

# Growth Hormone Stops Excessive Inflammation After Partial Hepatectomy, Allowing Liver Regeneration and Survival Through Induction of H2-B1/HLA-G

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**BACKGROUND AND AIMS:** Growth hormone (GH) is important for liver regeneration after partial hepatectomy (PHx). We investigated this process in C57BL/6 mice that express different forms of the GH receptor (GHR) with deletions in key signaling domains.

**APPROACH AND RESULTS:** PHx was performed on C57BL/6 mice lacking GHR (*Ghr*<sup>-/-</sup>), disabled for all GH-dependent Janus kinase 2 signaling (*Box1*<sup>-/-</sup>), or lacking only GH-dependent signal transducer and activator of transcription 5 (STAT5) signaling (*Gbr391*<sup>-/-</sup>), and wild-type littermates. C57BL/6 *Ghr*<sup>-/-</sup> mice showed striking mortality within 48 hours after PHx, whereas *Box1*<sup>-/-</sup> or *Gbr391*<sup>-/-</sup> mice survived with normal liver regeneration. *Ghr*<sup>-/-</sup> mortality was associated with increased apoptosis and elevated natural killer/natural killer T cell and macrophage cell markers. We identified H2-B1, a key immunotolerance protein, which is up-regulated by PHx through a GH-mediated, Janus kinase 2-independent, SRC family kinase-dependent pathway. GH treatment was confirmed to up-regulate expression of the human homolog of *H2-B1* (human leukocyte antigen G [*HLA-G*]) in primary human hepatocytes and in the serum

of GH-deficient patients. We find that injury-associated innate immune attack by natural killer/natural killer T cell and macrophage cells are instrumental in the failure of liver regeneration, and this can be overcome in *Ghr*<sup>-/-</sup> mice by adenoviral delivery of H2-B1 or by infusion of HLA-G protein. Further, H2-B1 knockdown in wild-type C57BL/6 mice showed elevated markers of inflammation after PHx, whereas *Ghr*<sup>-/-</sup> backcrossed on a strain with high endogenous *H2-B1* expression showed a high rate of survival following PHx.

**CONCLUSIONS:** GH induction of *H2-B1* expression is crucial for reducing innate immune-mediated apoptosis and promoting survival after PHx in C57BL/6 mice. Treatment with HLA-G may lead to improved clinical outcomes following liver surgery or transplantation. (HEPATOLOGY 2021;73:759-775).

The innate immune system has been shown to play a major role in liver regeneration, with key roles being attributed to hepatic natural killer (NK) and natural killer T cells (NKT), the major resident lymphoid cell populations that are located

*Abbreviations:* ad-GFP, adenovirus-expressing GFP; ad-H2-B1, adenovirus-expressing H2-B1 and GFP; DAMP, danger-associated molecular pattern molecule; ERK, extracellular signal-regulated kinase; GH, growth hormone; GHD, GH deficient; GHR, growth hormone receptor; HLA-G, human leukocyte antigen G; IFN $\gamma$ , interferon  $\gamma$ ; IGF-1, insulin-like growth factor 1; IHC, immunohistochemistry; IL, interleukin; JAK2, Janus kinase 2; MHC, major histocompatibility complex; NK, natural killer; NKT, natural killer T cells; nt, nontargeting; PHx, partial hepatectomy; rbGH, recombinant human growth hormone; RNAi, RNA interference; SFK, SRC family kinase; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

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particularly in the liver sinusoids. These cells normally protect against viral infection and tumor formation, but are now known to contribute to the progression of liver injury in rodent models. Partial hepatectomy (PHx) in mice results in an increase in hepatic NK, and particularly hepatic NKT cell number.<sup>(1)</sup> These inflammatory cells have complex roles in the regeneration process, in some cases promoting regeneration, but in most cases acting to delay or prevent regeneration, such as through NK or invariant NKT cell induction of interferon  $\gamma$  (IFN $\gamma$ ).<sup>(1,2)</sup> Both NK and NKT cells recognize damaged/stressed hepatocytes and kill them using Fas ligand (NKT cells) or tumor necrosis factor–related apoptosis-inducing ligand, as well as lysis through granzyme B and perforin.<sup>(1,2)</sup> These processes are amplified by activated NKT and Kupffer cells, as these enhance NK cell cytotoxicity and IFN $\gamma$  production.<sup>(2)</sup>

Previous studies found that PHx in transgenic mice overexpressing the growth hormone (GH) receptor antagonist G118R resulted in a high level of early mortality (43%) as well as a major and delayed reduction in hepatocyte proliferation.<sup>(3)</sup> Mortality rates of approximately 40% have been reported for mice with deletion of the key priming factors tumor necrosis factor (TNF) receptor and interleukin (IL)-6,<sup>(4,5)</sup> although mortality was more delayed than for

growth hormone receptor (GHR) antagonist mice that still retain significant GH action (hepatic insulin-like growth factor 1 [IGF-1] transcript level around 45% of wild type). To define the GH signaling roles following PHx, we used a panel of mutant GHR knock-in mice that sequentially remove the GH signaling elements in the receptor cytoplasmic domain (Fig. 1A).<sup>(6,7)</sup> These knock-in lines have been key in defining a second pathway used by the GHR, which initiates from a SRC family kinase (SFK) bound to the receptor rather than the conventional Janus kinase 2 (JAK2), which binds to the Box1 sequence within the receptor cytoplasmic domain. This second pathway activates extracellular signal–regulated kinase (ERK) through RAS, as opposed to the conventional JAK/signal transducer and activator of transcription (STAT) pathway.<sup>(6,8)</sup> We were able to identify that the key component responsible for the high mortality in mice deficient in GHR following PHx was the absence of GH-mediated SFK activation, which leads to a dysregulated inflammatory response. We have shown that GH induction of the immunotolerance protein H2-B1 (homolog of human leukocyte antigen G [HLA-G] in humans) through SFK signaling is vital in suppressing this inflammatory response and preventing mortality in C57BL/6 mice. A number

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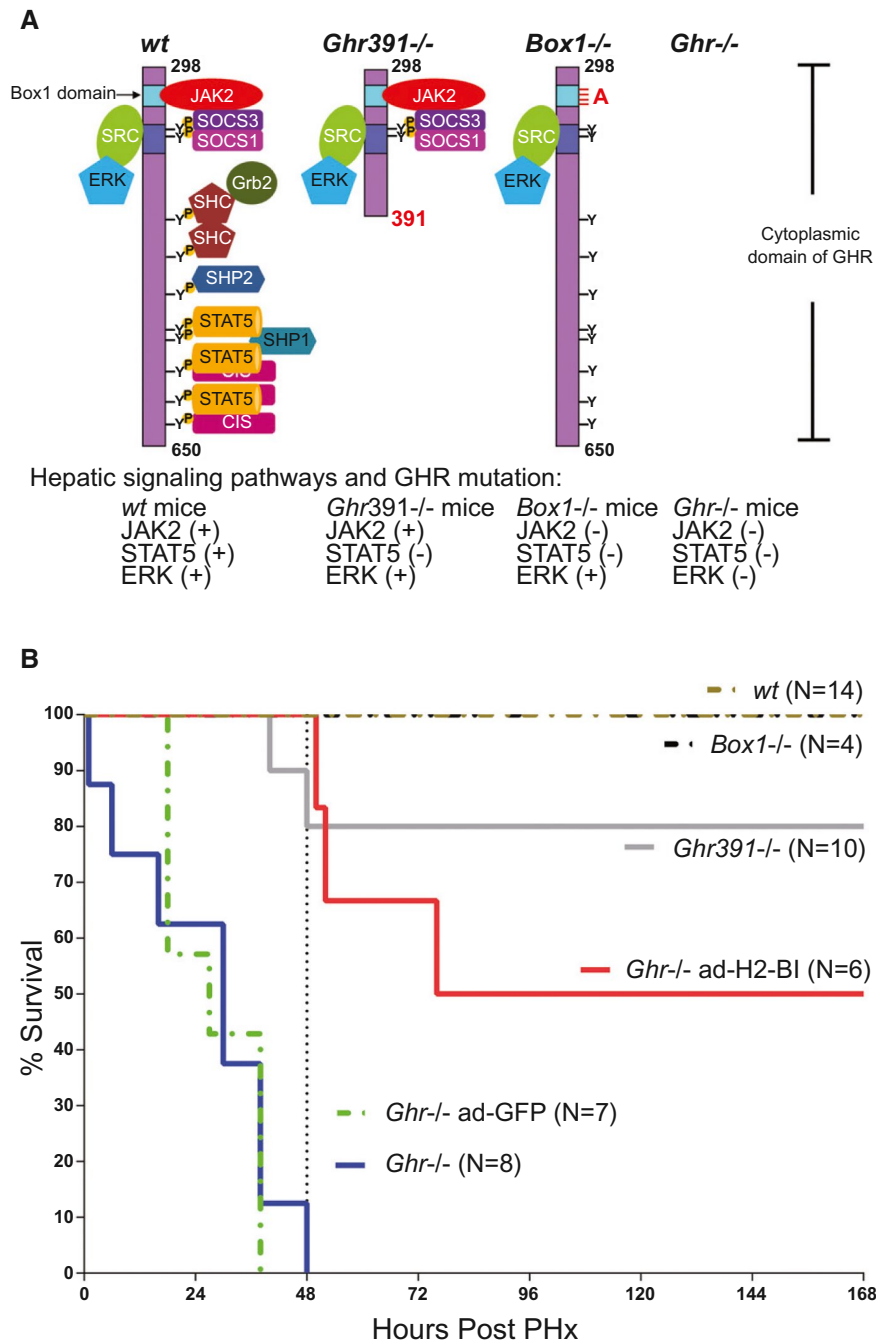
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**FIG. 1.** Survival of *Ghr* mutant C57BL/6 mouse strains after PHx. (A) Signaling capabilities of GHR mutant mice. The cytoplasmic domain of receptor is shown, with remaining signaling pathways for each mutant. *Ghr391*<sup>-/-</sup> eliminates STAT5 signaling; *Box1*<sup>-/-</sup> removes all JAK/STAT signaling while maintaining SFK signaling; and *Ghr*<sup>-/-</sup> removes all GH mediated signaling. (B) Survival of mouse strains after PHx (number of mice for each genotype shown), in which the mortality of *Ghr*<sup>-/-</sup> is partially rescued by adenoviral expression of *H2-BI*. Abbreviations: CIS, cytokine-inducible SH2 (Src homology 2) protein; SHC, Src Homology and Collagen protein; SHP1, Src homology region 2 domain-containing phosphatase-1; SHP2, Src homology region 2 domain-containing phosphatase-2.

