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1   **Inhibiting membrane rupture with NINJ1 antibodies limits tissue injury**

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37

38 **Plasma membrane rupture (PMR) in dying cells undergoing pyroptosis or apoptosis requires**

39 **the cell-surface protein NINJ1[1]. PMR releases proinflammatory cytoplasmic molecules,**

40 **collectively called damage-associated molecular patterns (DAMPs), that activate immune**

41 **cells. Therefore, inhibiting NINJ1 and PMR may limit the inflammation that is associated**

42 **with excessive cell death. Here we describe an anti-NINJ1 monoclonal antibody, specifically**

43 **targeting murine NINJ1, that blocks oligomerization of NINJ1 and prevents PMR. By**

44 **electron microscopy, this antibody prevented NINJ1 from forming oligomeric filaments. In**

45 **mice, inhibition of NINJ1 or *Ninj1* deficiency ameliorated hepatocellular PMR induced with**

46 **TNF plus D-Galactosamine, concanavalin A, Jo2 anti-Fas agonist antibody, or ischemia-**

47 **reperfusion injury (IRI). Accordingly, serum levels of lactate dehydrogenase (LDH), liver**

48 **enzymes alanine aminotransaminase (ALT) and aspartate aminotransferase (AST), and**

49 **DAMPs interleukin 18 (IL-18) and HMGB1 were reduced. Moreover, in the liver IRI model,**

50 **there was an attendant reduction in neutrophil infiltration. These data indicate that NINJ1**

51 **mediates PMR and inflammation in diseases driven by aberrant hepatocellular death.**

52

53 NINJ1 is a 16-kDa cell-surface protein predicted to have two transmembrane regions and N_{out}/C_{out}

54 topology[2,3]. Although dispensable for cell death induction, NINJ1 controls an important

55 consequence of apoptotic or pyroptotic cell death, mediating PMR that non-selectively releases

56 proinflammatory cytoplasmic contents from dying cells[1,4]. Whether NINJ1-dependent PMR

57 exacerbates tissue damage in disease is unclear, but *Ninj1* deficiency is reported to attenuate mouse

58 models of pulmonary fibrosis and multiple sclerosis[5,6]. Given that a conserved extracellular region

59 of NINJ1 is essential for its oligomerization and PMR[1], we hypothesized that extracellular anti-

60 NINJ1 antibodies might be used to inhibit NINJ1-dependent PMR in vivo.

61

62 **Identifying NINJ1 blocking antibodies**

63 To generate NINJ1 blocking antibodies, *Ninj1*$^{-/-}$ mice were immunized with extracellular vesicles

64 (EVs) expressing full-length mouse NINJ1 (Fig. 1a). We isolated approximately 15,000 NINJ1-

65 binding IgM$^-$ B cells by flow cytometric analysis and characterized single cell supernatants for

66 binding to NINJ1-expressing cells. Functional screening of 217 recombinant anti-mouse NINJ1

67 IgG2a monoclonal antibodies identified clone D1 (Ninj1-575) as an inhibitor of NINJ1-dependent

68 PMR (Fig. 1b). Mouse bone marrow-derived macrophages (BMDMs) were primed with the Toll-

69 like receptor 2 (TLR2) agonist Pam3CSK4 to up-regulate inflammasome components, including

70 *Nlrp3*, and then cultured with 1 µg/mL anti-NINJ1 antibodies for 15 min prior to stimulation with

71 nigericin to activate NLRP3- and GSDMD-dependent pyroptosis[7,8]. LDH release was used to

72 monitor NINJ1-dependent PMR[1]. The most potent anti-NINJ1 antagonist antibody, clone D1,

73 reduced PMR in wild-type BMDMs to levels observed in *Ninj1*$^{-/-}$ control BMDMs. The antigen-

74 binding fragment (Fab) of clone D1 also prevented nigericin-induced PMR in wild-type BMDMs,

75 suggesting that clone D1 can inhibit NINJ1 independently of binding to Fc receptors on the cell

76 surface (Extended Data Fig. 1a). Clone D1 or its Fab also suppressed membrane damage caused

77 by ectopic expression of mouse NINJ1, but not human NINJ1 in HEK293T cells (Fig. 1c and

78 Extended Data Fig. 1b).

79

80 We confirmed that clone D1 recognized mouse NINJ1, but not human NINJ1 by surface staining

81 and flow cytometric analysis of live HEK293T cells expressing N-terminally FLAG-tagged mouse

82 NINJ1 (Fig. 1d), or FLAG-tagged human NINJ1 (Extended Data Fig. 1c). Antibody binding to

83 mouse NINJ1 was abrogated by deletion of extracellular C-terminal residues 142-152 or by

84   substitution of residues 147-151 with alanines ($D_{147}VAPR \rightarrow A_{147}AAAA$). By contrast, deletion

85   of extracellular N-terminal residues 2-73 did not prevent the binding of clone D1. These data

86   indicate that clone D1 recognizes a C-terminal epitope in mouse NINJ1 (Extended Data Fig. 1d).

87   As a control, we used clone 25 anti-mouse NINJ1 antibody, which recognizes N-terminal residues

88   22-31[1]. As expected, clone 25 immunolabeling of NINJ1-expressing cells was abrogated by

89   deletion of residues 2-73 of NINJ1 (Fig. 1d).

90

91   To extend our analyses in BMDMs, we monitored NINJ1-dependent PMR by time-lapse live-cell

92   imaging. Pam3CSK4-primed BMDMs were loaded with fluorescein isothiocyanate (FITC)-

93   conjugated 150 kDa dextran (DD-150) and dye release after nigericin stimulation was used as

94   indicator of PMR (Fig. 2a). Clone D1 reduced PMR in BMDMs in a dose-dependent manner

95   compared to an isotype control antibody. The clone D1 Fab also inhibited the release of DD-150

96   from nigericin-treated BMDMs (Extended Data Fig. 1e). Clone 25 exhibited PMR-blocking

97   activity[1], but was not as potent as clone D1 (Fig. 2a). Clones D1 and 25 also exhibited dose-

98   dependent inhibition of NINJ1-dependent PMR when measured by LDH release (Fig. 2b, lower

99   graphs). As expected, neither clone prevented nigericin-induced cell death based on the

100   measurement of cellular ATP levels (Fig. 2b, upper graphs). Morphologically, wild-type BMDMs

101   undergoing pyroptosis develop bubble-like herniations that burst in a NINJ1-dependent manner to

102   yield shrunken cellular corpses[1] (Fig. 2c). However, wild-type BMDMs treated with nigericin in

103   the presence of clone D1 or 25 resembled *Ninj1*$^{-/-}$ BMDMs in that they exhibited a persistent

104   "bubble" morphology (Fig. 2c). Thus, NINJ1-blocking antibodies prevent PMR, but not formation

105   of membrane herniation during pyroptosis.

106

107   PMR is not limited to pyroptosis. BMDMs also undergo GSDMD-independent, but NINJ1-

108   dependent PMR after apoptotic blebbing and shrinkage, likely attributable to ATP depletion[1].

109   Accordingly, *Ninj1*^{−/−} BMDMs released less LDH than wild-type BMDMs following pyroptosis

110   induction with intracellular lipopolysaccharide (LPS) or flagellin, or after apoptosis induction with

111   doxorubicin, venetoclax, or tumor necrosis factor (TNF) plus actinomycin (Fig. 2d and Extended

112   Data Fig. 1f). Clone D1 also attenuated PMR, but not cell death, when wild-type BMDMs were

113   exposed to these pyroptotic or apoptotic stimuli. As expected[1], neither *Ninj1* deficiency nor clone

114   D1 reduced LDH release following necroptosis induction with TNF plus the pan-caspase inhibitor

115   zVAD (Extended Data Fig. 1f). These results establish anti-mouse NINJ1 antibody clone D1 as a

116   potent inhibitor of PMR associated with apoptosis or pyroptosis. A commercial anti-NINJ1

117   antibody (BD Bioscience clone 50) and a NINJ1$_{26-37}$ peptide have been used to block mouse NINJ1

118   in vivo[9-11], but neither blocked nigericin-induced PMR in BMDMs (Extended Data Fig. 2a, b, c).

119   Furthermore, BD clone 50 failed to bind to mouse NINJ1 expressed in HEK293T cells (Extended

120   Data Fig. 2d). Therefore, clone D1 appears to be unique in its ability to potently block NINJ1-

121   dependent PMR (Fig. 1b).

122

123   **Clone D1 blocks NINJ1 oligomerization**

124   We hypothesized that clone D1 blocked PMR in BMDMs by preventing oligomerization of

125   NINJ1[1]. In keeping with such a mechanism, nigericin induced speck-like assemblies of NINJ1 in

126   wild-type BMDMs, but these were less prevalent in the presence of a clone D1 Fab (Fig. 3a, b).

127   We also looked at the effect of clone D1 on N-terminally FLAG-tagged mouse NINJ1 purified

128   from transiently transfected human Expi293F cells. By size exclusion chromatography (SEC),

129   purified FLAG-NINJ1 migrated as a high molecular weight species (peak at 8.5 ml retention

130 volume) (Fig. 3c, Extended Data Fig. 3a, b). Negative stain electron microscopy (ns-EM) of the

131 FLAG-NINJ1 revealed that NINJ1 formed oligomeric structures with heterogeneous shapes,

132 including rings, filaments, clusters, and arcs up to 200 nm in size (Fig. 3d). In contrast, when

133 FLAG-NINJ1 was co-expressed with clone D1 Fab, the purified NINJ1-Fab complex migrated as

134 a lower molecular weight species (peak at 15 ml retention volume) (Fig. 3c, Extended Data Fig.

135 3a, b) and showed no high-order oligomeric structure formation in ns-EM (Fig. 3d). Thus, binding

136 of clone D1 Fab to NINJ1 prevents it from assembling into larger oligomeric structures.

137

138 Purified FLAG-NINJ1 added to synthetic liposome membranes caused the release of an

139 encapsulated cargo, whereas the NINJ1-D1 Fab complex did not (Fig. 3e). These data suggest that

140 NINJ1 forms lytic higher order oligomers, which can be prevented by clone D1. The formation of

141 higher order oligomeric filaments is not without precedent and has been reported for multiple

142 apoptotic, pyroptotic, and necroptotic molecules, including ASC and caspase-8[12,13]. We propose

143 that oligomeric assemblies of NINJ1 mediate PMR in cells and clone D1 binding to NINJ1

144 prevents these oligomers from forming. Clone 25 resembled clone D1 in preventing NINJ1-

145 dependent cargo release from liposomes (Fig. 3e) and NINJ1 filaments by ns-EM (Extended Data

146 Fig. 3c), but was not as efficient as clone D1 at preventing the oligomeric assembly of NINJ1 in

147 SEC analysis (Extended Data Fig. 3d). These data are consistent with clone 25 being a weaker

148 antagonist than clone D1 in cellular PMR assays (Fig. 2a, b). Neither the C-terminal residues of

149 NINJ1 recognized by clone D1, nor the N-terminal residues bound by clone 25, are resolved in the

150 cryo-EM structure of NINJ1 filaments reported by Degen et al (manuscript accepted at Nature),

151 suggesting that these regions of NINJ1 are flexible and potentially dispensable for oligomerization.

