Understanding the molecular pathogenesis of inflammatory liver disease is essential to design efficient therapeutic approaches. In hepatocytes, the dimeric transcription factor c-JUN/AP-1 is a major mediator of cell survival during hepatitis, although functions for other JUN proteins in liver disease are less defined. Here, we found that JUNB was specifically expressed in human and murine immune cells during acute liver injury. We analyzed the molecular function of JUNB in experimental models of hepatitis, including administration of concanavalin A (ConA) or α-galactosyl-ceramide, which induce liver inflammation and injury. Mice specifically lacking JUNB in hepatocytes displayed a mild increase in ConA-induced liver damage. However, targeted deletion of JUNB in immune cells and hepatocytes protected against hepatitis in experimental models that involved NK/NKT cells. The absence of JUNB in immune cells decreased IFN-γ expression and secretion from NK and NKT cells, leading to reduced STAT1 pathway activation. Systemic IFN-γ treatment or adenovirus-based IRF1 delivery to JUNB-deficient mice restored hepatotoxicity, and we demonstrate that Ifng is a direct transcriptional target of JUNB. These findings demonstrate that JUNB/AP-1 promotes cell death during acute hepatitis by regulating IFN-γ production in NK and NKT cells and thus functionally antagonizes the hepatoprotective function of c-JUN/AP-1 in hepatocytes.

Introduction

Inflammation of the liver (hepatitis) is mostly triggered by viral infections. Intoxications (notably alcohol), autoimmune diseases, metabolic disorders, fatty liver disease, and hereditary disorders are also important contributors to hepatitis and liver cancer development (1). Acute hepatitis is characterized by a strong innate inflammatory response (10). Studies using mouse models indicated that IFN-γ and NKT cells participate in viral clearance, thereby preventing progression to chronic hepatitis (11, 12). However, the beneficial potential of IFN-γ administration to hepatitis-infected patients is still uncertain (13). IFN-γ activates the STAT1 pathway in hepatocytes and induces cell death, which can result in liver failure (14). Therefore, IFN-γ can be considered to be a double-edged sword during hepatitis, as it is crucial for viral defense but can also lead to liver damage. Defining the cellular and molecular signals controlling IFN-γ production during hepatitis is therefore of particular relevance to understanding the complex function of this cytokine and developing therapeutic approaches.

Activator protein 1 (AP-1) represents a family of dimeric transcription factors composed of JUN, Fos, and ATF proteins, which are central to several biological processes from embryonic development to various diseases (15). In particular, AP-1 has been shown to regulate the expression of several cytokines in a tissue/cell-specific manner (16). For instance, during in vitro T cell differentiation, JUN-containing dimers regulate IL2, IL4, and IFNG (17–21), whereas JUN proteins control skin inflammation in vivo by affecting IL6 and GMCSF expression and TNF-α shedding in keratinocytes (22, 23). It is therefore likely that AP-1 transcription factors contribute to the inflammatory process during acute hepatitis. JUN proteins are very similar structurally, and members of the AP-1 transcription family can have specific or redundant functions (24). We have previously shown that c-JUN/AP-1 is necessary for the survival of hepatocytes during acute hepatitis. Surprisingly, c-JUN does not seem critical for immune response in this setting (25).

Here, we show that JUNB is strongly expressed in a subset of immune cells from liver samples from humans and mice with hepatitis. Using loss-of-function mouse models for JUNB, we unravel a novel function for JUNB/AP-1 in regulating the expression of Ifng in NK and NKT cells, thereby modulating acute liver damage and counteracting the protective function of c-JUN in hepatocytes.
Figure 1
JUNB expression in immune cells from livers of patients with hepatitis. (A) Liver sections from healthy patients or patients with hepatitis were stained with H&E (scale bar: 50 μm) or with an antibody against JUNB (brown). Insets show representative inflamed areas. Black arrowheads indicate JUNB-positive cells (n = 5). Representative areas are shown. (B) Quantification of JUNB-positive cells in liver sections from healthy patients and those with hepatitis (n = 5, *P < 0.05). (C) Hepatitis samples were stained for JUNB (brown) and with a marker for immune cells (CD45), monocytes/macrophages (CD68), T cells (CD3), or NK and NKT cells (CD56) (red). JUNB-single positive cells are indicated with black arrowheads and double-positive cells are indicated with pink arrowheads. (D) Liver sections from wild type-mice treated or untreated with ConA for 2 hours and from ConA-treated JunbΔli* mice were stained for JUNB (brown). Black arrowheads indicate JUNB-positive cells. Immune-fluorescence costaining of livers from ConA-treated mice for JunB (red) and CD45 (white). White arrows indicate double-positive cells. Scale bar: 20 μm. One representative experiment is shown (n > 5).
Results