of studies provide increasing evidence demonstrating the potent immunosuppressive effects mediated by HLA-G.<sup>(9,10)</sup> As we identified that HLA-G is

also GH-regulated in humans, these findings may have clinical applications in treatment of liver failure and transplantation.

## Materials and Methods

C57BL/6 mice with partial or total loss of GH-induced STAT5 or JAK2 activation by GH were derived as previously described.<sup>(6,7)</sup> Two-thirds partial hepatectomy on 8–13 week old male mice were performed as previously described.<sup>(11)</sup> Further details on mice including backcrossing, adenoviral expression, and osmotic pump delivery of HLA-G are detailed in the Supporting Information. Animal experimental protocols were approved by the University of Queensland Animal Ethics Committee and the Office of the Gene Technology Regulator, Canberra, ACT.

Details regarding expression and purification of HLA-G, construction of adenoviral delivery vectors, histology and TUNEL staining, western blot analysis, quantitative PCR (qPCR), quantification of HLA-G from serum, hepatocyte NK cell co-culture apoptosis assay, AML12 mouse hepatocyte culture, human hepatocyte culture, and statistical analysis are provided in the Supporting Information.

Blood and specimens for human study analysis were taken from patients after informed consent, approved by the human research ethics committee at the Princess Alexandra Hospital, Brisbane (HREC/12/QPAH/126 and SSA/12/QPAH/140). The culture of the human hepatocyte cells was approved by the Tokyo Metropolitan Medical Examiner's Office (No. 788, 1996).

## Results

### PHx IN C57BL/6 GHR NULL MICE IS LETHAL

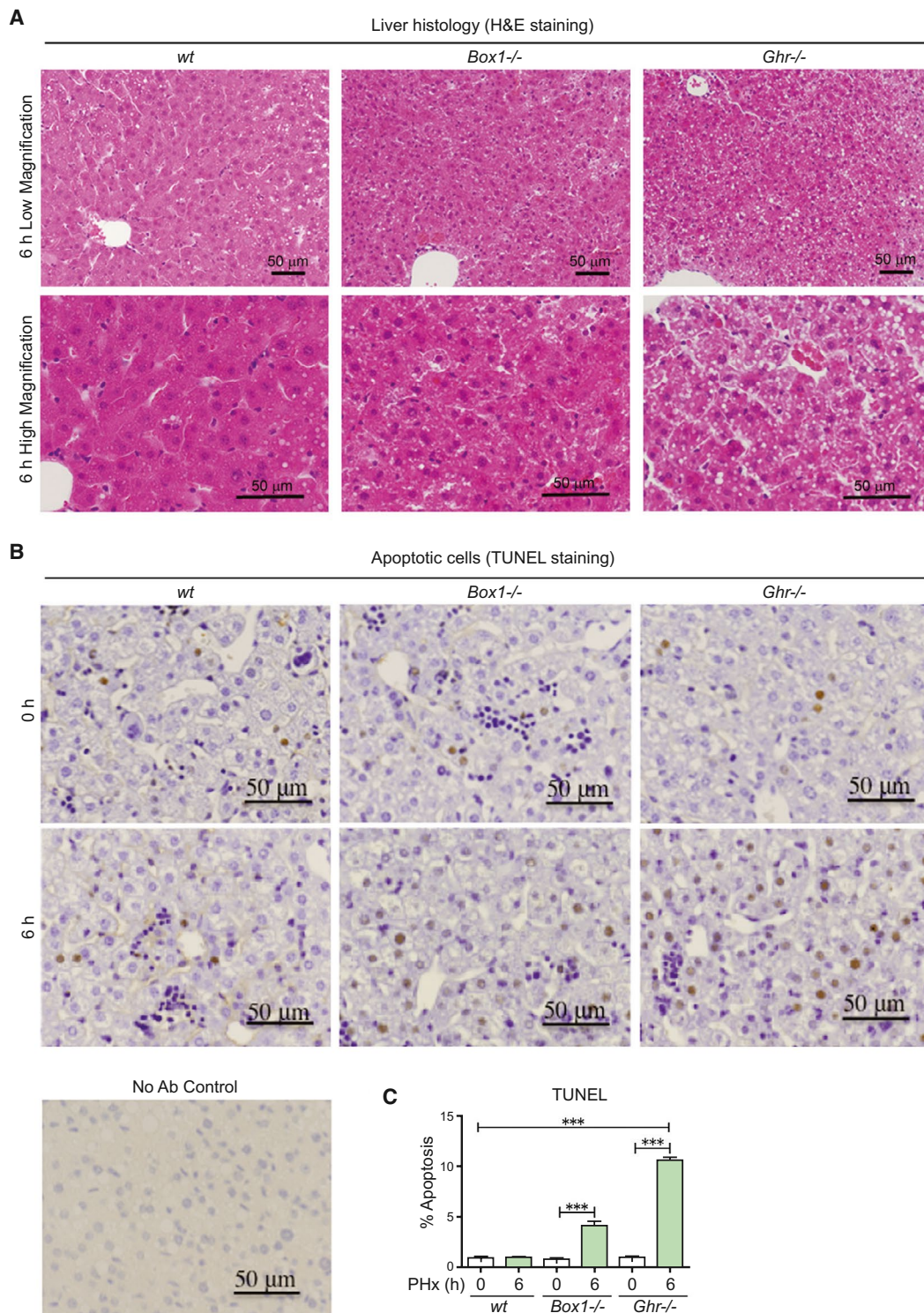
Initially we studied the survival of *Ghr* mutant (*Ghr391*<sup>-/-</sup>, *Box1*<sup>-/-</sup>, and *Ghr*<sup>-/-</sup>) and wild-type (*wt*) mouse strains over a 7-day period after two-thirds PHx. Strikingly, only *Ghr*<sup>-/-</sup> mice exhibited significant mortality, so that all but one of the eight *Ghr*<sup>-/-</sup> mice were dead within 38 hours (the remaining mouse had to be euthanized 48 hours following PHx) (Fig. 1B). Over half of the *Ghr*<sup>-/-</sup> mice were dead within 30 hours following PHx. All other *Ghr* mutant mouse strains appeared healthy 7 days later, except for the loss of two *Ghr391*<sup>-/-</sup> mice (2 of 10) around 48 hours. Importantly, the dwarf *Box1*<sup>-/-</sup> mice lacking ability to

activate the JAK/STAT pathway, but retaining SRC/ERK signaling, showed no mortality after PHx.

