $^{+/-}$	-/-
002778	*Alox15* $^{-/-}$	-/-
002781	*Cdkn1b* $^{-/-}$	-/-
002817	*Lck* $^{-/-}$	-/-
002867	*Icam1* $^{-/-}$	-/-
002928	*Cd40* $^{-/-}$	-/-
002952	*Il2ra* $^{-/-}$	-/-
002986	*Nos1* $^{+/-}$	-/-
002994	*Bax* $^{+/-}$	-/-
003173	*Cd47* $^{-/-}$	-/-
003175	*Il5* $^{-/-}$	-/-
003232	*Chrna7* $^{-/-}$	-/-
003288	*Ifngr1* $^{-/-}$	-/-
003608	*Cd86* $^{-/-}$	-/-
003643	*C4b* $^{+/-}$	-/-
003726	*Cd14* $^{-/-}$	-/-
004130	*Il18* $^{+/-}$	-/-
004201	*Selplg* $^{-/-}$	-/-
004319	*Mapk8* $^{-/-}$	-/-
004341	*Cxcr4* $^{+/-}$	-/-
004434	*Ccl2* $^{-/-}$	-/-
004525	*Bcl2l11* $^{-/-}$	-/-
004584	*Pparg* $^{-/-}$	-/-
004657	*Icosl* $^{-/-}$	-/-
004745	*Esr2* $^{-/-}$	-/-
004855	*Mmp12* $^{-/-}$	-/-
004912	*Akt1* $^{+/-}$	-/-
004936	*Spp1* $^{-/-}$	-/-
004999	*Ccr2* $^{-/-}$	-/-
005037	*Ticam1* $^{-/-}$	-/-
005090	*Ccl5* $^{-/-}$	-/-
005248	*Igfbp1* $^{-/-}$	-/-
005427	*Ccr5* $^{+/-}$	-/-
005530	*Ddit3* $^{-/-}$	-/-
005940	*Csf2rb* $^{-/-}$	-/-
006098	Il2/NFAT-*luc* Tg/0	-/-

Figure Legends

Figure S1**.** Genotyping results of genetically engineered mice shown in Table 1 containing the wild-type allele of *Nnt* (*Nnt*$^{+/+}$ and *Nnt*$^{+/-}$). DNA samples from C57BL/6J and C57BL/6NJ mice were used as positive controls for mice having *Nnt*$^{-/-}$ and *Nnt*$^{+/+}$ genetic markers, respectively.

Figure S2. Role of c-Jun N-terminal kinase 2 in liver injury caused by concanavalin A was dependent on the substrain of C57BL/6 wild-type mice paired with c-Jun N-terminal kinase 2 deficient mice. Mice were treated with concanavalin A (ConA, 10 mg/kg intravenously). Liver injury was assessed 24 hours after treatment by measurement of serum alanine aminotransferase (ALT) activity and by histopathologic examination of H&E stained liver sections from formalin-fixed livers; magnification 200x. (A) Serum ALT activities of mice treated with ConA represent the means ± SEM where $*P < 0.05$ when 6NJ (n=5) mice were compared to JNK2$^{-/-}$ (n=4), and 6J (n=5) mice. (D) Representative photomicrographs of liver sections of mice treated with ConA showed the severity of hepatic necrosis followed the order of 6NJ > JNK2$^{-/-}$ = 6J mice.