JUNB is upregulated in human and mouse liver hepatitis. Expression of JUNB was assessed on paraffin sections of liver needle biopsies from patients with hepatitis and compared to nonhepatitis samples. Very few JUNB-positive cells were observed in healthy livers compared with those in hepatitis samples (Figure 1A). Quantification confirmed the increase of JUNB-positive cells, which was more pronounced in the inflamed areas, in liver samples from patients with hepatitis (Figure 1, A and B). CD45 staining indicated that all JUNB-positive cells were immune cells and not hepatocytes (Figure 1C). In combination with the JUNB antibody, we next used CD68, a marker for monocytes; CD3, a marker for T cells; and CD56, a cell surface marker for NK and NKT cells. Double-positive cells, likely JUNB-expressing Kupffer, T, NK, and NKT cells, were found in liver samples from patients with hepatitis, in particular, in the inflamed areas (Figure 1C). This analysis revealed that JUNB is expressed in immune cells of the liver and strongly increased during hepatitis.

JUNB expression was next analyzed in experimental models of hepatitis. In mice, concanavalin A (ConA) injection is a paradigm for T cell–dependent hepatitis, although macrophages and NK and NKT cells are also essential (26–30). In nontreated wild-type mice, JUNB expression was not detectable in the liver by immunohistochemistry (IHC). However, 2 hours after ConA injection, JUNB was expressed strongly in nonparenchymal liver cells. Costaining with CD45 revealed that, similar to that in human hepatitis, JUNB-positive cells were also CD45 positive (Figure 1D). The specificity of the JUNB staining was confirmed using JunbΔli−/− mice (Junb fl/fl; MxCreT/− mice), which lack JUNB in all liver cells (Figure 1D). Furthermore, flow cytometry analysis confirmed that upon ConA treatment, JUNB was expressed in a subset of T, Kupffer, NK, and NKT cells and that JunbΔli−/− mice had decreased JUNB expression in all cell types (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI70405DS1). Importantly, the overall distribution of the different immune cells was not different between control and JunbΔli−/− mice, indicating that loss of JUNB did not impact on immune cell numbers (Supplemental Figure 1C). Finally, a similar increase of JUNB-positive immune cells was observed in 3 additional models of hepatitis: LPS/α-galactosamine (GalN), polyinosinic-polycytidylic acid (poly-I/C), and α-galactosyl-ceramide (αGalCer) administration (Supplemental Figure 1D). Collectively, our data indicate that JUNB is strongly expressed in immune cells of the liver during human and murine hepatitis, particularly in T, Kupffer, NK, and NKT cells.

Loss of JUNB in immune cells decreases liver damage in hepatitis mouse models involving NK/NKT cells. To investigate the functional relevance of JUNB in hepatitis, wild-type and Junb-deficient mice were treated with ConA and followed over 8 hours. Strikingly, JunbΔli−/− mice displayed increased survival rate (Figure 2A), which was also reflected by smaller necrotic areas observed by liver histology (Figure 2B) and by decreased cleaved caspase-3 protein (Figure 2C) and decreased Hmox1, Gad45b, and A20 mRNA (Figure 2D) in JunbΔli−/− livers. Serum alanine aminotransferase (ALT), a marker for liver damage, was also decreased in JunbΔli−/− mice (Figure 2E). Therefore, JunbΔli−/− mice are protected from liver damage and lethality induced by ConA treatment. We next used JunbΔli−/−
mice (JunbΔli*;AlfpCreT/+ mice) which lack JUNB specifically in liver parenchymal cells, but retain JUNB expression in immune cells upon ConA treatment (Supplemental Figure 2A). Importantly, serum ALT was modestly increased in JunbΔli* mice upon ConA treatment (Supplemental Figure 2B), while no changes in Hmox1, Gadd45b, A20, and Nos2 mRNA expression could be detected (Supplemental Figure 2C). This suggests that the decreased damage in JunbΔli* mutants is due to the loss of JUNB expression in immune cells, which is dominant over a possible hepatoprotective function of JUNB in liver parenchymal cells.