152 Indeed, alanine mutations within these regions did not suppress membrane damage caused by

153   ectopic expression of NINJ1[1]. Therefore, binding of clone D1 or clone 25 to NINJ1 may prevent

154   NINJ1 oligomerization through steric hindrance.

155

156   **Targeting PMR in mouse hepatitis models**

157   The role of NINJ1-dependent PMR in human disease and inflammation is unclear, but genome-

158   wide association studies[14] suggest a link between *NINJ1* and lower serum levels of the liver

159   enzymes ALT and AST, two clinically important biomarkers of hepatocellular injury or membrane

160   damage[15,16] (Extended Data Fig. 4). TNF-induced hepatocyte apoptosis causes liver inflammation,

161   and has been implicated in multiple diseases including hepatocellular carcinoma, ischemia, and

162   viral hepatitis[17]. To address the role of NINJ1 in apoptosis-associated PMR in vivo, we assessed

163   *Ninj1*^{fl/fl} *Rosa26*-CreER[T2] mice in a model of fulminant hepatitis. This mouse strain allows

164   tamoxifen-induced, systemic *Ninj1* deletion in adults (Extended Data Fig. 5a, b), and therefore

165   avoids the developmental hydrocephalus that is observed in a significant fraction of *Ninj1*^{−/−}

166   newborns[5]. After tamoxifen treatment, *Ninj1*^{fl/fl} *Rosa26*-CreER[T2] mice and *Rosa26*-CreER[T2]

167   control animals were dosed with TNF and the transcriptional inhibitor D-Galactosamine (D-Gal)

168   to induce hepatocyte apoptosis[18-20]. TNF plus D-Gal caused fulminant hepatocellular PMR in

169   *Rosa26*-CreER[T2] mice as measured by increased serum ALT, AST, and LDH (Fig. 4a). The sera

170   of *Ninj1*-deficient mice treated with TNF plus D-Gal contained significantly less ALT, AST, and

171   LDH. Histological analysis of control livers revealed that TNF plus D-Gal caused massive lesions

172   characterized by pyknotic hepatocellular death with hemorrhage (Fig. 4b, c). Immunolabeling

173   showed that these lesions were positive for cleaved caspase-3, a marker of apoptosis (Fig. 4d).

174   *Ninj1* deficiency did not abate TNF plus D-Gal-induced hepatocellular degeneration and caspase-

175   3 cleavage (Fig. 4b, c, d), consistent with the post-apoptotic role of NINJ1[1]. Although mortality

8

176   was not delayed in this acute liver injury model (Extended Data Fig. 6a), a greater proportion of

177   *Ninj1*-deficient hepatocytes exhibited the swollen morphology associated with PMR malfunction

178   (Fig. 4b, c). These data indicate that NINJ1 mediates apoptosis-related PMR in vivo.

179

180   Next, we determined whether clone D1 could limit liver injury induced by TNF plus D-Gal. Wild-

181   type mice dosed with an isotype control antibody 2 h prior to injection of TNF plus D-Gal exhibited

182   markedly elevated ALT, AST, and LDH serum levels, indicative of robust hepatocellular PMR

183   (Fig. 4e). Similar to *Ninj1* deficiency, pretreatment with clone D1 significantly attenuated TNF

184   plus D-Gal-induced increases in serum LDH, ALT, and AST (Fig. 4e). Clone D1 also caused TNF

185   and D-Gal-treated hepatocytes to have a ballooned morphology, whereas degeneration of the liver

186   and the extent of caspase-3 cleavage was comparable to that in livers pre-treated with the isotype

187   control antibody (Extended Data Fig. 6b, c, d). The presence of similar numbers of cleaved

188   caspase-3-positive cells in control and D1 treated (or *Ninj1*-deficient) mice suggests that NINJ1

189   inhibition does not alter the clearance of dead cells by phagocytes. Indeed, adult *Ninj1*-deficient

190   mice do not develop the spontaneous inflammation that is typical of mice with defective

191   efferocytosis[21].

192

193   TNF-dependent activation of apoptosis in D-Gal-sensitized hepatocytes relies on caspase-8[22],

194   which also cleaves and activates leaderless IL-18[23-25], but the mechanism by which IL-18, and

195   potentially other DAMPs, are released from apoptotic cells is ill-defined. We found that TNF plus

196   D-Gal increased IL-18 and HMGB1 DAMP[26] levels in the serum in a NINJ1-dependent manner

197   (Fig. 4f, g). *Ninj1* deficiency or pretreatment of mice with clone D1 both held serum IL-18 and

198   HMGB1 in check after TNF plus D-Gal dosing. These data strongly suggest that PMR mediated

199   by NINJ1 releases IL-18 into the serum in this apoptosis-driven liver injury model. This

200   mechanism differs from that in pyroptotic BMDMs, wherein GSDMD pores[27] suffice to release

201   IL-18 and other small DAMPs independently of NINJ1[1]. Although the role of GSDMD pores in

202   IL-18 release in vivo still remains uncertain, our data indicate that both NINJ1-dependent and -

203   independent mechanisms may release small DAMPs in disease.

204

205   We also investigated the effect of clone D1 in mouse hepatitis instigated by either the T cell

206   mitogen concanavalin A (ConA)[28], Jo2 anti-Fas agonistic antibody[29], or ischemia-reperfusion

207   injury (IRI)[30]. *Ninj1*-deficient mice or mice pre-treated with clone D1 exhibited less serum LDH,

208   ALT, AST, IL-18, or HMGB1 than control mice at 8 or 18 hours after ConA dosing (Fig. 4 h, i,

209   Extended Data Fig. 7a, b), after hepatic IRI (Extended Data Fig. 7c, d), and after Jo2 injection

210   (Extended Data Fig. 7e). As expected, clone D1 did not prevent hepatocellular degeneration and

211   the appearance of cleaved caspase-3-positive cells after ConA dosing (Extended Data Fig. 7f, g),

212   nor confluent necrosis after IRI injury (Extended Data Fig. 7h). Importantly, we observed NINJ1-

213   dependent neutrophil recruitment into the damaged liver after IRI (Extended Data Fig. 7i), in

214   agreement with a recent study[31] using *Ninj1*-deficient mice that was published during the revision

215   of our manuscript. Collectively, these data suggest that NINJ1 mediates hepatocellular PMR and

216   promotes inflammation in vivo.

217

218   Identification of antagonist anti-NINJ1 monoclonal antibodies that can prevent the assembly of

219   lytic NINJ1 oligomers, and thereby limit PMR and the release of proinflammatory DAMPs in vivo,

220   indicates that it is possible to target a post-cell death event. Although clone D1 treatment attenuated

221   PMR in vivo, its efficacy was evaluated in acute mouse hepatitis models. The impact of NINJ1

222 blockade in settings of chronic inflammation, where protracted DAMP release is expected to

223 exacerbate pathology, will be an exciting area of future investigation, but will require reagents

224 with improved pharmacokinetic properties. We were unable to sustain serum levels of clone D1

225 long-term by repeat dosing.

226

227

228   **Figure Legends**

229

230   **Figure 1 | Identification of NINJ1 blocking antibody clone D1**

231   **a**, Scheme of recombinant antibody screening. EV, extracellular vesicle. NGS, next-generation

232   sequencing.

233   **b,** Graph shows LDH released from Pam3CSK4-primed wild-type BMDMs after nigericin

234   stimulation for 16 h in the presence of 1 μg/mL antibody. The white circle non-stimulated wild-

235   type BMDMs, the black circle primed and stimulated $Ninj1^{-/-}$ BMDMs, the dark grey circle clone

236   25, the blue circle clone D1, and the light grey circles other anti-NINJ1 antibodies. The LDH score

237   is the LDH release normalized against the no antibody control.

238   **c**, Graphs indicate the percentage of YOYO-1$^+$ HEK293T cells expressing NINJ1 when cultured

239   with clone D1 or an isotype control antibody. Circles are the mean ± s.d. (shaded area) of 3

240   independent replicates.

241   **d,** Flow cytometry histograms of propidium iodide-negative HEK293T cells surface-stained with

242   anti-NINJ1 or anti-FLAG antibodies. Cells are mock transfected (light gray) or transfected with

243   the NINJ1 constructs indicated (dark gray). Results are representative of 3 independent

244   experiments (**c, d**).

245

246   **Figure 2 | Clone D1 potently inhibits NINJ1-dependent PMR**

247   **A**, Graphs show the release of DD-150 from Pam3CSK4-primed BMDMs after nigericin

248   stimulation. Data are means (circles) ± s.d. (shaded area) of biological replicates ($n = 3$ animals);

249   data were generated with bone marrow harvested from 3 different mice.

12

250 **b, d,** Graphs indicate cell viability (top) or LDH release (bottom) in BMDM cultures following

251 pyroptosis induction with nigericin (**b**) or cytoplasmic LPS for 3 h, apoptosis induction with

252 doxorubicin for 6 h, or TNF + actinomycin D for 6 h. Act, actinomycin D (**d**). Pyroptotic stimuli

253 were applied to Pam3CSK4-primed BMDMs. Bars are the mean of 3 biological replicates ($n = 3$

254 animals) (**b, d**) as in **a**.

255 **c**, Bright-field images of Pam3CSK4-primed BMDMs stimulated with nigericin for 8 h. Scale bar,

256 25 μm. Results representative of 3 independent experiments (**a, b, c, d**).

257

258 **Figure 3 | Clone D1 attenuates NINJ1 oligomerization**

259 **a**, Immunolabelling of endogenous NINJ1 in BMDMs after priming with Pam3CSK4 and then

260 stimulation with nigericin for 45 min in the presence or absence (cont) of clone D1 Fab.

261 Arrowheads highlight representative NINJ1 specks.

262 **b**, Quantification of the percentage of cells bearing NINJ1 specks in (**a**). Bars indicate the mean.

263 D1 Fab, $n = 10$ independent samples; the others, $n = 20$ independent samples. *P* value two-tailed

264 *t*-test, $P = 0.0000091$.

265 **c**, Size exclusion chromatography traces for purified NINJ1 or the NINJ1-clone D1 Fab complex.