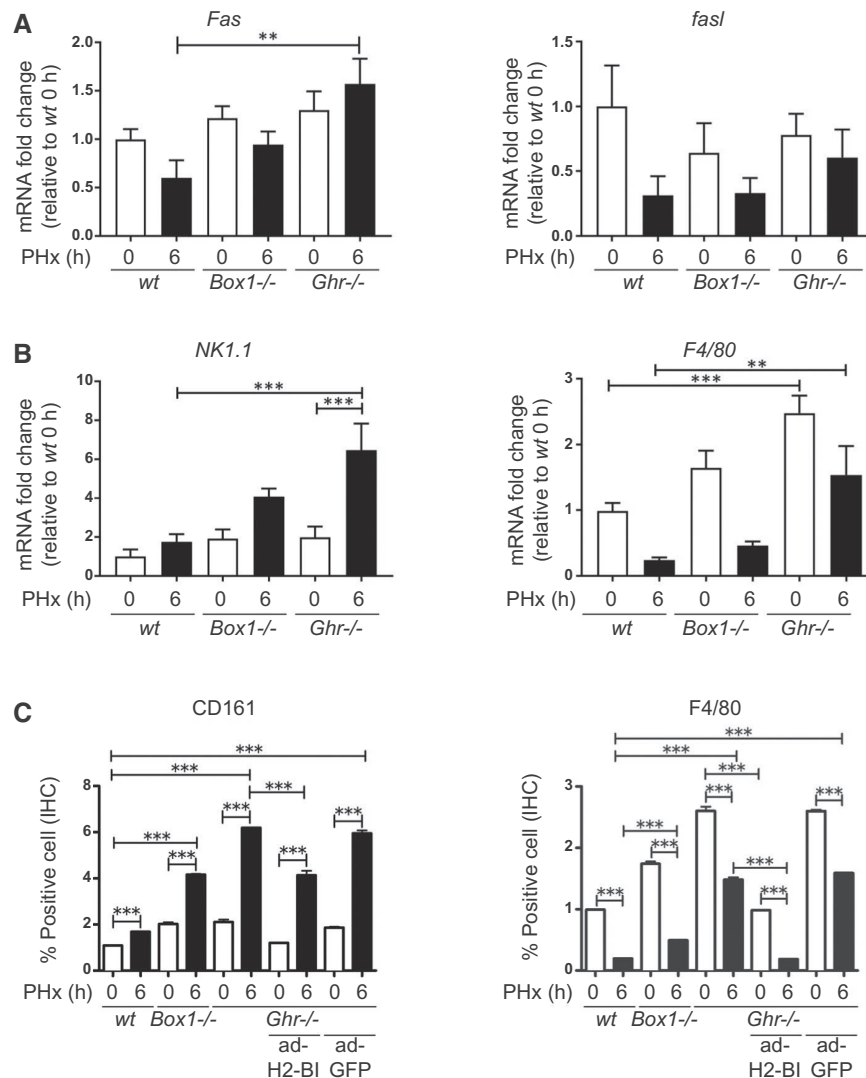
Analysis of liver sections by hematoxylin and eosin staining was undertaken at 6 hours after PHx because of the early mortality of *Ghr*<sup>-/-</sup> mice. No change was evident in *wt* and *Box1*<sup>-/-</sup> liver histology compared with the nonoperated controls, whereas *Ghr*<sup>-/-</sup> liver sections revealed numerous lipid droplets with ballooning of hepatocytes (Fig. 2A). At 6 hours after PHx there was a significant increase in apoptosis (by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling [TUNEL]) for *Box1*<sup>-/-</sup> mice and a striking increase in *Ghr*<sup>-/-</sup> mice (Fig. 2B,C), whereas there was no significant difference in apoptosis between genotypes before PHx. This was confirmed by cleaved caspase-3 staining (Fig. 7B). Increased apoptosis was accompanied by a significant rise in *Fas* transcript in *Ghr*<sup>-/-</sup> (but not *Box1*<sup>-/-</sup>) relative to *wt* mice, although not of *Fas* ligand (Fig. 3A).

### NK, NKT, AND MACROPHAGE CELL MARKERS ARE UP-REGULATED IN C57BL/6 GHR NULL MICE FOLLOWING PHx

Although bromodeoxyuridine (BrdU) incorporation or proliferating cell nuclear antigen (PCNA) expression analysis are common methods for analyzing liver regeneration following PHx, no significant increase in BrdU-positive cells or PCNA staining could be detected 24 hours following PHx in mice.<sup>(12)</sup> Given the extent of rapid mortality (Fig. 1B), and the increased apoptosis following PHx in the *Ghr*<sup>-/-</sup> mice (Fig. 2B,C), we hypothesized that immune attack was the central issue. Therefore, we measured transcripts for the NK/NKT marker NK1.1 (CD161) and the macrophage/Kupffer cell marker F4/80, and investigated their protein expression by immunohistochemistry (IHC). Transcript for NK1.1 and F4/80 were significantly elevated approximately 3-fold and 5-fold, respectively, in *Ghr*<sup>-/-</sup> mice relative to *wt* mice at 6 hours following PHx (Fig. 3B). This correlated with stronger staining for CD161 and F4/80 in liver sections from *Ghr*<sup>-/-</sup> mice 6 hours following PHx compared with *wt* mice (Fig. 3C and Supporting Fig. S1), indicating strongly increased infiltration or activation of NK/NKT cells and macrophages into the *Ghr*<sup>-/-</sup> liver following PHx.



**FIG. 2.** The livers of *Ghr<sup>-/-</sup>* mice 6 hours following PHx show lipid droplets, ballooning, and increased apoptosis compared with *wt* and *Box1<sup>-/-</sup>* mice. (A) Liver histology from *Box1<sup>-/-</sup>* and *Ghr<sup>-/-</sup>* mice compared with *wt* 6 hours after PHx, showing lipid droplets and ballooning in *Ghr<sup>-/-</sup>* liver. Lower panel shows high magnification images. (B) TUNEL staining in livers at 0 hours and 6 hours after PHx, showing increased apoptosis in *Box1<sup>-/-</sup>* mice compared with *wt*, but particularly in *Ghr<sup>-/-</sup>* mice compared with *wt*. Staining without primary antibody is shown (No Ab Control). (C) TUNEL quantification in livers at 0 hours and 6 hours after PHx. Six to nine sections were analyzed for each mouse at 0 hours and 6 hours following PHx, with n = 4–11 mice per group. Abbreviation: H&E, hematoxylin and eosin.