To dissect the cellular and molecular mechanisms affected by JUNB, JunbΔli* mice were next treated with LPS/GalN, poly-I/C, or αGalCer. LPS/GalN-induced hepatitis mainly involves TNF-α and Kupffer cells/macrophages (31) through activation of TLR4. Poly-I/C, a synthetic TLR3 ligand, mimics viral infection-associated and IL-2 are decreased in Junb-deficient mice during hepatitis. Cytokines previously associated with each experimental model were measured in the sera of JunbΔli* mice. While serum TNF-α was decreased at 4 hours after ConA treatment in mutants as compared with that in control mice, it was unaffected in the other models (Figure 3, A and B, and Supplemental Figure 4C), indicating that loss of JUNB does not significantly affect TNF-α production. This finding is also consistent with comparable liver damage observed in the LPS/GalN model, a paradigm for TNF-α-mediated acute liver failure (31). Interestingly, IFN-γ and IL-2 were significantly decreased in the sera of ConA-treated JunbΔli* mice (Figure 3A). A similar decrease in serum IFN-γ was observed in the 2 other experimental models where JUNB deletion is protective, i.e., in αGalCer-treated (Figure 3B) and poly-I/C–treated mice (Supplemental Figure 4A) but not in LPS/GalN-treated mice (Supplemental Figure 4A). Furthermore, JunbΔli* mice displayed a 2-fold reduction in Ifng and Il2 mRNA in the liver and the spleen 2 hours after ConA or αGalCer administration (Figure 3, C and D), indicating that decreased Ifng and Il2 transcription occurs in immune cells. IL-2 was unchanged in the 2 other experimental paradigms (Supplemental Figure 4B). Other cytokines, such as IL-4, IL-6, IL-10, or IL-12p40, were comparably induced in control and mutant JunbΔli* mice after ConA or αGalCer treatment (Supplemental Figure 5), and loss of JUNB did not alter type I IFN induction after poly-I/C or ConA treatment (Supplemental Figure 4, D and E). Interestingly, serum IL-2 was modestly decreased in c-Jun–deficient mice (c-JunΔli* or c-JunΔli*/MxCreT/+ mice) after ConA administration, while IFN-γ was unchanged (Supplemental Figure 5).

**Figure 3**

 JunbΔli* mice have decreased cytokine levels. (A) Serum cytokines in control and JunbΔli* mice at 0 (n = 3), 2 (n = 5), or 4 (n = 5) hours after ConA treatment. *P < 0.05. (B) Serum cytokines in control and JunbΔli* mice at 0 (n = 3), 2 (n = 6), or 4 (n = 6) hours after αGalCer treatment. *P < 0.05. (C) qRT-PCR for Ifng and Il2 in livers of control and JunbΔli* mice 2 hours after ConA or αGalCer treatment (n = 5; *P < 0.05). (D) qRT-PCR for Junb, Ifng, and Il2 in spleens of control and JunbΔli* mice 2 hours after ConA or αGalCer treatment (n = 5; *P < 0.05).
Collectively, these results indicate that reduced IFN-γ and possibly IL-2 production by immune cells might mediate the protective effect of JUNB deletion in the ConA, αGalCer, and poly-I/C experimental paradigms.

The STAT1 pathway is specifically altered in Junb-deficient mice. The STAT1 and STAT5 pathways are activated by IFN-γ and IL-2, respectively (38, 39). Phosphorylated STAT1 (pSTAT1) was decreased in liver extracts from ConA-treated ΔJunb α/α mice (Figure 4A), while it was unchanged in ConA-treated ΔJunb Δli mice, which lack JUNB in parenchymal cells (Supplemental Figure 6B). Despite reduced IL-2 in ConA-treated ΔJunb α/α mice, pSTAT5 was not significantly altered (Figure 4A). STAT3 phosphorylation was also unaffected, consistent with unchanged levels of IL-6 (Figure 4A, Supplemental Figure 5, and ref. 40). IHC analyses indicated that both hepatocytes and immune cells, which included F4/80-positive Kupffer cells, displayed nuclear pSTAT1 staining upon ConA treatment. The staining in ΔJunb α/α mice seemed reduced when compared with that in control mice (Figure 4B), while no staining was observed in samples from untreated mice (data not shown).