266 Molecular weight standard marker positions are pointed by arrows. Results representative of 3

267 independent experiments.

268 **d**, Negative stain electron microscopy of NINJ1 or the NINJ1-clone D1 Fab complex in (**c**).

269 **e**, Liposome cargo release by the NINJ1 or NINJ1-D1 Fab complex in (**c**) or NINJ1-clone 25 Fab

270 complex in Extended Data Fig. 3d. Bars are the mean of 3 independent replicates (circles). *P* value

271 two-tailed Mann–Whitney *U*-test, $P = 0.0000411$ (NINJ1 + clone 25 Fab v NINJ1 alone), $P =$

272 0.0000411 (NINJ1 + clone D1 Fab v NINJ1 alone). 100% cargo release is defined by total cargo

273 release mediated by 1% CHAPSO.

274

275 **Figure 4 | Clone D1 limits NINJ1-dependent PMR and DAMP release in vivo**

276 **a**, Mouse serum LDH, ALT, and AST. Where indicated, mice were dosed for 7 h with TNF and

277 D-Gal. a.u., arbitrary units. Untreated wild-type, $n = 5$ mice; tamoxifen-treated groups, $n = 6$ mice.

278 P value two-tailed unpaired t-test, $P = 0.0000000000665$ (LDH), $P = 0.00000296$ (ALT), $P =$

279 0.000000000067 (AST).

280 **b**, Representative haematoxylin and eosin-stained liver sections of the mice in (**a**). Scale bar, 25

281 μm.

282 **c**, Histological scoring of mouse livers. Untreated wild-type, $n = 3$ mice; tamoxifen-treated groups,

283 $n = 7$ (left) or 6 (right) mice. P value two-tailed Mann–Whitney U-test.

284 **d**, Mouse liver sections with immunolabeling of cleaved caspase-3 (brown). Graph indicates

285 qualitative scoring of cleaved caspase-3 labeling. Untreated wild-type, $n = 2$ mice; tamoxifen-

286 treated groups, $n = 7$ mice. P value two-tailed Mann–Whitney U-test. Scale bar, 100 μm.

287 **e, h,** Wild-type mouse serum LDH, ALT, and AST. Where indicated, mice were dosed with 50

288 mg kg^{-1} antibody for 2 h before dosing with TNF plus D-Gal for 6 h (**e**) or ConA for 8 h (**h**).

289 Untreated wild-type, $n = 5$ (**e**) or $n = 4$ mice (**h**); wild-type dosed with antibodies, $n = 10$ mice. P

290 value two-tailed unpaired t-test. **e,** $P = 0.00000915$ (LDH), $P = 0.000094$ (ALT).

291 **f, g, i,** Mouse serum IL-18 and HMGB1 of the mice in (**a**), (**e**), and (**h**), respectively. Untreated

292 wild-type, $n = 5$ mice; tamoxifen-treated $Ninj1^{+/+}$ $Rosa26$-CreER^{T2}, $n = 5$ (left), $n = 6$ (right) mice;

293 tamoxifen-treated $Ninj1^{fl/fl}$ $Rosa26$-CreER^{T2}, $n = 6$ (**f**). Untreated wild-type, $n = 5$ (**g**) or $n = 4$ (**i**)

14

294   mice; treated groups, n = 10 mice (**g, i**). P value two-tailed Mann-Whitney U-test (**f**), two-tailed

295   unpaired t-test (**g, i**). **g**, P = 0.00000682.

296   Lines indicate the mean, circles individual mice (**a, c, d, e, h, f, g, i**).

297

298

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384

Methods

Mice

All animal procedures were conducted under protocols approved by the Genentech Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals and applicable laws and regulations. All animal procedures related to hepatic ischemia-reperfusion injury were conducted under protocols approved by the Animal Care Committee at The Hospital for Sick Children and in accordance with animal care regulation and policies of the Canadian Council on Animal Care. *Ninj1*^{−/−} mice with a C57BL/6N background were described previously[1]. Wild-type mice (that have two alleles of wild-type *Ninj1, Ninj1*^{+/+}) were littermates. *Ninj1*^{fl/fl} with exon 3 floxed were generated by Ozgene (Australia) from C57BL/6 ES cells. *Ninj1*^{fl/+} mice were genotyped with PCR primers (5′-TAG TTA GTT CAA GCC AGA G and 5′-GCG GTCA GCA GAA TAG A, and 5′-CCA AGG AAG CAG GTA C) yielding 396 bp wild-type, 448 bp floxed, and 359 bp knockout DNA fragments. *Ninj1*^{fl/fl} mice were bred with *Rosa26*[Cre.ERT2/+] C57BL/6 mice[33].

For in vivo studies (described below), *Ninj1*^{fl/fl} mice rather than *Ninj1*^{−/−} mice were used to avoid the developmental hydrocephalus that is observed in a significant fraction of *Ninj1*^{−/−} newborns[5]. *Ninj1*^{fl/fl}*Rosa26*[Cre.ERT2/+] and *Ninj1*^{+/+}*Rosa26*[Cre.ERT2/+] siblings aged 6-9 weeks were dosed daily by intraperitoneal (ip) injection with 80 mg kg^{-1} body weight of tamoxifen in sunflower oil (MilliporeSigma) for 5 consecutive days. Experiments and analyses were performed two weeks after the final dose of tamoxifen.

407 Mice were housed in individually ventilated cages within animal rooms maintained on a 14:10-

408 hour, light:dark cycle with ad libitum access to food and water. Animal rooms were temperature

409 and humidity-controlled, between 68-79°F and 30-70% respectively, with 10 to 15 room air

410 exchanges per hour.

411

412 **Generation of EVs and recombinant mouse NINJ1**

413 Expi293F cells (Thermo Fisher Scientific) were co-transfected with pRK-FLAG-mNINJ1

414 (Genentech) and a mammalian expression vector encoding HIV-1/MLV Gag[34]. EVs were purified

415 from the supernatant 7 days post-transfection using ultracentrifugation[35].

416

417 For recombinant NINJ1 production, Expi293F suspension cells were cultured in Expi293

418 Expression Medium (Thermo Fisher Scientific) and transfected with pRK-FLAG-mNINJ1 using

419 the ExpiFectamine 293 transfection kit (Thermo Fisher Scientific). Frozen cell pellets (50 g) were

420 thawed and washed with a hypotonic buffer containing 20 mM HEPES pH 7.5, 1 mM EDTA,

421 supplemented with leupeptin, benzamidine and protease inhibitor tablets (Roche). Cells were

422 solubilized with 50 mM HEPES pH 7.5, 300 mM NaCl, 1% (w/v) lauryl maltose neopentyl glycol

423 (LMNG, Anatrace), 0.1% (w/v) cholesteryl hemisuccinate (CHS, Anatrace), supplemented with

424 leupeptin, benzamidine and Roche protease inhibitor tablets for 1.5 h at 4°C under gentle agitation.

425 After ultracentrifugation at 185,000 x g for 1 h, the supernatant was gently rotated with anti-FLAG

426 M2 affinity resin (Sigma) for 1 h at 4°C. Unbound proteins were washed away with 10 column

427 volumes (CV) of wash buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 0.1% (w/v) LMNG,

428 0.01% (w/v) CHS), followed by 10 CV of wash buffer B (50 mM HEPES pH 7.5, 150 mM NaCl,

429 0.005% (w/v) LMNG, 0.0005% (w/v) CHS). Recombinant NINJ1 was eluted with 5 CV of wash

430   buffer B supplemented with 300 µg/mL FLAG peptide (MilliporeSigma). Eluate was concentrated

431   in a 50 kDa MWCO concentrator and loaded onto a Superose6 Increase 10/300 GL column (GE

432   Healthcare) equilibrated with 20 mM HEPES pH 7.5, 150 mM NaCl, 0.005% (w/v) LMNG,

433   0.0005% (w/v) CHS. Recombinant NINJ1 was conjugated to Alexa Fluor 647 with a Lightning-

434   Link® Alexa Fluor 647 kit (Novus Biologicals).

435

436   **Anti-mouse NINJ1 antibody generation and screening**

437   *Ninj1*$^{-/-}$ mice were immunized biweekly with mouse NINJ1-expressing EVs (extracellular

438   vesicles) plus Ribi adjuvant (MilliporeSigma) and mouse plasmids encoding mouse NINJ1 (in

439   pCAGGS vector, Genentech), Flt3L (in pORF vector, Genentech) and mouse GM-CSF (in pORF

440   vector, Genentech). Sera from immunized mice were tested by flow cytometry for reactivity

441   towards mouse NINJ1-expressing BALB/3T3 cells (clone A31, ATCC CCL-163). B cells from

442   the lymph nodes and spleens of immunized mice were enriched using a cocktail of depletion

443   antibodies (biotinylated CD11b at 1:400, CD11c at 1:400, Ly-6G/C at 1:400, CD49b at 1:400,

444   Ter119 at 1:100, CD4 at 1:200, CD8b.2 at 1:200, CD11b at 1:400 from BD Biosciences) and

445   magnetic streptavidin beads (Miltenyi Biotec). Enriched cells were stained with PE-Cy7-

446   conjugated rat anti-mouse IgM (BD Pharmingen, 1:50-100), rat anti-mouse B220 eFluor 450

447   (Thermo Fisher Scientific, 1:50), and AF647-labeled mouse NINJ1. Approximately 15,000

448   NINJ1-bound IgM$^-$B220$^+$ B cells were single cell-sorted into 96-well plates containing

449   supplemented RPMI 1640 medium (Thermo Fisher Scientific) and EL-4-B5 feeder cells (Roche).

450   After 8 to 10 days in culture, supernatants were screened for mouse NINJ1-reactive IgGs using an

451   IgG ELISA (Rockland) and flow cytometry against NINJ1-expressing 3T3 cells. B cells producing

452   NINJ1-reactive IgGs were the starting point for the generation of 217 recombinant monoclonal

453   antibodies using published methods[36]. Briefly, RNA was extracted from the B cells using a

454   MagMax™-96 Total RNA Isolation Kit (Thermo Fisher Scientific). Variable light (VL) and

455   variable heavy (VH) domains were PCR-amplified from cDNA using a forward barcoded primer

456   set recognizing the leader sequence of most known mouse variable genes, and a barcoded reverse

457   primer recognizing the constant domain[37]. Individual purified VL and VH PCR products were

458   pooled for next-generation sequencing library preparation using the Ovation® Library System for

459   Low Complexity Samples kit (Nugen). A MiSeq sequencer (Illumina) was used for 2x300 bp

460   paired sequencing. VH and VL sequences were synthesized (Genscript) and cloned into pRK

461   mammalian expression vectors encoding the mouse γ2a and k constant domains, respectively[37].