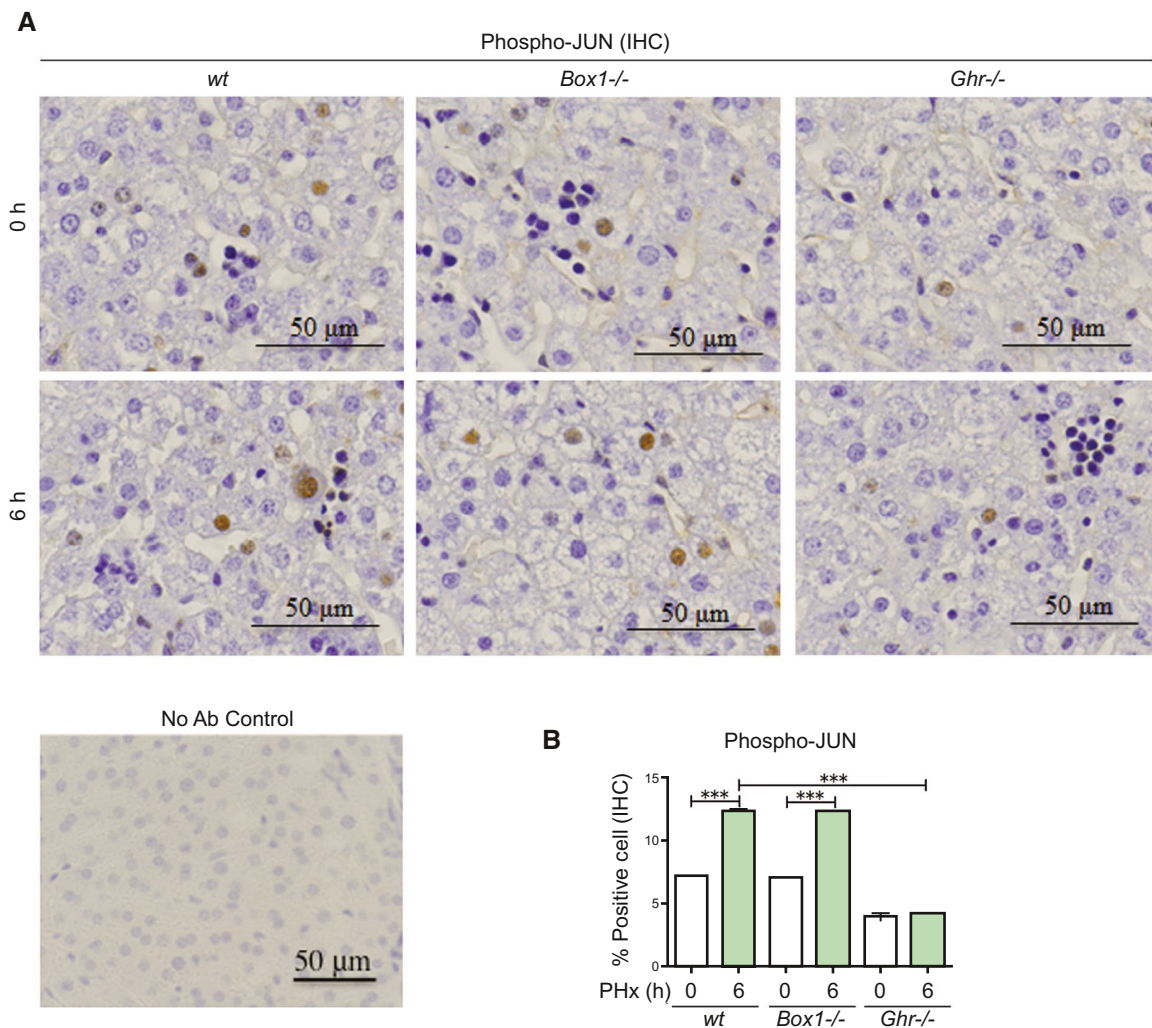


**FIG. 3.** Immune attack is elevated in *Ghr*<sup>-/-</sup> mice compared with *wt* mice following PHx, and this elevation can be ameliorated by expression of *H2-BI*. (A) *Fas* and *fasl* transcripts at 0 hours and 6 hours after PHx, showing significantly elevated *Fas* transcript relative to *wt* in *Ghr*<sup>-/-</sup> mice at 6 hours. (B) NK/NKT marker *NK1.1* and macrophage marker *F4/80* transcripts in livers at 0 hours and 6 hours after PHx, showing increased level of NK/NKT marker 6 hours after PHx in *Ghr*<sup>-/-</sup> mice, and elevated macrophage marker at both 0 hours and 6 hours in *Ghr*<sup>-/-</sup> relative to *wt*. (C) Protein expression of *NK1.1* and *F4/80* by IHC, showing correspondence with transcript levels. Also shown is the ability of ad-H2-BI, but not ad-GFP, to reverse these changes in *Ghr*<sup>-/-</sup> mice (n = 4–6 mice per group).

## ACTIVATION OF JUN KINASE IS IMPAIRED IN C57BL/6 GHR-NULL MICE

Hepatic *jun* expression is a key requirement for normal regeneration after PHx, with its deletion resulting in approximately 50% survival of mice together with impaired regeneration.<sup>(13)</sup> A striking decrease in hepatic phospho-JUN was evident in the *Ghr*<sup>-/-</sup>, but not in mice lacking the ability to activate

JAK2 by GH (i.e., *Box1*<sup>-/-</sup>), implying that JUN kinase is being largely activated by the SRC kinase pathway in *wt* mice and *Box1*<sup>-/-</sup>, but not in *Ghr*<sup>-/-</sup> mice (Fig. 4A,B and Supporting Fig. S2). The lack of GHR-mediated ERK1/2 activation by SRC is evident in *Ghr*<sup>-/-</sup> liver 6 hours after PHx compared with *wt* and *Box1*<sup>-/-</sup> mice (Fig. 5A). Accordingly, GH stimulated JUN phosphorylation in AML12 hepatocyte cells, and this was suppressed by SRC inhibitor PP2 (Fig. 5B).

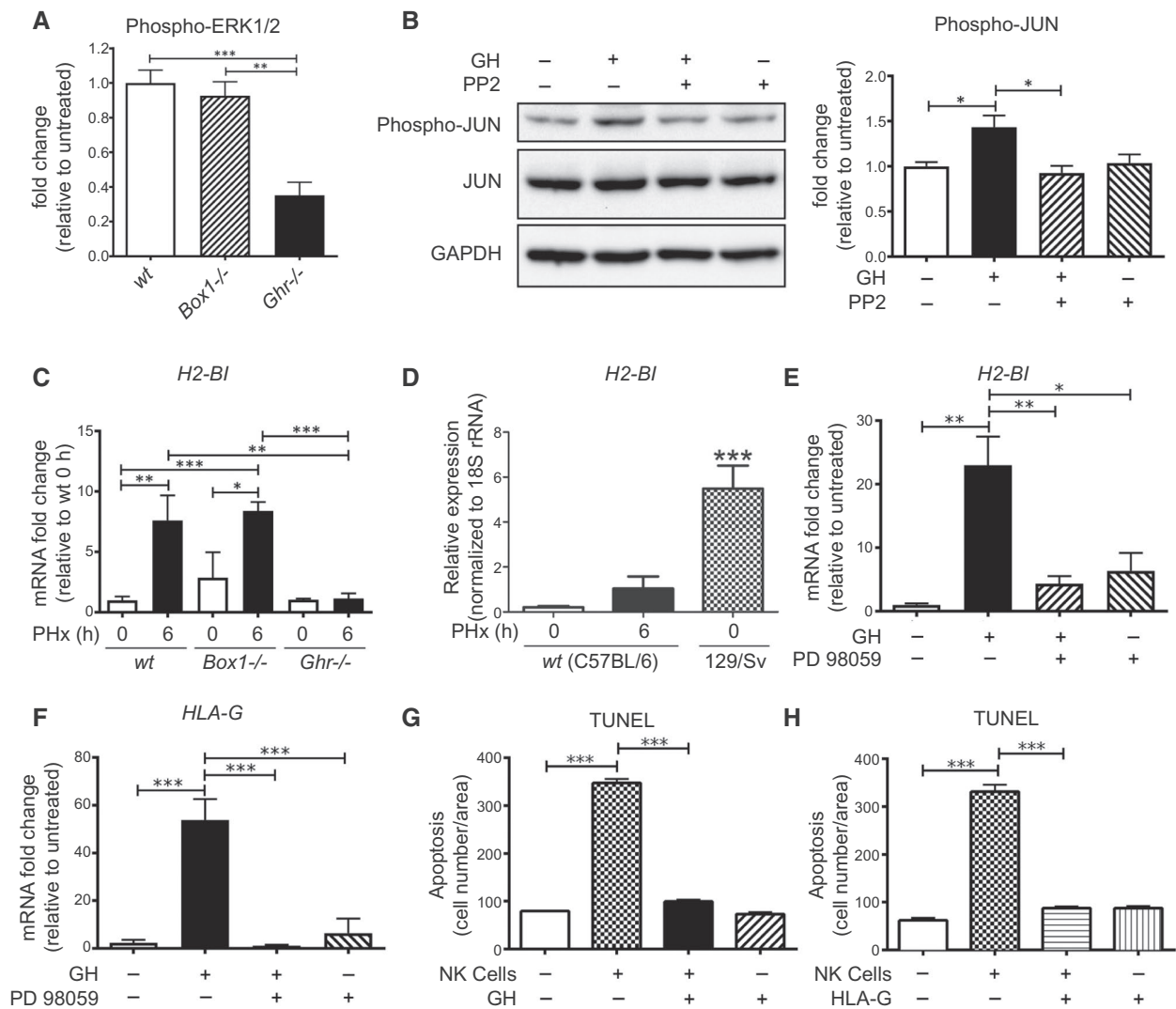


**FIG. 4.** JUN is activated in livers of *wt* and *Box1<sup>-/-</sup>* mice after PHx, but not in *Ghr<sup>-/-</sup>* mice. (A) JUN activation/phosphorylation is deficient in *Ghr<sup>-/-</sup>* mice, but not in *wt* or *Box1<sup>-/-</sup>* mice, 6 hours after PHx. Representative high-magnification IHC sections are shown. Staining without primary antibody is shown (No Ab Control). (B) Quantification of large nuclei with phospho-JUN immunoreactivity is shown (five sections per mouse,  $n = 3$  mice per group).