Transcriptional targets of STAT proteins were analyzed next. ΔJunb α/α mice displayed decreased mRNA expression of Ifg1, the main target of STAT1 downstream of IFN-γ, while Socs1, which is regulated both by STAT1 and STAT3, was unchanged together with the bona fide STAT3 targets Socs3 and Bel2l1 (refs. 36, 40, 41, and Figure 5A). Consistent with decreased IFN-γ levels, a similar impaired activation of STAT1 and Ifg1 was observed in ΔJunb Δli mice in the αGalCer model (Supplemental Figure 7, A and C). IHC analyses indicated that IRF1 was induced in hepatocytes and nonparenchymal cells after ConA (Figure 5B) and αGalCer treatment (Supplemental Figure 7B). Furthermore, the number of IRF1-positive cells appeared reduced in ΔJunb Δli livers (Figure 5B and Supplemental Figure 7B), and the decrease was confirmed by Western blot (Figure 5C). Importantly, IRF1 levels were unaffected in ConA-treated ΔJunb Δli mice (Supplemental Figure 6C).

During hepatitis, IFN-γ and activation of the STAT1 pathway are associated with hepatocyte death. STAT1 modulates Cxcl9, Cxcl10, and Nos2 expression in hepatocytes and immune cells, and IRF1 mediates further Nos2 induction (36, 42–44). Consistent with decreased IFN-γ, pSTAT1, and IRF1, mRNA expression of Nos2 and Cxcl9 was decreased in ΔJunb Δli mice after ConA or αGalCer treatment and Cxcl10 was additionally decreased upon αGalCer treatment (Figure 5D and Supplemental Figure 7C). IHC analysis suggested that inducible NOS (iNos) was mostly expressed in immune cells, including macrophages (Supplemental Figure 8A), 2 hours after ConA treatment, as previously reported (45). Interestingly, the number of iNos-positive cells appeared reduced in ΔJunb Δli livers (Supplemental Figure 8B). As the number of immune cells was unchanged in ΔJunb-deficient livers after ConA treatment (Supplemental Figure 1C), this indicates that, in the absence of JUNB, iNos expression is decreased in a subset of immune cells. Moreover, dot blot analyses revealed a significant decrease in nitrosylated proteins, a readout for iNos activity, in ΔJunb Δli livers 8 hours after ConA (Figure 5E).

All together, these data demonstrate that, during acute hepatitis, ΔJunb α/α mice, but not ΔJunb Δli mice, have decreased IFN-γ/pSTAT1 pathway activity in hepatocytes and immune cells, which is likely responsible for decreased hepatotoxicity.

Decreased IFN-γ expression in NK and NKT cells lacking JUNB. T, NK, and NKT cells are the main producers of IFN-γ, with NK and NKT cells being particularly relevant in the ConA or αGalCer hepatitis paradigms (31, 36). As the number of immune cells in the liver after ConA treatment appeared unaffected by JUNB deletion, cytokine expression was analyzed in the different immune cells. Flow cytometry measurements using immune cells isolated from the liver and the spleen after in vivo ConA treatment indicated reduced intracellular IFN-γ in NK and NKT cells and decreased IL-2 in T cells isolated from ΔJunb α/α mice when compared with control littermates (Supplemental Figure 9).

Next, T, NK, and NKT cells were isolated from the spleens of ΔJunb α/α mice using FACs. Molecular analyses revealed a 2-fold decrease in the expression of Ifng in ΔJunb-deficient NK and NKT cells but not in T cells (Figure 6, A and B). On the other hand, Il2 was decreased in ΔJunb-deficient T cells, while it was undetectable in NK and NKT cells (Figure 6, A and B). These data indicate that during acute hepatitis, JUNB controls Ifng expression, specifically in NK and NKT cells, while it modulates Il2 expression in T cells.