462   Recombinant antibodies were transiently expressed in CHO cells and purified on a protein A

463   column[38]. Briefly, CHO cells (Genentech) were seeded at $0.4\text{-}0.8 \times 10^6$ cells/ml. After three or

464   four days later, cells were transfected with plasmids by using PEIPro (Polyplus) according to

465   manufacture's instruction. Culture supernatants were harvested 14 days post transfection.

466   Recombinant antibodies were screened for NINJ1 inhibitory activity by LDH release assay in

467   BMDMs. Antibodies (1 μg/mL) were added to Pam3CSK4-primed BMDMs for 15 min prior to

468   nigericin stimulation. At 16 h post stimulation, supernatants were collected for LDH release assays.

469

470   **Reagents**

471   Ultra-pure LPS (*E. coli* O111:B4, InvivoGen), Pam3CSK4 (InvivoGen), nigericin (InvivoGen),

472   ultra-pure flagellin (from *P. aeruginosa*, InvivoGen), venetoclax (TOCRIS), doxorubicin (Enzo

473   Life Sciences), mouse TNF (Genentech), actinomycin D (Act, EMD Millipore), Z-VAD-FMK

474   (zVAD; Promega). Antibodies used for western blotting were rabbit anti-mouse NINJ1 clone 25

475   (Ninj1-25, Genentech, 0.2 μg/mL)[1], β-actin HRP (AC-15, Novus Biologicals, 0.1 μg/mL), HRP-

476   anti-rabbit F(ab')$_2$ fragment (Jackson Immunoresearch, 1:5000), and HRP-anti rabbit Fc fragment

477   (Jackson Immunoresearch, 1:5000). A list of all antibodies used in this manuscript is

478   provided in Supplementary Table 1. cDNAs encoding N-terminally FLAG-tagged NINJ1

479   (full-length, delta 2-73, delta 142-152 and mutant D$_{147}$VAPR → A$_{147}$AAAA) were cloned into

480   pcDNA3.1 Zeo(+) (Thermo Fisher Scientific).

481

482   **Cell line authentication and quality control**

483   Cell lines were authenticated by short tandom repeat (STR) profiling and regular single

484   nucleotide polymorphism (SNP) fingerprinting. STR profiles are determined for each line using

485   the Promega PowerPlex 16 System. This is performed once and compared to external STR

486   profiles of cell lines (when available) to determine cell line ancestry. SNP profiles are performed

487   each time new stocks are expanded for cryopreservation. Cell line identity is verified by high-

488   throughput SNP profiling using Fluidigm multiplexed assays. SNPs were selected based on

489   minor allele frequency and presence on commercial genotyping platforms. SNP profiles are

490   compared to SNP calls from available internal and external data (when available) to determine or

491   confirm ancestry. All cells are tested for mycoplasma prior to and after cell cryopreservation

492   using two methods are used to avoid false positive/negative results: Lonza Mycoalert and

493   Stratagene Mycosensor. Cell growth rates and morphology are also monitored for any batch-to-

494   batch changes.

495

496   **BMDM stimulations**

497   Mouse bone marrow cells were differentiated into macrophages in Dulbecco's modified Eagle's

498   medium (DMEM) supplemented with 10% (v/v) low-endotoxin fetal bovine serum (FBS; Omega

23

499 Scientific) and 20% (v/v) L929-conditioned medium for 5 days. For stimulation, cells were

500 replated overnight at 1.0×10^5 cells/well in 96-well plates. For inflammasome stimulations, cells

501 were primed with Pam3CSK4 (1 μg/mL) for 5 h and then stimulated with 10 μg/mL nigericin in

502 Opti-MEM I media (Thermo Fisher Scientific). For flagellin or LPS electroporation[39], 1.0×10^6

503 Pam3CSK4-primed BMDMs were electroporated with 1.0 μg LPS or 0.2 μg flagellin in 100 μL R

504 buffer using a neon 100 μL tip with 1,720 voltage, 10 width, 2 pulse settings. Electroporated cells

505 were added to 990 μL Opti-MEM I medium and cultured for 2 h. BMDMs treated with venetoclax

506 (25 μM for 6 h), doxorubicin (10 μM for 6 h), TNF plus ActD (20 ng/mL TNF, 500 ng/mL ActD

507 for 6 h), or TNF plus zVAD (100 ng/mL TNF, 20 μM zVAD for 16 h) were not primed. For lysis

508 controls, cells were lysed with 0.25% Triton-X100 in medium. Where indicated, BMDMs were

509 cultured with the indicated concentration of anti-NINJ1 clone 25[1] (mouse IgG2a; Genentech), anti-

510 NINJ1 clone D1 (mouse IgG2a; Genentech), anti-Ninjurin clone 50 (BD Biosciences BD610777,

511 raised against human NINJ1), or an isotype control mouse IgG2a (Thermo Fisher Scientific). Prior

512 to addition to cells, anti-Ninjurin clone 50 and isotype control mouse IgG2a antibodies were

513 dialyzed against PBS to remove sodium azide using Slide-A-Lyzer MINI Dialysis Device with a

514 10K MWCO (Thermo Fisher Scientific) according to manufacturer's instructions. Synthetic

515 peptides used were mouse NINJ1 aa 26-37 (PPRWGLRNRPIN, Genentech) and its sequence-

516 scrambled analogue (PWPPRRNRNGLI, Genentech). BMDMs were pretreated with antibody or

517 peptide for 10 min prior to addition of treatment.

518

519 **PMR and viability assays**

520 Culture medium was analyzed for LDH release with the CytoTox 96 Non-Radioactive

521 Cytotoxicity Assay (Promega). CellTiter-Glo reagent (ATP assay, Promega) was used for

24

522 detection of viable cells. Data for LDH and CellTiter-Glo assays was collected with an EnVision

523 2105 multimode plate using EnVision Manager 1.14.3049.1193 (PerkinElmer).

524

525 **Flow cytometry**

526 293T cells (ATCC, CRL-3216) cells were transfected with NINJ1 expression plasmids using

527 Lipofectamine 2000 (Thermo Fisher Scientific). Cells were stained with the following monoclonal

528 antibodies: anti-NINJ1 clone 25 mIgG2a (Genentech, 10 µg/mL), anti-NINJ1 clone D1 mIgG2a

529 (Genentech, 10 µg/mL), anti-Flag-M2 (Millipore Sigma, 1:100), anti-Ninjurin clone 50 (BD

530 Biosciences, 10 µg/mL). Primary staining was followed by APC-conjugated anti-mouse IgG

531 (Thermo Fisher Scientific, 1:300), and then propidium iodide (PI; 2.5 µg/mL; BD Biosciences).

532 Live PI$^-$ cells were analyzed in a FACSymphony (Becton Dickinson). Data was acquired using

533 BD FACSDiva Software v9.1, and analyzed using FlowJo v10.8.1. Representative FACS gating

534 strategies and contour plots with outliers are shown in Supplementary Figs. 2, 3, and 4.

535

536 **DD-150 dye release assay**

537 BMDMs were loaded with fluorescein isothiocyanate-dextran (DD-150, MilliporeSigma) using a

538 100 µL Neon tip (Thermo Fisher Scientific). 5.0×10^6 BMDMs were electroporated in 120 µL R

539 buffer plus 12 µL 50 mg/mL DD-150. Prior to plating, BMDMs were washed with high-glucose

540 DMEM. Following stimulation, BMDMs were imaged in an IncuCyte S3 (Essen BioScience) at

541 10X magnification.

542

Imaging of BMDM morphology

BMDMs were plated on glass-bottom 96 MicroWell Optical Plates (Thermo Fisher Scientific). Pam3CSK4-primed BMDMs were stimulated with 10 μg/mL nigericin in the presence or absence of 10 μg/mL anti-NINJ1 antibody. Plates were imaged using a 60× Plan Fluor objective on an ImageXpress Micro Confocal system running the MetaXpress v6.5.4.532 software and equipped with an environmental controller and gas mixer to maintain cells at 37°C and 5% CO_2. Images of the bright-field and transmitted light were imaged overnight every 15 min. Representative images at the 8 h timepoint were processed using the using the scikit-image 0.19.2 python package.

Immunofluorescence

Clone D1 variable domains were cloned into the human Fab expression vector 1AP39.hIgG1.D.Fab (Genentech). Protein was expressed in *E. coli* and purified with a low endotoxin level (<0.07 EU/mg). Pam3CSK4-primed BMDMs were cultured with 50 μg/mL D1 Fab and then stimulated with nigericin on glass-bottom 96 MicroWell Optical Plates (Thermo Fisher Scientific). Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.1% Tween-20. Cells were blocked in PBS supplemented with 0.2% fish gelatin (Millipore Sigma), 3% Bovine Serum Albumin and 0.1% Tween-20 for 1 h at room temperature, and then labelled with anti-mouse NINJ1 clone 80 (rabbit IgG2b raised against the N-terminal extracellular domain; Genentech, 2 μg/mL) at 4°C overnight. Bound antibody was revealed with an AF488-conjugated anti-rabbit secondary (Thermo Fisher Scientific, 1:200) at room temperature for 1 h. High resolution images were acquired with a Leica SP8X confocal laser scanning microscope running Leica LAS X v3.5.7 software and equipped with a white light laser and a HC PL APO CS2 oil immersion 40X lens of numerical aperture 1.3.

566

Transient expression of NINJ1 in HEK293T cells

cDNAs encoding untagged human or mouse NINJ1 were cloned into pcDNA3.1 Zeo(+). HEK293T cells (2.6×10^4) were transfected with 50 ng plasmid plus 0.16 μL Lipofectamine 2000 per well in 96-well plates in the presence of 20 or 200 μg/mL of isotype control antibody, anti-NINJ1 clone D1 (Genentech), or anti-NINJ1 clone D1 Fab (Genentech). YOYO-1 dye (Thermo Fisher Scientific) was added at a final concentration of 200 nM at the time of transfection. IncuCyte S3 images were scanned in the green channel every hour for at least 16 h and at ×10 magnification. Nuclear-ID Red DNA stain (Enzo Life Sciences) was added after the last time point and scanned in the red channel. IncuCyte S3 2019A software was used to determine the total number of YOYO-1^+ cells and Nuclear-ID$^+$ cells (total cells). % YOYO-1^+ positive was calculated as the number of YOYO-1^+ cells divided by the total number of Nuclear-ID$^+$ cells.