## GH UP-REGULATES THE EXPRESSION OF THE IMMUNOTOLERANCE GENE H2-BL IN MICE AND HLA-G IN HUMANS

We previously identified a restricted set of hepatic gene transcripts unique to *Ghr<sup>-/-</sup>* mice by comparison of Illumina (San Diego, CA) expression profiles for *Ghr391*, *Box1<sup>-/-</sup>*, and *Ghr<sup>-/-</sup>* mice.<sup>(6)</sup> This list included the transcript for a key immunotolerance gene, *H2-B1*.<sup>(14)</sup> Importantly, expression of the human homolog of *H2-B1*, *HLA-G*, correlates with

lower rates of rejection in liver transplant recipients.<sup>(15)</sup> We observed a 5-fold lower expression of *H2-B1* in *Ghr<sup>-/-</sup>* liver relative to *wt* and *Box1<sup>-/-</sup>* liver at 6 hours following PHx (Fig. 5C). *H2-B1* expression increased significantly following PHx in *wt* mice; however, the increase at 6 hours is not seen in *Ghr<sup>-/-</sup>* mice. A previous study in 129/Sv *Ghr<sup>-/-</sup>* mice reported no mortality after PHx<sup>(16)</sup>; therefore, we investigated the difference in expression of hepatic *H2-B1* between *wt* C57BL/6 and 129/Sv strains. We found that the *H2-B1* transcript is around 5-fold higher basally in 129/Sv mice, and this level is greater than that reached at 6 hours



**FIG. 5.** GH activates JUN and up-regulates *H2-BI* and *HLA-G* expression in a SRC-ERK pathway-dependent manner, protects hepatocytes from NK cell attack, and *H2-BI* expression is induced in livers of *wt* and *Box1<sup>-/-</sup>* C57BL/6 mice after PHx, but not in *Ghr<sup>-/-</sup>* C57BL/6 mice. (A) ERK1/2 activation in *Ghr<sup>-/-</sup>* liver 6 hours after PHx is impaired compared with *Box1<sup>-/-</sup>* and *wt* mice (quantification from western blot, n = 3 for each mouse line). (B) Immunoblot showing suppression of GH-stimulated JUN phosphorylation by SRC kinase inhibitor PP2 (10  $\mu$ M) in AML12 hepatocyte cells and its quantification (n = 3 wells per condition). (C) *H2-BI* transcript level does not increase after PHx in *Ghr<sup>-/-</sup>*, whereas *wt* and *Box1<sup>-/-</sup>* show a significant increase (n = 3-6 per group). (D) Basal expression of *H2-BI* transcript in the 129/Sv mouse strain is high compared with C57BL/6. (E) GH treatment increases *H2-BI* transcript in AML12 mouse hepatocyte cells, and this increase is blocked by the MEK inhibitor PD98059 (20  $\mu$ M) (n = 3 replicates per group). Inhibition of ERK1/2 is shown in Supporting Fig. S3. (F) GH treatment increases *HLA-G* transcript in human hepatocyte cells, and this increase is blocked by the MEK inhibitor PD98059 (20  $\mu$ M) (n = 3 replicates per group). Quantification of TUNEL staining of human hepatocytes following co-culture with human NK cells and treatment with either GH (G) or HLA-G (H) protein. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PP2, pyrazolopyrimidine (PP)2; and rRNA, ribosomal RNA.

following PHx in *wt* C57BL/6 mice (Fig. 5D). We also found that *Ghr<sup>-/-</sup>* mice on a mixed 129/Sv/C57BL/6 background did not exhibit significant mortality following PHx (Fig. 7A), in accord with the previous report.<sup>(16)</sup>

The SRC pathway stimulated by GH is the only signaling pathway remaining in *Box1<sup>-/-</sup>* mice.<sup>(6)</sup> This implies that *H2-BI* transcript expression is SRC-dependent, through ERK or additional SRC-dependent pathways such as JNK. We used



GH-responsive murine AML12 hepatocytes and showed that *H2-B1* transcript is induced by GH, and that this induction is blocked by the upstream MEK1 inhibitor PD98059 (Fig. 5E). We also found that *HLA-G* transcripts (Fig. 5F) and protein levels (Supporting Fig. S4) are induced by GH in primary human hepatocytes in a similar manner to that shown for AML12 cells (Fig. 5E).

## GH OR HLA-G BLOCKS NK CELL-MEDIATED APOPTOSIS OF HUMAN HEPATOCYTES *IN VITRO*

The importance of GH and HLA-G for hepatocyte viability was evident from the finding that hepatocyte apoptosis induced by co-culture of human NK-cells with primary human hepatocytes is prevented with GH (Fig. 5G) or HLA-G addition (Fig. 5H).

## ADENOVIRAL EXPRESSION OF H2-BL OR INFUSION OF HLA-G PROTEIN PERMITS LIVER REGENERATION AND SURVIVAL OF C57BL/6 GHR-NULL MICE FOLLOWING PHx

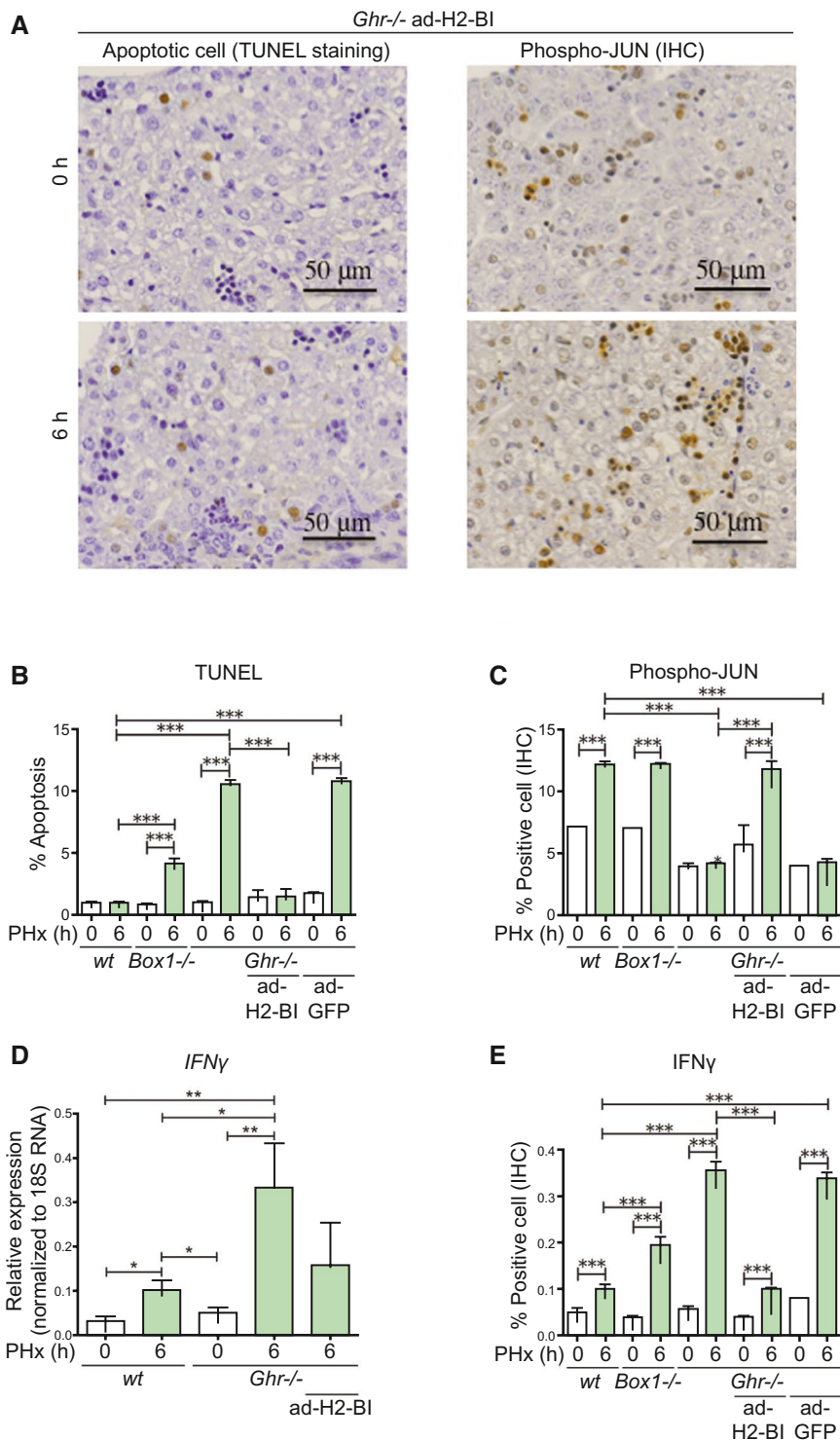
To establish that H2-B1 deficiency is the critical factor in the high mortality seen in C57BL/6 *Ghr*<sup>-/-</sup> mice, we used adenoviral expression of *H2-B1* and GFP, or GFP alone as a control, delivered by tail vein injection to determine whether this could confer increased survival of *Ghr*<sup>-/-</sup> mice after PHx (Supporting Fig. S5 showing H2-B1 transcript levels). Strikingly, the *H2-B1* vector was able to prevent 48-hour mortality, and 50% of all adenovirus-expressing H2-B1 and GFP (ad-H2-B1)-injected mice were alive after 7 days, whereas all control adenovirus-expressing GFP (ad-GFP) mice were dead within 48 hours of the PHx (Fig. 1B). In agreement with this, liver regeneration indexes (shown as liver to body mass ratio) were not significantly different for *Ghr391* or *Box1*<sup>-/-</sup> (Supporting Fig. S6A,B); however, for *Ghr*<sup>-/-</sup> mice (with ad-H2-B1), regeneration was significantly impaired (Supporting Fig. S6C). Accordingly, apoptosis in liver samples taken at 6 hours following PHx was markedly decreased in ad-H2-B1 mice compared with control

*Ghr*<sup>-/-</sup> mice or the ad-GFP controls (Fig. 6A,B). Moreover, phospho-JUN (a measure of regeneration) was restored at 6 hours (Fig. 6A,C), whereas the increase in IFN $\gamma$  evident in *Ghr*<sup>-/-</sup> mice was reduced to *wt* levels by ad-H2-B1 expression at both transcript and protein level, a reduction not seen with control ad-GFP-injected mice (Fig. 6D,E). This decrease in IFN $\gamma$  correlated with a decrease in NK/NKT and macrophage cell invasion (Fig. 3B,C). The decline in macrophage F4/80 expression at 6 hours following PHx, compared with basal levels, correlated well with survival (Fig. 3C).