JUNB binds to the IFN-γ promoter in vivo and in vitro. We next assessed whether JUNB directly controls Ifng using ChIP assays. The mouse and human proximal IFNG promoter harbors a con-
served TRE element (Figure 6C). ChIP analyses using whole liver extracts demonstrated that JUNB bound the chromatin region containing the TRE element in control livers but not in samples from JunbΔl/mice (Figure 6D). No enrichment was observed when using control immunoglobulins (IgG) or when analyzing Il2 or the ribosomal protein S16 promoters (Figure 6D). The YT human NK cell line, which produces IFN-γ, was used next. JUNB and IFNG were readily detectable in basal conditions, and treatment with PMA and ionomycin (P/I) further induced JUNB and IFNG expression. Low IL2 transcript levels were also measured under these conditions (Figure 6, E and F). JUNB efficiently bound to the proximal promoter of IFNG in basal conditions, and increased binding was observed after P/I stimulation, while no significant binding to the IL2 promoter was detected (Figure 6, G and H). This indicates that JUNB controls IFN-γ expression in vivo and in a human NK cell line through direct promoter binding.

Figure 5
STAT1 targets, IRF1 and iNos, are altered in JunbΔl/mice. Control and JunbΔl/mice were treated with ConA or αGalCer, and liver samples were analyzed. (A) qRT-PCR for Junb and STAT-regulated genes in the livers of saline-injected mice (n = 3) and in mice at 2 (n = 5) and 4 (n = 5) hours after ConA. *P < 0.05. (B) Liver sections stained for IRF1 (brown) in nontreated wild-type mice or 4 hours after ConA treatment of control and JunbΔl/mice. Arrowheads indicate positive immune cells (red) and hepatocytes (black). n = 3. A representative experiment is shown. Scale bar: 20 μm. (C) Western blot for IRF1 at 2 and 4 hours after treatment with either with ConA or αGalCer (αGal). The dotted line marks cropped samples. Vinculin is included to control for equal loading. Quantification is shown (n = 5–7; *P < 0.05). (D) qRT-PCR for Cxcl9, Cxcl10, and Nos2 in the livers of saline-injected mice (n = 3) and in mice at 2 (n = 5) and 4 (n = 5) hours after ConA treatment. *P < 0.05. (E) Dot blot for nitrosylated proteins (nitrotyrosine) 8 hours after ConA treatment. Vinculin is included to control for equal loading. Quantification is shown (n = 5; *P < 0.05).
Restoring the pSTAT1 pathway dampens the effects of JUNB loss. To address whether alterations in the IFN-γ/STAT1/IRF1 pathway are the main contributor to decreased hepatocyte death in JunbΔli mice, we treated Junb-deficient mice with recombinant IFN-γ. The timing and amount of IFN-γ were adjusted to achieve a level of pSTAT1 upon ConA treatment comparable to that in wild-type treated mice (Figure 7A). IFN-γ treatment did not lead to significant liver damage in either genotype when used alone (Figure 7B). Importantly, IFN-γ restored ConA-induced liver damage in Junb-deficient mice to levels comparable to those of wild-type mice, as assessed by serum ALT (Figure 7B) or by analysis of stress-induced markers (Figure 7C). Furthermore, liver damage was also restored by adenovirus-based expression of IRF1 in livers of JUNB mutant mice prior to ConA treatment (Figure 7D). These data indicate
that decreased IFN-γ production in Junb-deficient immune cells during acute hepatitis and subsequent decreased STAT1/IRF1 signaling are largely responsible for decreased liver damage.

In conclusion, our data demonstrate that IFN-γ production in NK and NKT cells and IL-2 production in T cells are regulated by JUNB/AP-1 during experimental hepatitis and unravel a novel function of JUNB as an important modulator of IFN-γ hepatotoxicity in vivo (Figure 7E).

Discussion

Mouse models are instrumental in advancing our understanding of the basic mechanisms of liver homeostasis and disease (1). Here, we show a selective increase in JUNB expression in immune cells in the livers of patients with hepatitis. By combining mouse genetics with established experimental models of liver inflammation, we have unraveled a novel function of JUNB/AP-1 in regulating IFN-γ expression in NK and NKT cells during acute hepatitis, which could potentially benefit the development of new therapies.

In mouse models, loss of JUNB decreases IFN-γ production upon ConA, poly-I/C, and αGalCer treatment but not upon LPS/GalN treatment. Consistent with impaired levels of IFN-γ, reduced active STAT1, decreased Irf1 and Nos2 expression, and attenuated hepatocyte death was observed. We provide evidence that Ifng expression is specifically decreased in NK and NKT cell–derived IFN-γ induces phosphorylation of STAT1 and the expression of STAT1 targets in hepatocytes, leading to cell death.
acute inflammatory response, similar to what has been reported in the context of *Burkholderia pseudomallei* infection (46).