578

Size exclusion chromatography (SEC)

The C-terminally His6-tagged heavy chain Fab region (VH-CH1) and the untagged light chain of anti-NINJ1 clone D1 and clone 25 were cloned into the mammalian expression vector pRK5J (Genentech). Expi293F cells were transfected with pRK-FLAG-mNINJ1 alone, or co-transfected with pRK-FLAG-mNINJ1, pRK5J-His6-VH-CH1 clone D1 (or pRK5J-His6-VH-CH1 clone 25), and pRK5J-light chain clone D1 (or pRK5J-light chain clone 25) in a 2:1:2 plasmid ratio. NINJ1, the NINJ1-clone D1 Fab complex, or the NINJ1-clone 25 Fab complex was purified with anti-FLAG M2 resin (described above). For comparative SEC analysis, similar protein amounts and volume of NINJ1, NINJ1-clone D1 Fab complex, or NINJ1-clone 25 Fab complex were injected

27

588   onto a Superose6 Increase 10/300 GL column (GE Healthcare). Data was collected with Unicorn

589   7.6 (Cytiva).

590

Negative stain electron microscopy

592   EM grids (400 mesh copper with continuous carbon, Electron Microscopy Sciences) were glow

593   discharged for 15 s at 10 mA using a GloQube glow discharge system (Quorum) before applying

594   4 μL of sample diluted to approximately 0.1 mg/mL in SEC buffer (20 mM HEPES pH 7.5,

595   150 mM NaCl, 0.005% (w/v) LMNG, 0.0005% (w/v) CHS). After 1 min of incubation, the grid

596   was washed with distilled, deionized water, and stained with 1% uranyl acetate. After drying

597   completely, the grids were imaged in a Talos F200C equipped with a 4k × 4k Ceta 16M Camera

598   (Thermo Fisher Scientific) using SerialEM Version 3.9.0. Images were recorded at a nominal

599   magnification of 45,000x (3.2 Å per pixel).

600

Liposomal cargo release assay

602   Stocks of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, Avanti Polar Lipids) and 1-

603   palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS, Avanti Polar Lipids)

604   were prepared in chloroform from dry powder. A lipid mixture of 80% POPC and 20% POPS was

605   generated, freeze dried, and hydrated with a solution of 10 mM Tris pH 8.0, 150 mM NaCl

606   containing the cargo, LANCE Eu-W1024 Biotin (PerkinElmer). The suspension was sonicated in

607   a water bath, freeze-thawed, and extruded using a Mini Extruder (Avanti Polar Lipids) fitted with

608   a Nucleopore 0.4 μm membrane (Whatman) to yield large unilamellar vesicles. The liposomes

609   were purified by eluting through a column packed with Pierce Streptavidin Agarose resin (Thermo

610   Fisher Scientific). Liposomes were destabilized by addition of 0.0005% LMNG/0.00005% CHS

611   (Anatrace). Cargo release assay was set up by mixing destabilized liposomes (25 μM in lipid

612   concentration, diluted from 6.4 mM stock) with 8 μM NINJ1 purified according to the protocol

613   above or 0.4 μM Melittin peptide (Anaspec). Streptavidin-Alexa Fluor 647 (Thermo Fisher

614   Scientific) was added to each well at a final concentration of 1 μM. Liposomes mixed with

615   0.0005% LMNG in ddH2O were used as a control. All samples were loaded into wells of a

616   ProxiPlate (PerkinElmer). TR-FRET readout was recorded on an EnVision 2105 multimode plate

617   reader (PerkinElmer) using EnVision Manager 1.14.3049.1193 (PerkinElmer). A pre-read of each

618   plate was taken immediately after loading the wells, and each plate was subsequently incubated

619   overnight at room temperature. After ~15 hours of incubation, another reading was taken, followed

620   by full digestion of the liposomes by addition of 1% Cholamidopropyl]dimethylammonio)-2-

621   hydroxy-1-propanesulfonate (CHAPSO, Anatrace) to each well. A final read was recorded

622   representing 100% cargo release. Results were converted to percentage cargo released per well

623   and background control subtracted.

624

625   **Stimulant-induced liver injury models**

626   In the TNF plus D-Gal model, liver injury was induced in females aged 8 to 14 weeks by ip

627   injection of 700 mg kg^{-1} D-Gal (MilliporeSigma) and 30 μg kg^{-1} mouse TNF (Genentech) unless

628   otherwise indicated. Serum was collected after 6-7 h. Where indicated, 8 to 14 weeks old age

629   matched C57BL/6J female (Jackson Labs) mice were dosed by ip injection with 50 mg kg^{-1} body

630   weight of mouse anti-NINJ1 clone D1 antibody or isotype control anti-gp120 mouse IgG2a

631   monoclonal antibody (Genentech) at 2 h before TNF plus D-Gal. For ConA or anti-Fas mAb (Jo2)

632   treatment, liver injury was induced in males aged 9 to 11 weeks by intravenous injection with

633   either 20 mg kg^{-1} body weight of Concanavalin A (Millipore Sigma) or 0.5 μg g^{-1} body weight of

634   anti-Fas (anti-CD95 clone Jo2, BD Biosciences), respectively, unless otherwise indicated. Serum

635   was collected after 8 or 18 h for ConA, and after 5 h for anti-Fas. For antibody pre-treatment, 9 to

636   11 weeks old C57BL/6N male mice (Charles River Labs) were treated with the indicated mAbs as

637   described above. Serum ALT and AST were measured in a serum chemistry analyzer (Beckman

638   Coulter AU480). LDH in serum was measured with the CytoTox 96 Non-Radioactive Cytotoxicity

639   Assay. Enzyme-linked immunosorbent assays (ELISAs) were used to assay IL-18 (MBL

640   International) and HMGB1 (IBL). LDH assay and IL-18 and HMGB1 ELISA data was collected

641   with an EnVision 2105 multimode plate using EnVision Manager 1.14.3049.1193 (PerkinElmer).

642

643   **Histology**

644   Hematoxylin and eosin stained sections of $Rosa26^{Cre.ERT2/+}$ livers were scored for hepatocellular

645   degeneration on a four-point scale based on the amount of viable tissue present as follows: (1)

646   multifocal hepatocellular injury with preservation of bridging portal tracts, (2) intermixed

647   populations of viable and degenerate cells throughout the liver, (3) bridging hepatocellular injury

648   with only the preservation of peri-portal hepatocytes, or (4) panlobular hepatocellular degeneration

649   with loss of lobular architecture. For scoring hepatocellular degeneration in antibody-treated mice,

650   six lobes were scored according to the following criteria: (0) no significant hepatocellular

651   degeneration, (1) multifocal cell death without loss of architecture, (2) non-bridging lobular

652   hepatocellular degeneration and loss of architecture, (3) bridging hepatocellular degeneration with

653   loss of architecture, or (4) diffuse hepatocellular degeneration. Scores from the 6 lobes were

654   averaged for a final score. To assess the persistence of swollen, degenerate hepatocytes, livers

655   were scored based on the predominant features of either cell loss with sinusoidal hemorrhage or

656   persistence of swollen degenerate hepatocytes. A three-tier scoring system was applied: (1)

30

657 predominant hepatocellular loss with sinusoidal hemorrhage, (2) mix of hepatocyte loss and

658 hemorrhage with regional aggregates of swollen, degenerate hepatocytes, or (3) predominant

659 preservation of swollen, degenerate hepatocytes. Scoring was performed in a random, blinded

660 manner.

661

662 **Immunohistochemistry**

663 Formalin-fixed, paraffin-embedded sections of liver were immunolabelled with rabbit anti-cleaved

664 caspase-3 antibody (Asp175, Cell Signaling Technologies, 0.05 μg/mL) or rabbit anti-NINJ1 clone

665 80 (5 μg/mL) using a discovery IHC platform (Roche). Conditions included CC1 standard antigen

666 retrieval (Roche), OmniMap detection (Roche) with diaminobenzidine, and hematoxylin

667 counterstain. Immunohistochemistry and histology images were acquired with Leica Application

668 Suit v4.6.0. Cleaved caspase-3 immunolabeling was scored according to the following matrix: (1)

669 multifocal individual or aggregates of labeled cells, (2) either extensive intermix of labeled and

670 unlabeled cells or centrilobular labeling with variable bridging, (3) extensive bridging cleaved

671 caspase 3 expression with only rims of unlabeled periportal hepatocytes, (4) diffuse hepatocellular

672 labeling. Scoring was performed in a random and blinded manner. NINJ1 immunolabeling was

673 qualitatively assessed in an unblinded manner.

674

675 **Ischemia-reperfusion liver injury**

676 Mixed-sex cohorts of 6 to 10 weeks old age C57BL/6J mice (Jackson Labs) were used in a 70 %

677 segmental ischemia-reperfusion model[40]. Under isoflurane anesthesia, a sagittal midline

678 laparotomy was made, and a clamp placed on the portal vein and the hepatic artery to block blood

679 flow to the left and medial lobes of the liver. The clamp was removed after 1 h to allow for

680   reperfusion and the animal returned to their home cage. Following 6 hrs of reperfusion, the animal

681   was euthanized by cardiac puncture under general anesthesia and tissue collected for analysis.

682   Sham laparotomy where the vascular pedicle was exposed but not clamped was used as a negative

683   control. Where indicated, mice were dosed by ip injection with 50 mg kg^{-1} antibodies 4 h before

684   induction of ischemia as described above. Animals were randomized to group and analyses

685   blinded. Serum LDH, ALT, AST levels were measured as described above. For histology, all

686   ischemic and reperfused liver lobes were collected, paraffin-embedded, sectioned at a thickness of

687   4 µm prior to staining with haematoxylin and eosin. Neutrophils were with rabbit anti-mouse Ly6G

688   (Cell Signaling Technology, 1:100) followed by biotinylated anti-rabbit secondary antibody

689   (Vector Laboratories, 1:200) and ABC (Vector Laboratories). DAB (Vector Laboratories) was

690   used to detect Ly6G staining. Tissue specimen processing and staining were conducted at the

691   Spatio-Temporal Targeting and Amplification of Radiation Response (STTARR) Innovation

692   Centre (Toronto, Canada). Slides were imaged using a 3DHistech Pannoramic Flash II Slide

693   Scanner and visualized using either 3DHISTECH CaseViewer (RRID: SCR_017654) or HALO

694   Image Analysis Platform (RRID: SCR_018350; indica labs). To evaluate confluent necrosis within

695   the liver samples, the DenseNet classifier supervised machine learning algorithm (HALO Image

696   Analysis Platform) was trained to detect substantial areas of liver cell death using the haematoxylin

697   and eosin stain and applied to the entire sample. Neutrophils (Ly6G-positive) were counted using

698   QuPath (RRID: SCR_018257). Statistical testing was calculated using Prism 9.5.1 (GraphPad

699   Software Inc). Presented data are representative of at least three independent experiments. All

700   collected data was analyzed and a *P* value < 0.05 was considered statistically significant.