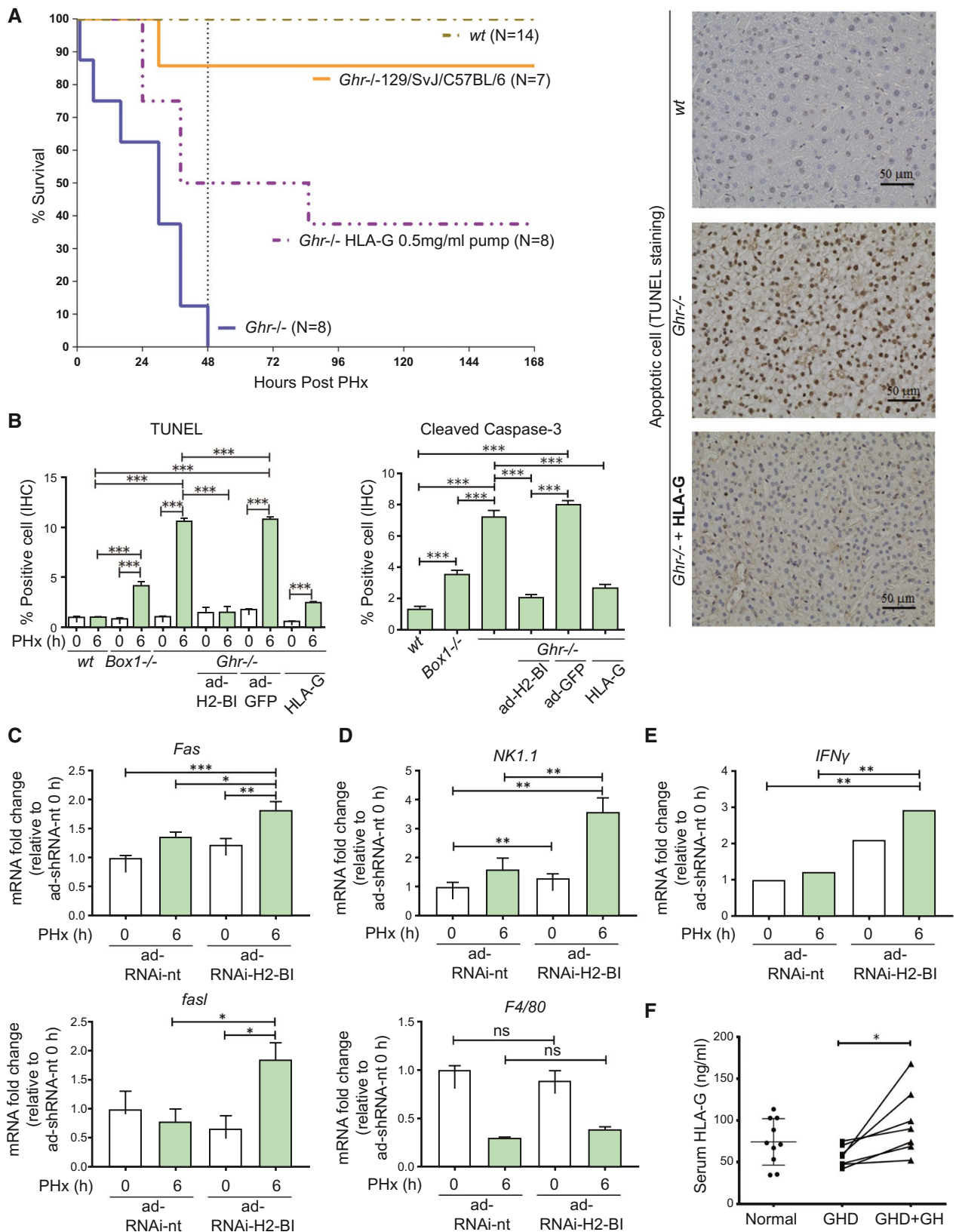
In light of the therapeutic potential for H2-B1/HLA-G, we infused homogeneous recombinant HLA-G<sup>(17)</sup> (shown to bind PIR-B,<sup>(18)</sup> the mouse homolog of LILRB1 [ILT2] and LILRB2 [ILT4]) by osmotic minipump into the *Ghr*<sup>-/-</sup> C57BL/6 mice before and after PHx, and observed that a similar percentage of mice survived (Fig. 7A) and apoptosis was reduced (Fig. 7B), as was observed with the adenovirus delivery of H2-B1.

## RNA-INTERFERENCE KNOCKDOWN OF H2-BL IN C57BL/6 MICE SHOWS ELEVATED MARKERS OF INFLAMMATION FOLLOWING PHx

To further demonstrate that H2-B1 plays an important role in modulating inflammation following PHx we used adenovirus expression of microRNA RNA interference (RNAi) to knock down the expression of H2-B1 in *wt* C57BL/6 mice. Increased transcripts of *Fas* and *Fasl* were shown with ad-RNAi-H2-B1 6 hours following PHx compared with the nontargeting (nt) control, ad-RNAi-nt (Fig. 7C). The NK/NKT marker *NK1.1* also showed elevated transcription at 6 hours after PHx with ad-RNAi-H2-B1 compared with ad-RNAi-nt (Fig. 7D), which correlated with an increase in IFN $\gamma$  (Fig. 7E), although no significant change was identified for *F4/80* (Fig. 7D). In addition, liver indices showed reduced liver regeneration with ad-RNAi-H2-B1 compared with ad-RNAi-nt in *wt* C57BL/6 mice following PHx (Supporting Fig. S7), supporting the important role of H2-B1 in liver regeneration following surgery.



**FIG. 6.** Indicators of immune attack are reversed by adenoviral delivery of *H2-BI* in *Ghr*<sup>-/-</sup> mice. (A) TUNEL and phospho-JUN staining shows ability of ad-*H2-BI* to prevent apoptosis and restore phospho-JUN level in *Ghr*<sup>-/-</sup> mice at 6 hours following PHx. Quantification of TUNEL (B) and phospho-JUN staining (C), five sections per mouse (n = 3 mice/group). (D) The increase in *IFN*γ transcript levels after PHx is higher in *Ghr*<sup>-/-</sup> mice compared with *wt* mice, and this increase in *IFN*γ transcript in *Ghr*<sup>-/-</sup> is reduced by adenoviral expression of *H2-BI*. (E) *IFN*γ expression by IHC shows increased expression at 6 hours in *wt*, but 3.5-fold higher expression in *Ghr*<sup>-/-</sup>, which is markedly decreased by ad-*H2-BI* but not by ad-GFP (five sections per mouse, n = 3 mice/group).



**FIG. 7.** Treatment with HLA-G or mixed background mice with high expression of *H2-B1* improves survival of *Gbr*<sup>-/-</sup> mice, while markers of immune attack are elevated in C57BL/6 mice by knockdown of *H2-B1* following PHx. (A) Survival of mouse strains after PHx (number for each genotype shown) and osmotic pump infusion of HLA-G. (B) Infusion of HLA-G protein markedly reduced apoptosis levels in *Gbr*<sup>-/-</sup> mice. TUNEL staining showing ability of HLA-G to prevent apoptosis in *Gbr*<sup>-/-</sup> mice at 6 hours PHx with representative images shown (right). Quantification of TUNEL and cleaved caspase-3, 5 sections per mouse (n = 3 mice per group). (C) Transcripts analysis in liver at 6 hours after PHx with H2-B1 RNAi show elevated levels of *Fas* and *fasl* compared to non-targeting control. (D) NK/NKT marker NK1.1 is elevated at 6 hours following PHx with H2-B1 RNAi compared to non-targeting RNAi while macrophage marker F4/80 transcripts showed no significant change. (E) *IFN $\gamma$*  transcript levels in the liver are increased with H2-B1 RNAi 6 hours after PHx compared with nontargeting RNAi (n = 6 ad-RNAi-H2-B1 and n = 4 ad-RNAi-nt). (F) HLA-G levels in serum from patients with GHD, patients with GDH following GH treatment, and healthy controls. Abbreviation: ns, not significant.