We also observed that JUNB deletion leads to decreased IL-2 production in T cells from ConA- or αGalCer-treated mice, consistent with in vitro experiments demonstrating that AP-1 modulates IL-2 transcription in T cells (18, 19, 47). IL-2 triggers NK cell proliferation and activation (48) and increases IFN-γ expression in T cells (49) and NK cells (48). Therefore, decreased circulating IL-2 in *JunbΔ* mice could contribute to decreased IFN-γ production and reduced liver damage. Our data suggest that the contribution of IL-2 is likely marginal, as NK cell numbers in the liver were comparable between control and *junb*-deficient mice and IFN-γ was unaffected in c-JUN–deficient mice, despite a reduction in circulating IL-2.

Strikingly, the function of JUNB appears both overlapping and distinct from the hepatoprotective role of its close homolog c-JUN. Hepatocytes lacking c-JUN are more prone to cell death (25, 50), and mice lacking JUNB in hepatocytes specifically also display a mild increase in ConA-induced liver damage. However, additional deletion of JUNB, but not c-JUN, in resident and circulating immune cells protects hepatocytes against ConA-induced death, indicating that the role of JUNB in immune cells is dominant over its function in hepatocytes. Interestingly, the third member of the JUN family, JUND is also important for TNF-α–induced liver damage (21, 51), but the cell-specific function of JUND has not been addressed. These studies show that JUN proteins are important for the survival of hepatocytes, although the underlying mechanisms are likely cell-context and stress-signal specific.

No2 is rapidly upregulated in macrophages followed by hepatocytes during acute hepatitis, leading to increased NO production. The function of No2 is complex, as NO has been shown to have both cytotoxic and anti-inflammatory effects in the liver (52). We and others have shown that c-JUN/AP-1 regulates No2 expression (25, 53, 54), and impaired No2 induction upon ConA treatment in hepatocytes lacking c-JUN correlated with decreased NO and increased hypoxia, oxidative stress, and liver damage (25). Our data indicate that the function of JUNB/AP-1 during acute hepatitis is distinct from c-JUN and mediated, at least in part, by IFN-γ/pSTAT1-triggered No2 induction in immune cells of the liver. In addition, mice with genetic loss of IRF1 are protected from acute inflammation (36), and our adenoviral gene-delivery experiment indicates that decreased IFN-γ/pSTAT1–triggered IRF1 induction in *junb*-deficient livers is likely the major contributor to the phenotype.

Besides No2 and IRF1, IFN-γ/pSTAT1 induces Cxcl9 and Cxcl10 expression in macrophages (42, 43), and mice deficient for Cxcr3, the Cxcl9/10 receptor, display more severe ConA-induced liver injury (55). Therefore, the induction of cell death by IFN-γ/pSTAT1 in hepatocytes is counteracted by upregulation of protective chemokines by the same pathway in immune cells. Consistent with attenuated IFN-γ/pSTAT1 signaling, Cxcl9, and to a lesser extent Cxcl10, were decreased in *junb*-deficient livers. Further work will address whether this decrease occurs specifically in macrophages and whether a direct transcriptional control of Cxcl9/10 by AP-1 proteins contributes to decreased chemokine production.

While JNK activity has been shown to be an essential mediator of ConA-mediated liver injury (31), c-JUN is likely not a crucial target downstream of JNK in this model (25). Furthermore, experiments using JNK1 conditional alleles indicated that JNK activity is specifically required in hepatopoietic cells but not in hepatocytes for the development of hepatitis and that loss of JNK in immune cells results in a marked reduction of cytokines produced by NKT cells, including IFN-γ (56). As JUNB can be phosphorylated and activated by JNK in immune cells (20), our data imply that JUNB, rather than c-JUN, might be the relevant target downstream of JNK in immune cells modulating IFN-γ expression during acute hepatitis. Hepatitis viruses have evolved to suppress the IFN antiviral response (57), and impaired IFN signaling is a common immune defect in human cancer (58). This might explain the rather limited benefit of IFN-based therapies in the clinic (14). However, recent data indicate that NK and NKT cells are part of the immune suppressive tumor microenvironment (59, 60) and that T cell–derived IFN-γ and TNF-α efficiently induce tumor arrest and senescence (61), prompting interest in exploring innovative cancer therapies based on the manipulation of T, NK, or NKT cells. Since a better understanding of the functions of NK and NKT cells is crucial for their rational use in tumor immunotherapies, our findings could well be relevant for cancer research.