701

702   **Western blots**

703 Tissues were lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40,

704 0.5% SDS, 1× cOmplete Protease Inhibitor (Roche Applied Science) and PhosSTOP phosphatase

705 inhibitor (MilliporeSigma)) at 4 ℃ for 30 min. Tissues were mechanically disrupted with a bead

706 mill homogenizer (Omni International) and insoluble material was removed by centrifugation at

707 20,000 x g before addition of NuPAGE LDS sample buffer 4X (Thermo Fisher Scientific). Raw

708 images of uncropped gels are provided in Supplementary Fig. 1.

709

710 **GWAS**

711 Regional plots were generated using LocusZoom[41] and GWAS data that used UK Biobank random

712 participant samples (n = 363, 228) and 35 blood and urine biomarkers[14,32]

713 (https://doi.org/10.1038/s41588-020-00757-z).

714

715 **Statistics and figure preparation**

716 Unless otherwise specified, presented data are representative of at least two independent

717 experiments and means are of at least three biological replicates. Statistical analyses and number

718 of samples (n) are given in each figure panel. Mann–Whitney U-tests, t-tests and log-rank tests

719 were performed using GraphPad Prism 9.5.1 (GraphPad Software Inc).

720

721 No statistical methods were used to predetermine sample size. Sample sizes were chosen based

722 on prior experience and pilot experiments for detecting statistically significant differences

723 between conditions. For in vivo studies involving tamoxifen-treated animals, groups were

724 determined by genotype rather than treatment, and therefore not randomized. For TNF+D-Gal,

725 anti-Fas JO2, and ConA in vivo studies involving wt mice, animals were age- and sex- matched

726 and randomized to group. Experimental groups were assessed in the same experiment with

727 control groups to eliminate covariates. For animal procedures related to hepatic ischemia-

728 reperfusion injury mixed sex cohorts were used; animals were randomized to group and analyses

729 blinded.

730

731 **Data Availability**

732 The datasets generated during and/or analysed during the current study are available

733 from the corresponding authors upon reasonable request. Source data for animal studies are

734 provided with this paper. GWAS data was obtained from the UK Biobank study

735 (https://doi.org/10.1038/s41588-020-00757-z).

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769

Author contributions

771 N.K., I.B.S., O.S.K., and B.L.L. designed and performed experiments. K.A. and I.D. performed

772 protein purification, size exclusion chromatography and liposomal cargo release assay. M.C.J

773 performed negative stain electron microscopy. J.Z., J.L., E.S., and W.P.L. performed in vivo acute

774 hepatitis studies. S.W., Z.L., K.S., W.L., D.S., and R.L.K. generated antibodies. T.B. provided

775 GWAS analysis. I.B.S., O.S.K., and C.C. performed microscopy. J.D.W. analyzed tissue histology.

776 P.J. performed protein expression. J.M., S.Z., D.A., N.M.G., B.A.S., B.E.S., performed the hepatic

777 ischemia-reperfusion injury model. K.N. and V.M.D. contributed to experimental design and paper

778 editing. N.K. wrote the paper with input from all authors.

779

Competing interests

781 The following authors are employees of Genentech Inc.: Nobuhiko Kayagaki, Irma B. Stowe,

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783 Juan Zhang, John Liu, Eric Suto, Wyne P. Lee, Kellen Schneider, WeiYu Lin, Dhaya Seshasayee,

784 Tushar Bhangale, Cecile Chalouni, Matthew C. Johnson, Prajakta Joshi, Kim Newton, Joshua D.

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790

Extended Data Fig. 1 | Clone D1 inhibits PMR

a, Graphs indicate LDH release in cultures of wild-type BMDMs after priming, and then stimulation with nigericin for 16 h in the presence or absence (cont) of clone D1 Fab. Non-stim, non-stimulated control. Bars indicate the mean. Circles indicate 3 biological replicates. Results representative of 3 independent experiments.

b, Graphs indicate the percentage of YOYO-1$^+$ HEK293T cells expressing NINJ1 when cultured with or without (cont) clone D1 Fab. Circles are the mean ± s.d. (shaded area) of 3 independent replicates. Results are representative of 2 independent experiments.

c, Flow cytometry histograms of propidium iodide-negative HEK293T cells surface-stained with anti-NINJ1 or anti-FLAG antibodies. Cells are mock transfected (light gray) or transfected with human NINJ1 (dark gray). Results representative of 3 independent experiments.

d, Amino acid sequence alignment of human and mouse NINJ1. Predicted epitope of clone 25 (grey box) and clone D1 (blue box) are highlighted.

e, Graph shows the release of DD-150 from Pam3CSK4-primed BMDMs in (**a**) after nigericin stimulation. Data are means (circles) ± s.d. (shaded area). Circles indicate 3 biological replicates ($n = 3$ animals). Results representative of 3 independent experiments.

f, Graphs indicate cell viability (top) and LDH release (bottom) in BMDM. Non-stim, non-stimulated control. Bars indicate the mean. Circles indicate 3 biological replicates ($n = 3$ animals). Results representative of 2 independent experiments.

810

Extended Data Fig. 2 | Commercially available anti-NINJ1 antibody

a, c, Graphs indicate LDH release in cultures of wild-type or *Ninj1*$^{-/-}$ BMDMs after priming, and then stimulation with nigericin for 4 h in the presence or absence (non) of 10 μg/mL indicated

814   antibodies or 200 nM of peptides. Non-stim, non-stimulated control. Bars indicate the mean. BD

815   clone 50, BD Bioscience anti-Ninjurin clone 50. Circles indicate biological replicates ($n = 3$

816   animals); data were generated with bone marrow from 3 different mice. Results representative of

817   3 independent experiments.

818   **b**, Graph shows the release of DD-150 from Pam3CSK4-primed wild-type or *Ninj1$^{-/-}$* BMDMs

819   after nigericin stimulation in the presence or absence (non) of 10 μg/mL indicated antibodies. Data

820   are means (circles) ± s.d. (shaded area). Circles indicate biological replicates ($n = 3$ animals).

821   Results representative of 3 independent experiments.

822   **d,** Flow cytometry histograms of propidium iodide-negative HEK293T cells surface-stained with

823   indicated antibodies. Cells are mock transfected (light gray) or transfected with mouse NINJ1

824   (dark gray). Results representative of 3 independent experiments.

825

826   **Extended Data Fig. 3 | Biochemical analysis for NINJ1-anti-NINJ1 Fab complex**

827   **a**, Schematic of the procedures in Fig. 3c, d, e, and Extended Data Fig. 3b, c, d.

828   **b**, Coomassie blue staining of NINJ1 alone (retention volume 8.5-9 ml) and NINJ1-clone D1 Fab

829   complex (retention volume 15-15.5 ml) of Fig. 3c or NINJ1-clone 25 Fab complex of (**d**) (retention

830   volume 15.5-16 ml). Results representative of 2 independent experiments.

831   **c**, Negative stain electron microscopy of NINJ1-clone 25 Fab complex. Results representative of

832   2 independent experiments.

833   **d**, Size exclusion chromatography traces for purified NINJ1-clone 25 Fab complex. Results

834   representative of 3 independent experiments. Molecular weight standard marker positions are

835   pointed by arrows.

836

Extended Data Fig. 4 | A link between *NINJ1* and lower levels of serum AST and ALT in Genome-wide Association Studies (GWAS)

Regional plot of GWAS (UK Biobank random participants, n = 363, 228)[14,32] showing correlation of a *NINJ1* single nucleotide polymorphism (rs7018885, purple diamond in chromosome 9 locus) with lower serum AST (bottom) or ALT (top). *P* values were obtained from a two-sided test for the null hypothesis that the effect of genotype (at each single nucleotide polymorphism) on the ALT or AST values in a linear regression model was zero. *P* values are not adjusted for multiple tests across the genome.

845

Extended Data Fig. 5 | Characterization of tamoxifen-treated *Ninj1^fl/fl^ Rosa26*-CreER^T2^ mice.

a, Immunoblots of mouse tissues at 2 weeks after tamoxifen dosing. Lanes represent different mice. n = 3 mice per genotype.

b, Immunolabeling of NINJ1 (brown) in mouse liver sections. Scale bar, 25 μm. Results representative of 3 mice of each genotype.

852

Extended Data Fig. 6 | Clone D1 pretreatment limits hepatocellular degeneration and caspase-3 cleavage in mice treated with TNF plus D-Gal

a, Kaplan–Meier survival plots of mice injected with 25 μg kg⁻¹ TNF plus D-Gal. n = 10 mice per group. *P* value two-tailed log-rank test. Results representative of 3 independent experiments.

b, Representative haematoxylin and eosin-stained liver sections of the mice in Fig. 4e. Scale bar, 50 μm. Arrows indicate swollen cells.

859   **c**, Histological scores of hepatocellular degeneration (left) and liver cell swelling (right) for the

860   mice in Fig. 4e. Untreated wild-type, $n = 5$ mice; wild-type dosed with antibody, TNF plus D-Gal,

861   $n = 10$ (left), n= 9 (right) mice. Lines represent the median, circles individual mice. P value two-

862   tailed Mann-Whitney U-test.

863   **d**, Liver sections from the mice in Fig. 4e after immunolabeling of cleaved caspase-3 (brown).

864   Graph indicates qualitative scoring of cleaved caspase-3 labeling. Untreated wild-type, $n = 5$ mice;

865   wild-type dosed with isotype control antibody, TNF plus D-Gal, $n = 10$ mice; Wild-type dosed

866   with clone D1 and TNF plus D-Gal, $n = 10$ mice. Lines represent the median, circles individual

867   mice. P value two-tailed Mann-Whitney U-test. Scale bar, 100 μm.

868

869   **Extended Data Fig. 7 | Clone D1 limits NINJ1-dependent PMR and inflammation in hepatitis**

870   **models.**

871   **a**, Mouse serum LDH, ALT, and AST. Where indicated, mice were dosed for 8 h with ConA.

872   Untreated wild-type, $n = 4$ mice; tamoxifen-treated $Ninj1^{+/+}$ $Rosa26$-CreER^{T2}, $n = 7$ mice;

873   tamoxifen-treated $Ninj1^{fl/fl}$ $Rosa26$-CreER^{T2}, $n = 9$ mice. a.u., arbitrary units. Lines represent the

874   mean, circles individual mice. P value two-tailed unpaired t-test, $P = 0.00000112$ (LDH), $P = $

875   0.0000427 (ALT), $P = 0.0000319$ (AST).