## CIRCULATING HLA-G LEVELS ARE DECREASED IN GH-DEFICIENT PATIENTS AND RESTORED WITH GH REPLACEMENT

Finally, we showed that serum HLA-G is significantly increased in GH-deficient patients by 6 months of GH replacement to restore normal IGF-1 levels (Fig. 7F). Thus, GH induces expression of both *H2-B1* in mice and its homolog *HLA-G* in humans.

## Discussion

We sought to elucidate the molecular mechanisms by which GH deficiency compromises survival after PHx in C57BL/6 mice by using a panel of mouse models with different GHR signaling domains deleted. Early studies in rats showed that GH (but not IGF-1) administration increased hepatocyte proliferation and final liver size after PHx.<sup>(19)</sup> Furthermore, the increase in *Hgf* transcript seen approximately 5 hours after PHx was delayed in hypophysectomized rats until 10–18 hours following PHx, unless GH was administered previously.<sup>(20)</sup> The first study to examine the role of GH in recovery from PHx in transgenic mice used mice expressing the GH antagonist G118R, which decreases, but does not eliminate, GH signaling. That study reported a high mortality (approximately 43%, most within 48 hours) in a mixed genetic background after PHx. In addition, the study found that liver *Igf-1*-deleted mice showed minimal mortality, while extensive areas of necrosis were evident at 24 following PHx only in the GH-antagonist transgenic mice.<sup>(3)</sup> GH levels decrease with age, and GH treatment of aged mice has been shown to stimulate

hepatocyte proliferation in a FOXM1B-dependent manner; this was associated with an increase in expression of CDC25A/B phosphatases and cyclin B1, while p27<sup>Kip1</sup> was decreased.<sup>(21)</sup> These results imply a specific action of GH to reduce mortality and to increase hepatocyte proliferation after PHx in mice.

We demonstrated almost complete mortality of *Gbr*<sup>-/-</sup> C57BL/6 mice within 48 hours following PHx, whereas mice that lacked canonical GH-mediated JAK/STAT signaling but retained the GH-mediated SRC/ERK pathway showed complete survival 7 days following PHx. This allowed us to establish that a GHR-dependent SRC/ERK pathway<sup>(6)</sup> confers resistance to mortality after liver resection in mice. Through hepatic transcript profiling with Illumina arrays, we identified the immunotolerance protein H2-B1 as a likely candidate crucial for mouse survival following PHx.<sup>(6)</sup> The central role of GH induction of *H2-B1* was supported by demonstrating survival and allowing liver regeneration in *Gbr*<sup>-/-</sup> mice following PHx only when deficient *H2-B1* expression had been overcome either with an *H2-B1* adenoviral expression vector or by infusion of the homogeneous human ortholog, HLA-G.<sup>(22)</sup>

*H2-B1* (blastocyst major histocompatibility complex [MHC]) is an MHC class 1b gene.<sup>(14)</sup> Transcripts of *H2-B1* were first identified in mouse blastocysts and proposed to induce tolerance to maternal NK cells at the maternal–fetal interface in a similar manner to its human ortholog, HLA-G.<sup>(9,22)</sup> As for HLA-G,<sup>(9)</sup> H2-B1 can be secreted as a soluble form consequent to alternative splicing of its transcript. Expression of *H2-B1* was shown to protect a T-cell line lacking surface MHC class Ia molecules from NK cell attack,<sup>(22)</sup> demonstrating the same function as HLA-G.<sup>(23)</sup> The potent immunosuppressive properties of HLA-G act by binding to two separate immune inhibitory receptors, LILRB1 on monocytes, macrophages, dendritic,

NK, B cells and T cells, and LILRB2 on NK cells, monocytes, macrophages, and dendritic cells.<sup>(9,17,24)</sup> Transgenic expression of HLA-G in mice allows skin allografts to survive substantially longer than in *wt* controls, with delaying of maturation of dendritic macrophages.<sup>(25)</sup> Similarly, transgenic HLA-G expression on swine endothelial cells was shown to suppress human macrophage-mediated cytotoxicity.<sup>(26)</sup> Moreover, human CD4+HLA-G+T cells, characteristic of an important T<sub>reg</sub> population, act as potent suppressors of graft-versus-host disease *in vivo*,<sup>(10)</sup> while increased levels of soluble HLA-G correlate with reduced graft-versus-host disease.<sup>(27)</sup> These findings are congruent with *in vitro* data showing that HLA-G is also able to decrease T-cell responses, presumably by inhibiting the development of antigen-presenting cells such as macrophages.<sup>(28)</sup> Importantly, expression of HLA-G has been shown to correlate with lower rates of rejection in liver, kidney, and heart transplant recipients.<sup>(9)</sup>

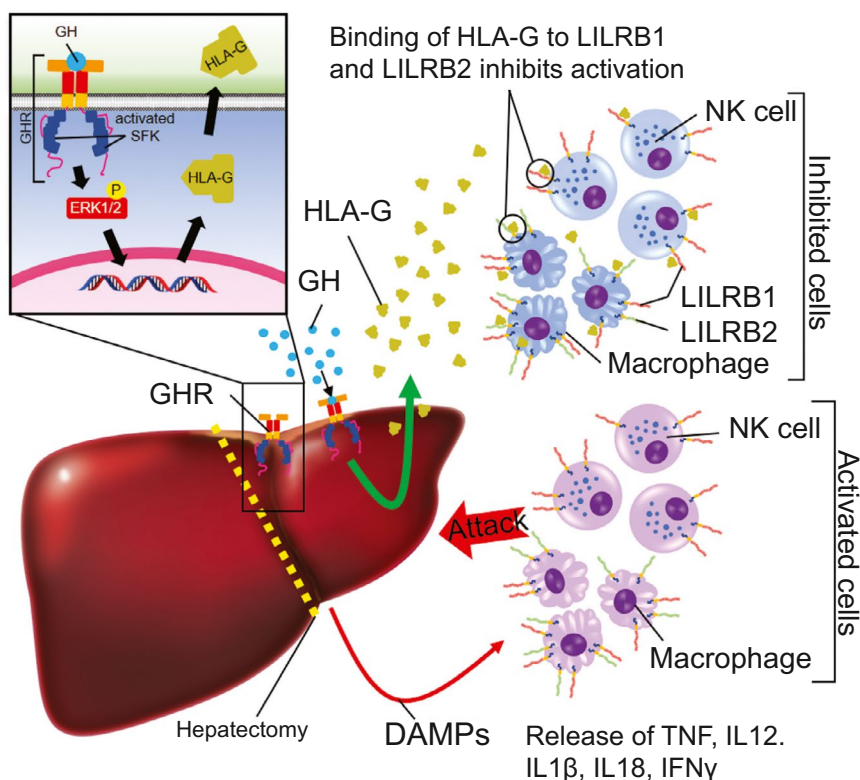
Given the strong correlation between elevated HLA-G and absence of transplant rejection, a key question remains as to why some patients have high levels of HLA-G and others do not. Therefore, it is indeed relevant that GH is able to induce expression of *H2-B1* in mice and that the serum level of HLA-G in GH-deficient (GHD) patients is increased by GH replacement. In addition, patients with GHD have an elevated prevalence of nonalcoholic steatohepatitis (NASH), which is characterized by elevated inflammation.<sup>(29)</sup> GH replacement therapy has been shown to improve NASH in patients with GHD,<sup>(30)</sup> and our study reveals that GH induction of HLA-G may play a role in this process.

Expression level of *H2-B1* is critical for survival in mice following PHx. This is illustrated by our finding of almost total mortality in C57BL/6 *Ghr*<sup>-/-</sup> mice by 48 hours after PHx, in stark contrast to a report showing zero mortality after PHx in *Ghr*<sup>-/-</sup> mice,<sup>(16)</sup> associated with a striking decrease in hepatocyte proliferation, cyclin A, and cyclin D expression at 40–48 hours following PHx. That study was performed in mice on a Sv129Ola background, whereas our study used C57BL/6 mice, and the study using GHR antagonist mice showing partial mortality following PHx used an undefined mix of C57BL/6, 129/Sv, and FVB/N strains.<sup>(3)</sup> In agreement with previous reports,<sup>(14,31)</sup> we confirmed that *H2-B1* expression is significantly higher in the 129/Sv strain relative to

the hypomorphic C57BL/6 strain (Fig. 5D), and that PHx in *Ghr*<sup>-/-</sup> mice on a mixed 129/SvJ/C57BL/6 background did not result in significant mortality (Fig. 7A). This supports the importance of differential expression of *H2-B1* between these strains as a key survival factor. The finding that C57BL/6 mice are much more susceptible to ischemia reperfusion damage than 129/Sv mice is potentially a consequence of the strain difference in *H2-B1* expression.<sup>(32)</sup> Strain differences in *H2-B1* expression may recapitulate the variations of *HLA-G* expression in patients and their outcomes from liver transplants. These differences would be significant, given the normally low level of HLA-G expression in liver.<sup>(9)</sup>