**Methods**

**Human samples.** Hepatitis samples had Metavir scores of A1 or A2 (necroinflammatory) and between F0 and F2 (fibrosis index).

**Mice and treatments.** *JunbΔ*, *MxCre*, and *AlfpCre* mice were backcrossed to C57BL/6 for a minimum of 5 generations. Four-week-old control and mutant *JunbΔ* mice were injected intraperitoneally with 13 mg/kg poly-I/C twice, with a 5-day interval, to delete the JUNB floxed allele. All subsequent experiments were carried out using 8-week-old animals. 12.5 mg/kg or 25 mg/kg ConA (Sigma-Aldrich) was injected intravenously. 30 mg/kg poly-I/C was injected intraperitoneally. 35 μg + 0.1 g/kg LPS/GalN (Sigma-Aldrich) was injected intraperitoneally. αGalCer (Funakoshi) was dissolved in 1% DMSO/PBS and delivered either intravenously or intraperitoneally at 100 μg/kg. The analysis time points, which are different between experimental models, correspond to the commonly used time points in the field. Recombinant murine IFN-γ (Tebu-Bio) was dissolved in 0.1% BSA/PBS and injected intraperitoneally. 3 × 105 PFUs of either Ad-IRF1 (SignaGen) or Ad-GFP (Iowa University) adenoviruses were injected intravenously 4 days prior to ConA treatment.

**Cell culture.** The human YT NK cell line was kept in RPMI with 10% FCS. Cells were left untreated or treated with 1 μM PMA and 0.5 μM ionomycin for 2 hours before processing for further analysis.

**Immunohistochemistry.** Tissues were fixed in 4% PFA and embedded in either paraffin or OCT. H&E staining was performed according to standard procedures. For paraffin-embedded sections, antigen retrieval was performed with a pressure cooker using citrate buffer, pH 6. The following antibodies were used for IHC: JUNB (CS3753, Cell Signaling Technology Inc.), CD68 (KP1, Dako), F4/80 (catalogue no. 123101; Biolegend), pSTAT1 (CS9171, Cell Signaling Technology Inc.), and IRF1 (SC-640, Santa Cruz Biotechnology Inc.), together with matching secondary antibodies coupled with a fluorescent dye (Invitrogen) or using the VECTA-STAIN Elite ABC Kits (Vector Laboratories). iNos (catalog no. 610332, BD Biosciences) staining was performed on frozen sections, and secondary anti-rabbit coupled to AF594 or HRP was used. Counterstaining was performed with DAPI or hematoxylin.

**Flow cytometry and cell sorting.** Immune cells were isolated from the spleen or the liver 2 hours after treatment with either ConA or αGalCer. The whole organ was presssed through a 70-μm (spleen) or 100-μm (liver) cell strainer. Liver immune cells were further isolated by 38% to 70% Percoll gradient centrifugation. Red blood cells were lysed (R7757, Sigma-Aldrich). Cells were next incubated with FC-Block (BD) and the following antibodies against immune cell surface markers: F4/80 (Biolegend) and CD3, CD4, CD8, and NK1.1 (all from BD Bioscience). For JUNB intracellular staining, cells were next fixed in 2% PFA, permeabilized with
0.1% Triton X-100, and incubated with primary (anti-JUNB, CS7535) and secondary (anti-rabbit AF594, Invitrogen) antibody. For IFN-γ and IL-2 intercellular staining, single cells were treated with Golgi Stop (BD) but not restimulated. Cells were stained with cell surface markers and then fixed and permeabilized with BD Bioscience fixation and permeabilization buffers. Data were acquired on a BD LSRII Fortessa and analyzed using FlowJo 9.5.3. At least 5,000 individual living cells were collected. The different immune cell populations were gated as follows: T cells: CD3+, NK1.1−; NK cells: CD3+, NK1.1+; NKT cells: CD3+, NK1.1+; CD4+ T cells: CD4+, CD8−; CD8+ T cells: CD4+, CD8+; and macrophages and monocytes: F4/80+. For RNA isolation, splenocytes were stained with CD3 and NK1.1 antibodies, and single-positive (T or NK) cells and double-positive (NKT) cells were sorted using a BD INFLUX sorter.

