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End-Organ Damage in a Mouse Model of Fulminant Liver Inflammation Requires CD4⁺ T Cell Production of IFN- γ but Is Independent of Fas¹

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Abstract

Fulminant inflammation in the liver is often accompanied by the accumulation of IFN- γ -producing T cells. The BALB/c-*Tgfb1*^{-/-} mouse exhibits extensive, spontaneously developing necroinflammation in the liver, accompanied by the accumulation of IFN- γ -producing CD4⁺ and CD8⁺ T cells. Liver damage depends on the presence of an intact *Ifng* gene. We determined the relevant cellular source(s) of IFN- γ . In *Tgfb1*^{-/-} liver, CD4⁺ T cells were more numerous than CD8⁺ T cells and NK cells, and produced more IFN- γ . Depletion of CD4⁺ T cells eliminated both the elevation in plasma IFN- γ and aspartate aminotransferase, whereas depletion of CD8⁺ T cells did not. *Rag1*^{-/-}*Tgfb1*^{-/-} mice exhibited neither IFN- γ elevation nor tissue damage, indicating that NK cells are not sufficient. IFN- γ was required for strong overexpression of class II genes but not for CD4⁺ T cell activation, oligoclonal expansion, or accumulation in the liver. The T cell inhibitory molecule PD-L1 was strongly expressed in *Tgfb1*^{-/-} livers, ruling out a lack of PD-L1 expression as an explanation for aberrant liver T cell activation. Finally, whereas *Tgfb1*^{-/-} CD4⁺ T cells overexpressed Fas ligand, hepatocellular damage was observed in *Fas*^{lpr/lpr}*Tgfb1*^{-/-} mice, indicating that liver pathology is Fas independent. We conclude that liver damage in this model of fulminant autoimmune hepatitis is driven by CD4⁺ T cell production of IFN- γ , is independent of both CD8⁺ T cells and the Fas ligand/Fas pathway, and is not explained by a lack of PD-L1 expression.

Hepatocellular damage mediated by CD4⁺ T cell is an important pathological component of several inflammatory liver diseases, including autoimmune hepatitis (AIH),³ chronic hepatitis due to hepatotropic viruses, and posttransplantation ischemia-reperfusion injury (1–5). CD4⁺ T cells carry out their effector functions principally by elaborating cytokines such as IFN- γ and IL-17. Expression of the Th1 cytokine IFN- γ is characteristic of acute liver injury. In AIH patients, IFN- γ expression correlates strongly with disease activity (6). In vivo neutralization

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Disclosures

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³Abbreviations used in this paper: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD4SP, CD4⁺ single positive; FasL, Fas ligand; NPC, nonparenchymal cell.

of IFN- γ in mice is protective in acute liver injury due to either Con A (7) or acetaminophen (8). Pretreatment with IFN- γ exacerbates liver damage in murine experimental hepatic posttransplantation ischemia-reperfusion injury (9).

AIH is considered to be mediated by T cells that recognize one or more liver-specific Ags (10–12). Analysis of TCR usage in liver T cells from AIH patients demonstrates oligoclonality (13,14), a hallmark of Ag-specific responses. Prominent features of AIH include a strong genetic influence (15,16) and the accumulation in liver of CD4⁺ T cells and CD8⁺ T cells (17). The prominence of CD4⁺ T cells distinguishes AIH from viral hepatitis, dominated by CD8⁺ T cells (1). In liver biopsies from AIH patients, CD4⁺ T cells are found located in portal tracts and, upon isolation and stimulation *ex vivo*, produce IFN- γ (2,18). IFN- γ can injure the liver through a variety of mechanisms, including direct cellular injury (19,20), modulation of Ag presentation, and the recruitment and activation of other immune cells that participate in liver damage. It is likely that IFN- γ produced by the CD4⁺ T cell compartment is important for the development of hepatocellular damage. However, this hypothesis has not been directly tested in patients or in an appropriate animal model.

The BALB/c-*Tgfb1*^{-/-} mouse model recapitulates certain features of human AIH, including a strong genetic influence, extensive necroinflammation in the liver, the spontaneous development of pathology, and the accumulation of CD4⁺ and CD8⁺ T cells in the liver (21, 22). Disease is CD4⁺ T cell dependent (23) and liver CD4⁺ T cell populations are oligoclonal (24). Further supporting an Ag-specific basis for disease, experimental restriction of the CD4⁺ TCR repertoire is protective (25).

IFN- γ is critical to the pathogenesis of liver disease in this model system. *Tgfb1*^{-/-} mice rendered doubly deficient in IFN- γ (*Ifng*^{-/-}*Tgfb1*^{-/-} mice) exhibit portal inflammation but not the widespread parenchymal loss associated with *Ifng*^{+/+}*Tgfb1*^{-/-} mice. The measured concentration of IFN- γ in circulation correlates with the extent of tissue damage in *Tgfb1*^{-/-} mice, underscoring the importance of determining which cell types are its relevant source(s). In this study, we show that CD4⁺ T cells are the sole source of IFN- γ relevant to pathology, with no significant contribution from either CD8⁺ T cells or NK cells. We also show that tissue damage is independent of CD8⁺ T cells and of Fas.