Figure S1

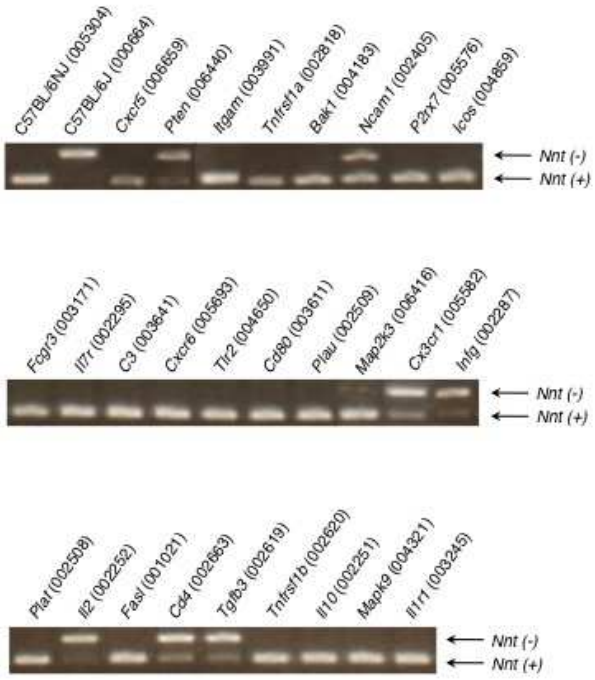
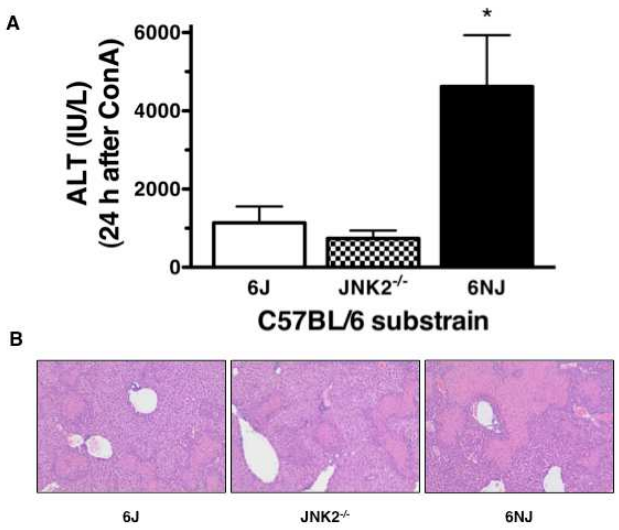

C57BL/6NJ (005304)
C57BL/6J (000664)
Cxcl5 (006559)
Ptprn (006440)
Itgam (003991)
Tnfrsf1a (002818)
Bak1 (004183)
Ncam1 (002405)
P2rx7 (005576)
Icos (004859)
Nnt (-)
Nnt (+)
Fcgr3 (003171)
Il7r (002295)
C3 (003641)
Cxcr6 (005693)
Tlr2 (004650)
Cd80 (003611)
Plau (002509)
Map2k3 (006416)
Cx3cr1 (005582)
Ifng (002287)
Nnt (-)
Nnt (+)
Pla1 (002508)
Il2 (002252)
Fasl (001021)
Cd4 (002663)
Tgfb3 (002619)
Tnfrsf1b (002620)
Il10 (002251)
Mapk9 (004321)
Il1r1 (003245)
Nnt (-)
Nnt (+)

Figure S2



Conflicting Findings on the Role of JNK2 in Concanavalin A-Induced Liver Injury. Concanavalin A-induced liver injury (CAILI) is an extensively studied T cell-mediated model of liver injury. In one study, researchers found that JNK2$^{-/-}$ mice were less susceptible than WT mice to CAILI and concluded that JNK2 had a hepatotoxic role in CAILI (*4*), while another group reported that JNK2$^{-/-}$ and WT mice did not differ in susceptibility to CAILI and therefore did not have a pathologic role in this model (*5*). The major difference in these studies is that the mice used in the latter study were from JAX, while the former group used the same JNK2$^{-/-}$ mice and matched C57BL/6 WT controls that were discussed earlier in studies of JNK2 in AILI (*6*). We have confirmed that JNK2$^{-/-}$ and C57BL/6J WT mice from JAX do not differ in susceptibility to CAILI and have found when the study was repeated with C57BL/6NJ WT mice, JNK2$^{-/-}$ mice were now less susceptible than WT mice to CAILI as determined biochemically by measurement of serum ALT activity and histopathological extent of perivenous necrosis (Fig. S2A and B, respectively).

Additional Mispairings of C57BL/6 Substrains of GEM and WT Controls. Several other examples of C57BL/6 subtrain that could have been mispaired with JNK2$^{-/-}$ mice from JAX have been published (*7-13*). Additionally, *Ccr2*$^{-/-}$, *Ccr5*$^{-/-}$ and *Ncf1*$^{-/-}$ mice from Taconic Farms on a C57BL/6NTac (*Nnt*$^{+/+}$) background have been studied with C57BL/6J mice from JAX as controls (*14, 15*), while *Ggta1*$^{-/-}$ mice backcrossed to C57BL/6J were used with C57BL/6JBomTac controls from Taconic Farms (*16*), which are *Nnt*$^{+/+}$ and do not carry the *Nnt* mutation (http://www.taconic.com/user-assets/Documents/Nnt.pdf), raising the question as to whether these and other similar mismatches of C57BL/6 substrains have produced errors in the literature.