Blood analysis. Blood was collected by heart puncture. Serum parameters were measured using a VetScan Chemistry Analyzer (Abaxis) or a Reflouvet plus blood chemistry analyzer (Sci Diagnostics) according to the manufacturer’s instructions. Serum cytokines were measured using the CBA Flex Set Cytometric Bead Array (BD Bioscience) for IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-γ, TNF-α, and Cxcl9 and the ELISA Kit for IFN-β (Vekirine).

Protein isolation, Western blot, and dot blot. Tissues were disrupted using a Precellys 24 device (Bertin Technologies) in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich), 0.1 mM NaVO4, 40 mM B-glycerophosphate, 40 mM NaPPi, and 1 mM NaF. For Western blot analysis, 50 μg protein per sample was loaded, and membranes were blocked with 2.5% BSA in TBS-T before incubation with primary antibody. The following antibodies were used: pSTAT1 (CS9171, Cell Signaling Technology Inc.), pSTAT3 (CS3131, Cell Signaling Technology Inc.), pSTAT5 (CS9351, Cell Signaling Technology Inc.), STAT1 (SC-346, Santa Cruz Biotechnology Inc.), STAT3 (CS9132, Cell Signaling Technology Inc.), STAT5 (SC-835, Santa Cruz Biotechnology Inc.), cleaved caspase-3 (CS9661, Cell Signaling Technology Inc.), IRF1 (SC-640, Santa Cruz Biotechnology Inc.), and GFP (ab290-50, abcam). For dot blots, 15 μg protein was spotted on a PVDF membrane and blocked with 5% milk in TBS-T before incubation with anti-Nitrotyrosine antibody (06-284, Millipore). HRP-linked secondary antibody was purchased from Amersham and DAKO.

qRT-PCR. RNA was isolated with TRIzol (Sigma-Aldrich) and complementary DNA synthesized with Ready-To-Go-First-Strand Beads (GE Healthcare), using 1 μg DNAse-pretreated total RNA and random hexamers. Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega) and an Eppendorf fluorescence thermocycler. The comparative cycle threshold method was used for quantification. Expression levels were normalized using at least one housekeeping gene (Actb, Gapdh). See Supplemental Table 1 for primer sequences.

ChIP. ChIP was performed as previously described (25). Chromatin was immunoprecipitated using JUNB antibody (CS7535) or IgG (Millipore). qPCR was used to monitor amplified fragments.

Statistics. All experiments were performed at least 3 times, and data in bar graphs represent mean ± SD of the indicated sample numbers. For ALT measurement, randomized block design was applied to account for variation among litters. Statistical analysis was performed using non directional 2-tailed Student’s t test, except for analysis of Kaplan-Meier curves, for which a log-rank (Mantel-Cox) test was used. P < 0.05 was considered as significant.

Study approval. Paraflin sections of needle biopsies from healthy patients and patients with chronic hepatitides were provided by the CNIO tumor bank in accordance with the ethical guidelines of the Helsinki Declaration. Mouse handling and experimentation was done in accordance with and with the approval of local and institutional guidelines and regulations (the ethics commission for animal research and welfare under Institución Carlos III).

Acknowledgments

We are grateful to G. Luque and G. Medrano for technical help with mouse procedures and the CNIO tumor bank for patient samples. This work was supported by the Banco Bilbao Vizcaya Argentaria Foundation (F-BBVA), a grant from the Spanish Ministry of Economy (BFU2012-40230), and a European Research Council–advanced grant (ERC-FCK-2008/37) to E.F. Wagner. M.K. Thomsen is supported by a Juan de la Cierva postdoctoral fellowship. S.C. Hasenfuss was the recipient of a Boehringier Ingelheim Fonds PhD fellowship and an European Molecular Biology Organization short-term fellowship (ASTF 198–2012). R. Hamacher was supported by Deutsche Forschungsgemeinschaft (HA 6068/1-1).

Received for publication August 2, 2013, and accepted in revised form September 5, 2013.

Address correspondence to: Erwin F. Wagner, Genes, Development and Disease Group, National Cancer Research Centre (CNIO), F-BBVA Cancer Cell Biology Programme, Melchor Fernandez Almagro 3, 28029 Madrid, Spain. Phone: 34.91.224.69.12; Fax: 34.91.224.69.14; E-mail: ewagner@cnio.es.