876   **b**, **e**, Wild-type mouse serum LDH, ALT, and AST. Where indicated, mice were dosed with 50

877   mg kg^{-1} antibody for 2 h before dosing with 15 mg kg^{-1} ConA for 18 h (**b**) or Jo2 anti-Fas for 5 h

878   (**e**). Untreated wild-type, $n = 5$ mice; wild-type dosed with isotype control antibody, $n = 8$ mice;

879   wild-type dosed with clone D1, $n = 7$ mice. Lines indicate the mean, circles individual mice. P

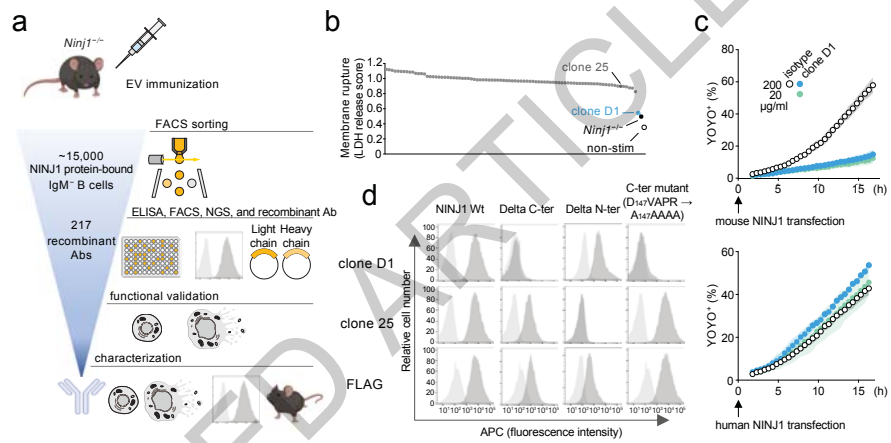880   value two-tailed Mann-Whitney U-test (**b**) and two-tailed unpaired t-test (**e**).

881   **c**, Schematic of the IRI procedure in (**d**, **h**, **i**).

882   **d,** Wild-type mouse serum LDH, ALT, and AST. Where indicated, mice were dosed with 50 mg

883   kg^{-1} antibody for 4 h before IRI for 6 h. Untreated sham, n = 6 mice; wild-type dosed with isotype

884   control antibody, n = 9 mice; wild-type dosed with clone D1, n = 8 mice. Lines indicate the mean,

885   circles individual mice. P value two-tailed unpaired t-test.

886   **f, g,** Liver sections with haematoxylin and eosin-staining (**f**) and immunolabeling of cleaved

887   caspase-3 (brown) (**g**) of mice dosed with 50 mg kg^{-1} antibody for 2 h before dosing ConA for 6

888   h. Scale bar, 100 μm. Graphs indicate qualitative scoring of hepatocellular degeneration (**f**) and

889   cleaved caspase-3 labeling (**g**). Untreated sham, n = 4 mice; wild-type dosed with isotype control

890   antibody, n = 8 mice; wild-type dosed with clone D1, n = 8 mice. Lines indicate the median, circles

891   individual mice. P value two-tailed Mann-Whitney U-test.

892   **h, i**, Liver sections with representative haematoxylin and eosin (**h**) and Ly6G (**i**) staining of the

893   mice in (**d**). Scale bar, 100 μm. Graphs indicate histological scoring of % confluent necrosis (**h**)

894   and Ly6G-positive cells (neutrophils) (**i**). Untreated sham, n = 6 mice; wild-type dosed with

895   isotype control antibody, n = 9 mice; wild-type dosed with clone D1, n = 8 mice. Lines indicate

896   the median, circles individual mice. P value two-tailed unpaired t-test.
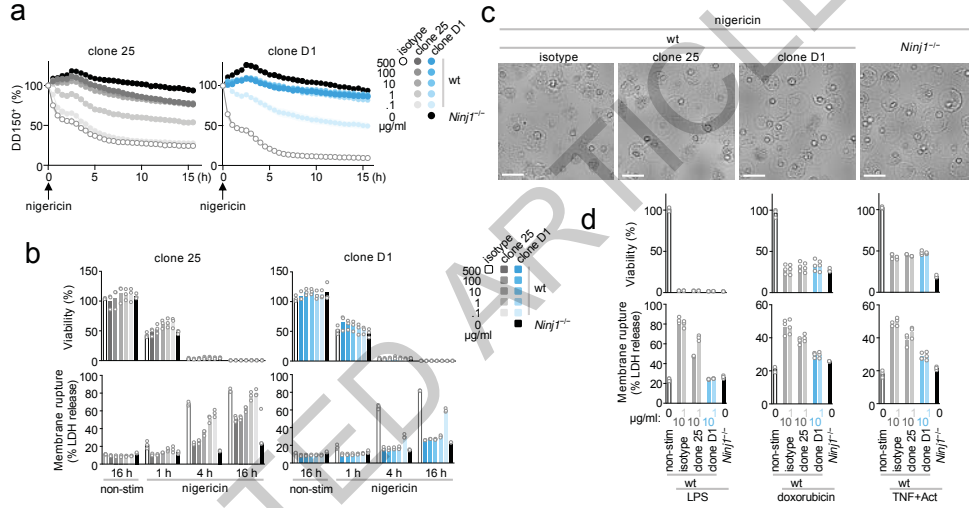
897

a

Ninj1⁻/⁻

EV immunization

~15,000
NINJ1 protein-bound
IgM⁻ B cells

FACS sorting

217
recombinant
Abs

ELISA, FACS, NGS, and recombinant Ab

Light   Heavy
chain   chain

functional validation

characterization

b

Membrane rupture
(LDH release score)

1.2
1.0
0.8
0.6
0.4
0.2

clone 25

clone D1

Ninj1⁻/⁻

non-stim

c

YOYO⁺ (%)

80
60
40
20

isotype clone D1
200 ○ ●
20   ○ ●
μg/ml

0   5   10   15 (h)

mouse NINJ1 transfection

YOYO⁺ (%)

60
40
20

0   5   10   15 (h)

human NINJ1 transfection

d

NINJ1 Wt   Delta C-ter   Delta N-ter   C-ter mutant
(D147VAPR →
A147AAAA)

clone D1

clone 25

FLAG

Relative cell number

APC (fluorescence intensity)

a

clone 25

clone D1

DD150⁺ (%)

nigericin

nigericin

b

clone 25

clone D1

Viability (%)

Membrane rupture (% LDH release)

non-stim   nigericin

non-stim   nigericin

c

d

Viability (%)

Membrane rupture (% LDH release)

LPS   doxorubicin   TNF+Act

a

	non-stim		nigericin	

NINJ1

cont

Wt

clone D1 Fab

Ninj1−/−

cont

scale bar = 10 µm

b

<0.0001

% speck⁺ cells

nigericin: − + − +

pretreat: cont   clone D1 Fab

c

(kDa)
670 158 44

NINJ1 alone

Abs (280 nm)

Retention volume (ml)

NINJ1 +
clone D1 Fab

Abs (280 nm)

Retention volume (ml)

d

NINJ1 alone

NINJ1 +
clone D1 Fab

scale bar = 200 nm   scale bar = 40 nm

e

<0.0001

<0.0001

% Cargo release

cont
NINJ1 alone
NINJ1 + clone 25 Fab
NINJ1 + clone D1 Fab
Melittin

a

cont
clone D1 Fab] wt
$Ninj1^{-/-}$

Membrane rupture
(% LDH release)

non-stim   nigericin

b

YOYO$^+$ (%)

cont
20
200 µg/ml

mouse NINJ1 transfection

YOYO$^+$ (%)

20
cont
200 µg/ml

human NINJ1 transfection

c

human
NINJ1

clone D1

FLAG

Relative cell number

APC (fluorescence intensity)

e

DD150$^+$ (%)

cont
clone D1 Fab] wt
$Ninj1^{-/-}$

nigericin

d

hNINJ1   MESGTEEYELNGGEPGFPGSPDASPARMGWRHGPINVNHYASKKSAAESMLDIALLMAN
mNINJ1   MESGTEEYELNGDLRGPGSPDALPDRWGLENRPINVNHYANKKSAAESMLDIALLMAN
clone 25

hNINJ1   ASQLKAVVEQGPSPAFYVPLVVLISISLVLQIGVGVLLIFLVKYDLNNPAKHAKLDFLNN
mNINJ1   ASQLKAVVEQGNDFAFSVPLVVLISISLVLQIGVGVLLIFLVKYDLNNPAKHAKLDFLNN

hNINJ1   LATGLVFIIVVVNIFITAFGVQKPLMDMAPQQ
mNINJ1   LATGLVFIIVVVNIFITAFGVQKPLMDMAPRQ
clone D1

f

Viability (%)

Membrane rupture
(% LDH release)

µg/ml

non-stim
isotype
clone 25
clone D1
$Ninj1^{-/-}$

wt

non-stim   flagellin   venetoclax   TNF + zVad

Extended Data Fig. 1

a

Membrane rupture
(% LDH release)

antibody:

non	BD clone 50	clone D1	non

wt

Ninj1⁻/⁻

non-stim

nigericin

b

DD150⁺ (%)

antibody:
● non ─ Ninj1⁻/⁻
● clone D1

○ non ─ wt
● BD clone 50

nigericin

8   16 (h)

c

Membrane rupture
(% LDH release)

Pre-treatment:

non
Scramble peptide
NINJ126-37 peptide
isotype Clone D1
non

wt

Ninj1⁻/⁻

non-stim

nigericin

d

mouse
NINJ1

FLAG

Relative cell number

clone D1

BD clone 50

APC (fluorescence intensity)

Extended Data Fig. 2

a

Expi293F cells

NINJ1
-/+ clone D1 Fab or clone 25 Fab

purified NINJ1
(by anti-FLAG/FLAG peptide elution)

size exclusion chromatography

negative stain
electron microscopy

Cargo release assay

b

NINJ1 alone
NINJ1 + clone 25 Fab
NINJ1 + clone D1 Fab

(kDa)
62
49
38
28
18

c

NINJ1 +
clone 25 Fab

scale bar = 200 nm

scale bar = 40 nm

d

NINJ1 +
clone 25 Fab

670   158   44 (kDa)

Abs (280 nm)

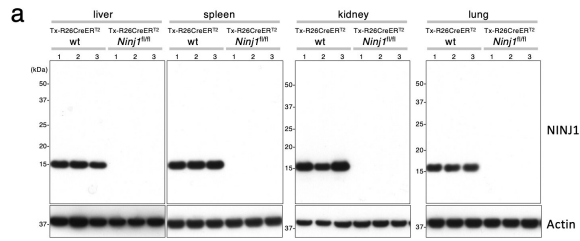Retention volume (ml)