REFERENCES

(1) Bourdi, M., Korrapati, M. C., Chakraborty, M., Yee, S. B., and Pohl, L. R. (2008) Protective role of c-Jun N-terminal kinase 2 in acetaminophen-induced liver injury. *Biochem.Biophys.Res.Commun.*, *374*, 6-10.
(2) Mekada, K., Abe, K., Murakami, A., Nakamura, S., Nakata, H., Moriwaki, K., Obata, Y., and Yoshiki, A. (2009) Genetic differences among C57BL/6 substrains. *Exp.Anim*, *58*, 141-149.
(3) Huang, T. T., Naeemuddin, M., Elchuri, S., Yamaguchi, M., Kozy, H. M., Carlson, E. J., and Epstein, C. J. (2006) Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. *Hum.Mol.Genet.*, *15*, 1187-1194.
(4) Maeda, S., Chang, L., Li, Z. W., Luo, J. L., Leffert, H., and Karin, M. (2003) IKKbeta is required for prevention of apoptosis mediated by cell-bound but not by circulating TNFalpha. *Immunity.*, *19*, 725-737.
(5) Ni, H. M., Chen, X., Ding, W. X., Schuchmann, M., and Yin, X. M. (2008) Differential roles of JNK in ConA/GalN and ConA-induced liver injury in mice. *Am.J.Pathol.*, *173*, 962-972.
(6) Nakagawa, H., Maeda, S., Hikiba, Y., Ohmae, T., Shibata, W., Yanai, A., Sakamoto, K., Ogura, K., Noguchi, T., Karin, M., Ichijo, H., and Omata, M. (2008) Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. *Gastroenterology*, *135*, 1311-1321.
(7) Nagelin, M. H., Srinivasan, S., Nadler, J. L., and Hedrick, C. C. (2009) Murine 12/15-lipoxygenase regulates ATP-binding cassette transporter G1 protein degradation through p38- and JNK2-dependent pathways. *J.Biol.Chem.*, *284*, 31303-31314.
(8) Osto, E., Matter, C. M., Kouroedov, A., Malinski, T., Bachschmid, M., Camici, G. G., Kilic, U., Stallmach, T., Boren, J., Iliceto, S., Luscher, T. F., and Cosentino, F. (2008) c-Jun N-terminal kinase 2 deficiency protects against hypercholesterolemia-induced endothelial dysfunction and oxidative stress. *Circulation*, *118*, 2073-2080.

(9) Ma F.Y., Flanc R.S., Tesch G.H., Han Y., Atkins R.C., Bennett B.L., Friedman G.C., Fan J.H., Nikolic-Paterson D.J. (2007) A pathogenic role for c-Jun amino-terminal kinase signaling in renal fibrosis and tubular cell apoptosis. *J Am Soc Nephrol*., 18,472-484.
(10) Barnes MJ, Krebs P, Harris N, Eidenschenk C, Gonzalez-Quintial R, Arnold CN, Crozat K, Sovath S, Moresco EM, Theofilopoulos AN, Beutler B, Hoebe K. (2009) Commitment to the regulatory T cell lineage requires CARMA1 in the thymus but not in the periphery. *PLoS Biol*., 7, e51.
(11) Theruvath TP, Snoddy MC, Zhong Z, Lemasters JJ. (2008) Mitochondrial permeability transition in liver ischemia and reperfusion: role of c-Jun N-terminal kinase 2. *Transplantation*. 85, 1500-1504.
(12) Saito, C., Lemasters, J. J., and Jaeschke, H. (2010) c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol. Appl. Pharmacol., 246*, 8-17.
(13) Zhu J, Krishnegowda G, Gowda DC. (2005) Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: the requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-kappaB pathways for the expression of proinflammatory cytokines and nitric oxide. *J Biol Chem*., 280, 8617-8627.
(14) Chantakru, S., Miller, C., Roach, L. E., Kuziel, W. A., Maeda, N., Wang, W. C., Evans, S. S., and Croy, B. A. (2002) Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J.Immunol*., *168*, 22-28.
(15) Miriyala, S., Gongora Nieto, M. C., Mingone, C., Smith, D., Dikalov, S., Harrison, D. G., and Jo, H. (2006) Bone morphogenic protein-4 induces hypertension in mice: role of noggin, vascular NADPH oxidases, and impaired vasorelaxation. *Circulation*, *113*, 2818-2825.
(16) Dahl, K., Buschard, K., Gram, D. X., d'Apice, A. J., and Hansen, A. K. (2006) Glucose intolerance in a xenotransplantation model: studies in alpha-gal knockout mice. *APMIS*, *114*, 805-811.