A key question is the mechanism of action of H2-B1/HLA-G in promoting survival after PHx. The strongest correlate with survival following adenoviral expression of *H2-B1* was the decreased level of the macrophage/Kupffer cell marker F4/80 and IFN $\gamma$  expression at 6 hours following PHx. Decreasing Kupffer and macrophage cell number pharmacologically before PHx with gadolinium chloride has been reported to enhance liver regeneration.<sup>(33)</sup> A time-series microarray analysis of the Kupffer cell transcriptome following PHx shows a decline in immune function expression soon after the procedure,<sup>(34)</sup> correlating with our observed loss of the F4/80 marker. We suggest that HLA-G binding to inhibitory LILRB1 and LILRB2 receptors present on macrophage cells or recruited dendritic cells blocks their activation by DAMPs (danger-associated molecular pattern molecules) (Fig. 8). DAMPs such as HMGB1, hyaluronic acid, and S100 proteins released from necrotic parenchymal cells can activate TLR4 on Kupffer and dendritic cells, and this has been implicated in sterile innate inflammatory responses to ischemia/reperfusion injury, which may be expected during PHx.<sup>(35,36)</sup> Additionally, histones and HMGB1 released from necrotic parenchymal cells activate TLR9 on Kupffer cells, with a similar outcome through the NLRP3 inflammasome.<sup>(37)</sup> Excessive activation of these inflammatory cells would release dangerous levels of TNF, IL-12, cleaved IL-1 $\beta$  and IL-18, which would not only induce apoptosis of parenchymal cells, but also serve to recruit and activate NK and NKT cells to release IFN $\gamma$  and further TNF.<sup>(38)</sup>

As H2-B1/HLA-G exists in a membrane-bound form and as a soluble ectodomain, both forms would



**FIG. 8.** Growth hormone protects the liver from excessive inflammation through SFK-dependent induction of HLA-G (H2-B1 in mice) after PHx. Following PHx surgery, immune cells such as NK cells and macrophages are activated and release inflammatory cytokines, which may contribute to liver failure and mortality. GH induces the expression of HLA-G, which inhibits immune cell activation, keeping inflammation in check, which permits regeneration.

be expected to dampen the innate immune response to damaged cells. We instead used the ectodomain, supplied both as an adenoviral construct or by continuous infusion of the protein. In further support of the immunosuppressive actions of H2-B1/HLA-G, we have shown that RNAi knockdown of H2-B1 in *wt* C57BL/6 mice results in elevation of markers of inflammation 6 hours following PHx (Fig. 7C-E). It has been reported that soluble HLA-G impairs NK/dendritic cell crosstalk by inhibiting dendritic cell maturation and IL-12 secretion, as well as inhibiting associated NK/NKT cell IFN $\gamma$  cell secretion, all of which would contribute to inhibition of cytotoxicity and apoptosis.<sup>(9,23)</sup> Similarly, it has been shown that soluble HLA-G is able to inhibit the production of TNF and IFN $\gamma$  from decidual mononuclear cells.<sup>(39)</sup> Human liver stem cells (HLSCs) have been shown to express HLA-G and suppress NK cell activation, and this suppression of NK cells was able to be blocked with an antibody against HLA-G.<sup>(40)</sup> In further support, HLSCs

reduced liver necrosis and apoptosis and enhanced liver regeneration in a model of fulminant liver failure.<sup>(41)</sup> In a liver transplant animal model, recombinant human growth hormone (rhGH) has been shown to attenuate ischemic injury of intrahepatic bile ducts,<sup>(42)</sup> and a clinical study has shown HLA-G to be expressed in biliary epithelial cells in liver allografts of patients with combined liver-kidney transplants.<sup>(9)</sup> Moreover, studies using carbon tetrachloride-treated mice showed that transplantation of HLA-G-expressing human amniotic epithelial cells reduces hepatic fibrosis and that soluble HLA-G1 (extracellular domain of HLA-G) reduced TGF- $\beta$ 1 protein levels and reduced the intracellular collagen content of hepatic stellate cells.<sup>(43)</sup> A study investigating HLA-G expression in liver transplantation found that cirrhotic explant livers showed robust HLA-G expression in hepatocytes, whereas most of the noncirrhotic livers and graft biopsies taken before or after liver transplantation showed no or weak HLA-G expression. In addition, the study suggested

that HLA-G expression in hepatocytes in end-stage liver diseases may be due to a negative feedback to protect the liver against immunological damage.<sup>(44)</sup> This mechanism is supported by investigations that show how end-stage liver disease is associated with an immunosuppressed state with reduced HLA-DR expression and reduced capacity for lipopolysaccharide stimulation of TNF production, and that HLA-G reduces HLA-DR expression.<sup>(45,46)</sup> Based on our data and supportive studies, we propose that the anti-inflammatory action of H2-B1 on macrophage cells and dendritic cells is the primary mechanism responsible for enhanced survival after PHx, that this is enhanced by direct actions on NK/NKT cells, and that these actions are initiated by GH through its ability to induce expression of *H2-B1* (Fig. 8). Accordingly, we show that both GH and HLA-G are able to block apoptosis of human hepatocytes by human NK cells *in vitro*.

Our previous studies<sup>(7,47)</sup> suggest several other factors could contribute to impaired regeneration in *Ghr*<sup>-/-</sup> mice, resulting in significantly impaired regeneration even in H2-B1 replaced mice. First, *egfr* (epidermal growth factor receptor) transcripts are 8-fold decreased in *Box1*<sup>-/-</sup> (and *Ghr391*<sup>-/-</sup>) mice lacking STAT5 activation by GH, and this decrease is accompanied by strongly decreased EGFR protein expression. We also found 2-fold elevated phospho-STAT1 in livers of *Ghr*<sup>-/-</sup> (and *Ghr391*<sup>-/-</sup>) mice, owing to loss of STAT5-dependent suppressor of cytokine signaling expression.<sup>(47)</sup> STAT1 is a major signaling element used by IFN $\gamma$  and has been proposed to contribute to impaired liver regeneration in hepatic *stat5a/b*<sup>-/-</sup> mice, as concomitant *stat1* deletion restored proliferative ability of the liver.<sup>(48)</sup> The elevation of IFN $\gamma$  transcript we observe in *Ghr*<sup>-/-</sup> mice following PHx, presumably as a result of NK/NKT cell activation, would exacerbate this action of phospho-STAT1. We also note that p21 (*cdkn1a*) transcript is 3-fold-elevated in *Ghr*<sup>-/-</sup> mice relative to *Box1*<sup>-/-</sup> mice,<sup>(6)</sup> and this is an inhibitor of hepatocyte proliferation.<sup>(49)</sup> We suggest that these additional elements would act to reduce liver regeneration in *Ghr*<sup>-/-</sup> mice, while survival depends critically on the level of *H2-B1* expression and its role in regulating the inflammatory response. These other elements may account for the death of about 50% of *Ghr*<sup>-/-</sup> mice following PHx, even with H2-B1 or HLA-G replacement, although effectiveness of adenoviral tail vein injection or an incompletely

optimized replacement dose of HLA-G would also be important factors.

In conclusion, our study demonstrates an important role for H2-B1/HLA-G in suppressing inflammatory responses to facilitate liver regeneration (Fig. 8). Our study suggests that previous treatment of liver-transplant patients with HLA-G or with rhGH or both will be beneficial in suppressing inflammation, and this together with the other positive hepatic actions of GH may also account for the striking decrease in mortality reported in patients with liver failure following administration of rhGH.<sup>(50)</sup>

*Author Contributions:* M.I. was responsible for the study design, conducting the experiments, data acquisition, and analysis and interpretation of data. A.J.B. was responsible for the study design, conducting the experiments, data acquisition, analysis and interpretation of data, drafting the manuscript, obtaining funding, and study supervision. M.A.F. was responsible for the study design, conducting experiments, data acquisition, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. J.M. was responsible for conducting the experiments, data acquisition, and analysis and interpretation of data. Y.C. was responsible for data acquisition, and analysis and interpretation of data. K.A.T. was responsible for the design and construction of adenoviral vectors for RNAi knock-down. S.M. was responsible for obtaining funding, and technical and material support. G.A.R. was responsible for critical revision of the manuscript for important intellectual content. R.P. was responsible for critical revision of the manuscript for important intellectual content, obtaining funding, and technical and material support. J.V. and J.R. were responsible for the production of purified HLA-G. V.C. and K.K.Y.H. were responsible for technical support and collection of patient samples. M.J.W. was responsible for the study concept and design, analysis and interpretation of data, drafting of the manuscript, obtaining funding, and study supervision.

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## Supporting Information

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