Extended Data Fig. 3

Extended Data Fig. 4

a

liver | spleen | kidney | lung

Tx-R26CreER^T2 wt | Tx-R26CreER^T2 Ninj1^fl/fl | Tx-R26CreER^T2 wt | Tx-R26CreER^T2 Ninj1^fl/fl | Tx-R26CreER^T2 wt | Tx-R26CreER^T2 Ninj1^fl/fl | Tx-R26CreER^T2 wt | Tx-R26CreER^T2 Ninj1^fl/fl

1 2 3 1 2 3   1 2 3 1 2 3   1 2 3 1 2 3   1 2 3 1 2 3

(kDa)
50-
37-
25-
20-
15-   NINJ1

37-   Actin

b

NINJ1 IHC

Tx-R26CreER^T2 wt   Tx-R26CreER^T2 Ninj1^fl/fl

Extended Data Fig. 5

ACCELERATED ARTICLE PREVIEW

a

Survival (%)

○ Tx-R26CreER^T2 wt
● Tx-R26CreER^T2 *Ninj1^fl/fl* } TNF-α/D-Gal

n = 10
P = >0.999

b

H & E

non | TNF-α/D-Gal
| isotype | clone D1

c

Hepatocellular degeneration (score)

0.0077

Cell swelling (score)

0.0078

pre-treatment:
○ non
● isotype
● clone D1 } TNF-α/D-Gal

d

Cleaved caspase-3 IHC

non | TNF-α/D-Gal
| isotype | clone D1

Cleaved Caspase-3 (score)

0.5321

pre-treatment:
○ non
● isotype
● clone D1 } TNF-α/D-Gal

Extended Data Fig. 6

a

b ConA (18 h/later time point)

c

d IRI

e Jo2 anti-Fas

f ConA

g ConA

h IRI

i IRI

Extended Data Fig. 7

nature portfolio

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n/a	Confirmed	
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☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Imaging (live and fixed) data captured using MetaXpress v6.5.4.532, Incucyte S3 2019A, or Leica LAS X v3.5.7. Immunohistochemistry and histology images were acquired with Leica Application Suite v4.6.0. Flow cytometry data were acquired with BD FACSDiva software v9.1 on a BD FACSymphony. Serum ALT and AST were measured in a serum chemistry analyzer (Beckman Coulter AU480). CellTiter-Glo, LDH, HMGB1 ELISA and IL-18 ELISA data were acquired with PerkinElmer EnVision Manager 1.14.3049.1193. Size exclusion chromatography data was collected with Unicorn 7.6 (Cytiva). Negative stain data collection was done using SerialEM Version 3.9.0. For hepatic IRI studies, slides were imaged using 3DHISTECH CaseViewer v2.4 (RRID: SCR_017654; indica labs, Albuquerque, US).
Data analysis	Plots were generated with Prism 9.5.1 (GraphPad Software Inc, La Jolla, CA; RRID:SCR_002798). Imaging data were analysed and prepared using scikit-image 0.19.2. Flow cytometry data were analysed with FlowJo version 10.8.1. Incucyte data was analysed with Incucyte S3 2019A. LocusZoom v0.12 was used to generate regional association plots. For hepatic IRI studies, slides were analysed with the HALO Image Analysis Platform 3.5.3577 (RRID: SCR_018350; indica labs, Albuquerque, US). To evaluate necrosis within the hepatic I/R liver samples, the DenseNet classifier supervised machine learning algorithm (HALO Image Analysis Platform 3.5.3577) was trained to recognize necrotic tissue using the haematoxylin and eosin stain and applied to the entire sample. To quantify immune cell infiltration, neutrophils (Ly6G-positive) were counted using QuPath v0.2.1 (RRID: SCR_018257).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

> The datasets generated during and/or analysed during the current study are available from the corresponding authors upon reasonable request. Source data for animal studies are provided with this paper. GWAS data was obtained from the UK Biobank study (https://doi.org/10.1038/s41588-020-00757-z).

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	*Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data where this information has been collected, and consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.*
Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences   ☐ Behavioural & social sciences   ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes are reported in the figure legends or the Methods section. No prior sample size calculation was performed for in vitro studies. For in vitro experiments involving BMDMs, bone marrow from at least 3 animals per genotype were analyzed for reproducibility. For in vitro experiments involving HEK 293T cells or liposome assays, three technical replicates were chosen per experiment. A minimum of three independent experiments was done for all experiments. This sample size was chosen to match previously published work by our group (Kayagaki et al, Nature 2015; Lee et al, J. Exp. Med. 2018, Kayagaki et al, Sci. Signal.) and is the norm in our field. No prior sample size calculation was performed for in vivo studies. To account for greater variability in the in vivo studies, larger sample sizes (n=6-10) were used in the animal challenge studies (TNF+D-Gal, anti-Fas JO2, and ConA experiments) based on previous experience with the models used. These larger numbers were used to account for the greater variability between wild-type controls in these experiments. Sample sizes were chosen based on standards in the field and are sufficient based on the relatively large quantified differences between groups.
Data exclusions	For analysis of IL-18 in Fig 4f, serum from one animal was excluded in the tamoxifen-treated group due to to insufficient serum quantity. For analysis of ALT & AST serum levels in Fig 4h, one sample was excluded from the isotype control treated group due to marked icterus.
Replication	All experiments were performed independently at least twice with similar results, as described in figure legends. All attempts at replication were successful. Independent experiments and biological replicates were used to ensure reproducibility of results.
Randomization	For in vivo studies involving tamoxifen-treated animals, groups were determined by genotype rather than treatment, and therefore not

| Randomization | randomized. For TNF+D-Gal, anti-Fas JO2, and ConA in vivo studies involving wt mice, animals were age- and sex- matched and randomized to group. Experimental groups were assessed in the same experiment with control groups to eliminate covariates.

For animal procedures related to hepatic ischemia-reperfusion injury mixed sex cohorts were used; animals were randomized to group and analyses blinded.

For all in vitro experiments, samples were not randomized because samples were not allocated into experimental groups. |
|---|---|
| Blinding | Imaging was performed blindly and automatically using an ImageXpress Micro Confocal or Incucyte system. Histological scoring and evaluation and serum analyses were performed blinded. For other experiments, mice and cell lines were picked and treated by the same individual, so blinding to genotype and treatment as well as during data collection and analysis was not possible. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Supplementary Table 1 describes all antibodies used in this study.
Validation	Any antibody validation of commercial primary antibodies is indicated in Supplementary Table 1 and can be found on the manufacturer's websites. Non-commercial antibodies generated for this study were validated as indicated below and within the manuscript.

Clone D1-575 anti-NINJ1 antibody was validated for flow cytometry by comparing 293T cells transiently transfected with NINJ1 expression plasmids (this study, Fig1d)
Clone 80 anti-NINJ1 antibody was validated for immunohistochemistry and immunofluorescence by comparing tissues or cells from WT and Ninj1 KO mice (this study)
Clone 25 anti-NINJ1 antibody was previously validated for WB by comparing lysates from wild-type and NINJ1-/- BMDMs (Kayagaki et al . 2021 Nature 591(7848):131-136) |

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	HEK293T cells (ATCC CRL-3216), Expi293F cells (Thermo Fisher Scientific, cat#A14527), BALB/3T3 clone A31 (ATCC CCL-163), CHO (Genentech), EL-4-B5 feeder cells (Roche)
Authentication	Cell lines were authenticated by short tandom repeat (STR) profiling and regular single nucleotide polymorphism (SNP) fingerprinting. STR profiles are determined for each line using the Promega PowerPlex 16 System. This is performed once and compared to external STR profiles of cell lines (when available) to determine cell line ancestry. SNP profiles are performed each time new stocks are expanded for cryopreservation. Cell line identity is verified by high-throughput SNP profiling using Fluidigm multiplexed assays. SNPs were selected based on minor allele frequency and presence on commercial genotyping platforms. SNP profiles are compared to SNP calls from available internal and external data (when available) to determine or confirm ancestry.
Mycoplasma contamination	Cells negative for mycoplasma.
Commonly misidentified lines (See ICLAC register)	Not used.

Animals and other research organisms

Laboratory animals	Mice (Mus musculus) strains including Ninj1-/- and WT littermates (Ninj1+/+) (Kayagaki et al . 2021 Nature 591(7848):131-136), and Ninj1 fl/fl Rosa26.Cre.ERT2/+ and WT littermates (Ninj1 +/+ Rosa26.Cre.ERT2/+) (this study) were maintained on a C57BL/6N genetic background. Ninj1 fl/fl Rosa26.Cre.ERT2/+ and WT littermates (Ninj1 +/+ Rosa26.Cre.ERT2/+) were dosed with tamoxifen at 6 to 9 weeks of age. For TNF plus D-Gal studies 8 to 14 week old female mice were used. For TNF plus D-gal studies antibody treatment studies, 8 to 14 week old age matched C57BL/6J mice were used (Jackson Labs, strain #000664). For ConA and anti-Fas(JO2) studies, 9 to 11 week old male mice were used. For ConA and anti-Fas (JO2) studies involving antibody treatment, 9 to 11 week old age matched C57BL/6N male mice were used (Charles River Labs). For hepatic IRI studies, mixed-sex cohorts of 6 to 10 week old C57Bl/6J wild-type animals were purchased from Jackson Laboratories (strain #000664). Mice were housed in individually ventilated cages within animal rooms maintained on a 14:10-hour, light:dark cycle with ad libitum access to food and water. Animal rooms were temperature and humidity-controlled, between 68-79°F and 30-70% respectively, with 10 to 15 room air exchanges per hour.
Wild animals	The study did not involve wild animals.
Reporting on sex	TNF/Dgal in vivo studies were performed on 8 to 14 week old female mice. ConA and anti-Fas(JO2) studies were performed on 9 to 11 week old male mice. Hepatic IRI studies were perfomed on mixed-sex cohorts of 6 to 10 week old mice.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	All animal procedures were conducted under protocols approved by the Genentech Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in accordance with the Guide for the Care and Use of Laboratory Animals and applicable laws and regulations. All animal procedures related to hepatic ischemia-reperfusion injury were conducted under protocols approved by the Animal Care Committee at The Hospital for Sick Children and in accordance with animal care regulation and policies of the Canadian Council on Animal Care.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	HEK293T cells (ATCC) cells were transfected with NINJ1 expression plasmids using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were stained with monoclonal antibodies, followed by APC-conjugated anti-mouse IgG (Thermo Fisher Scientific) and then propidium iodide (PI; 2.5 µg/mL; BD Biosciences). Live PI– cells were analyzed in a FACSymphony (Becton Dickinson).
Instrument	BD FACSymphony
Software	Data was acquired using BD FACSDiva Software v9.1, and analyzed using FlowJo 10.8.1
Cell population abundance	No sorting was performed.
Gating strategy	Dead cells that stained with PI (BD Biosciences) were excluded from analyses of cells.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.