Materials and Methods

Mice

Mice were bred at Dartmouth Medical School (Lebanon, NH) and treated humanely according to National Institutes of Health guidelines. All experiments were done according to protocols approved by Dartmouth's Institutional Animal Care and Use Committee. BALB/c background *Tgfb1*^{-/-} mice, *Ifng*^{-/-}*Tgfb1*^{-/-} mice, and *Rag1*^{-/-}*Tgfb1*^{-/-} mice were generated as published (23,26). *Fas*^{lpr/lpr}*Tgfb1*^{-/-} mice were generated by intercrossing BALB/c-*Tgfb1*^{+/-} mice with BALB/c-*Fas*^{lpr/lpr} mice (27). Litters were screened by PCR of tail DNA for *Tgfb1*, *Ifng*, *Rag1*, and *Fas* genotypes as described (22,27). Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and IFN- γ were measured as published (26) from day 11 and 12 mice. Mice were depleted of CD4⁺ T cells via i.p. injections of the mAb GK1.5 according to the previously published dose and schedule (23). *Tgfb1*^{-/-} mice were similarly depleted of CD8⁺ T cells using the mAb T1B-210. Cell subset depletions were confirmed by flow cytometry.

Isolation of liver lymphocytes and thymocytes

Liver nonparenchymal cells (NPC) were prepared from 11 to 12-day-old mice as described (24). NPC were counted and either stained for flow cytometric analysis or used for positive

selection of CD4⁺ cells (for liver) or CD8⁺ negative selection (for thymus) to obtain CD4⁺ single positive (CD4SP) thymocytes using the Miltenyi system.

Flow cytometry

Analyses were performed as described by Robinson et al. (25). Ficoll-purified NPC were washed with FACS buffer (2% FCS in PBS) and incubated with Abs (all from BD Pharmingen) to label CD3ε, CD4, CD8, DX5, CD11b, CD11c, class II MHC, CD69, CD62L, CD44, and/or PD-L1, washed, fixed in 1% methanol-free formaldehyde in FACS buffer, and analyzed using a FACSCanto cytometer (BD Biosciences). For each analyte, positive staining was established using appropriate isotype controls. PMA/ionomycin stimulations and intracellular IFN-γ analyses were performed as described (28).

mRNA and expression analyses

Expression levels of mRNAs encoding class II MHC genes, CIITA, PD-L1, and β-actin were determined by mining a database of liver mRNA expression. In brief, total liver RNA was prepared from 11 day old mice of the following genotypes (three mice per genotype): *Ifng*^{+/+}*Tgfb1*^{+/+}, *Ifng*^{+/+}*Tgfb1*^{-/-}, *Ifng*^{-/-}*Tgfb1*^{+/+}, and *Ifng*^{-/-}*Tgfb1*^{-/-}. Gene expression analysis used a total of twelve GeneChip Mouse Genome 430 2.0 microarrays (Affymetrix), which were processed with standard labeling, 1-cycle amplification, hybridization, washes, and scanning, completed at the Dartmouth Genomics and Microarray Laboratory. Resulting data were analyzed using GeneTraffic version 3.2 (Stratagene). The accession number for the microarrays is GSE9892 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jjmtrseosyqownu&acc=GSE9892>).

A more complete description of these analyses has been submitted for publication (M. W. Milks, J. G. Cripps, H. Lin, J. Wang, R. T. Robinson, J. L. Sargent, M. L. Whitfield, and J. D. Gorham, submitted). Expression of Fas ligand (FasL) from purified liver CD4⁺ T cells was done by RT-PCR. Total RNA was isolated using the RNeasy method (Qiagen). cDNA was synthesized using the Omniscript RT-PCR kit (Qiagen) with oligo(dT) primers. FasL was detected by RT-PCR as described (29), and spectratype analyses were performed as described (24).

Graphing and statistics

Figures were prepared using Graph Pad Prism version 4.0. Statistical analyses used the bundled software. Bars in the figures show mean + SD. Numbers shown above horizontal lines or next to vertical lines represent *p* values for the comparisons indicated. Statistical comparisons involving AST levels used the nonparametric Mann-Whitney *U* test. Other statistical comparisons used Student's *t* test. Fig. 1B used linear regression.

Results

CD4⁺ T cells are the principal IFN-γ-producing lymphocyte subset

Compared with *Ifng*^{+/+}*Tgfb1*^{-/-} mice, *Ifng*^{-/-}*Tgfb1*^{-/-} mice developed less tissue damage as measured by either AST (Fig. 1A), or ALT (data not shown) confirming our previous study (26). A significant correlation was observed between the extent of tissue damage, measured by plasma AST, and concentration of circulating IFN-γ (Fig. 1B; *n* = 12; *r*² = 0.49; *p* = 0.01). IFN-γ production is typically associated with T cells and with NK cells. We therefore measured cell number and IFN-γ production for three liver lymphoid cell types: CD4⁺ T cells, CD8⁺ T cells, and NK (CD3⁻DX5⁺) cells. NKT cells could potentially be a source but are nearly absent in *Tgfb1*^{-/-} livers (23) and so were not examined for IFN-γ. The numbers of CD4⁺ T cells, CD8⁺ T cells, and NK cells were expanded in *Tgfb1*^{-/-} livers compared with *Tgfb1*^{+/+} livers

(27-, 18-, and 5-fold, respectively; Fig. 2A). To identify the source of IFN- γ , liver lymphocytes were stained for intracellular IFN- γ production upon fresh isolation, without additional ex vivo stimulation. In a representative experiment, 15.0% of CD4⁺ T cells stained for IFN- γ production, compared with 4.2% of CD8⁺ T cells and 1.4% of NKT cells (Fig. 2B). In littermate control *Tgfb1*^{+/+} liver lymphocytes, IFN- γ production was low for all three cell types. After pharmacologic stimulation with PMA/ionomycin, for all three subsets, a significant proportion (30–60%) produced IFN- γ (data not shown), ruling out the possibility that *Tgfb1*^{-/-} liver CD8⁺ T cells and NK cells are incapable of producing IFN- γ . Thus, among the three populations, liver CD4⁺ T cells are both most numerous and produce the most IFN- γ .

To definitively test which lymphocyte subset contributes to IFN- γ in vivo, we depleted specific lymphocyte subsets. *Tgfb1*^{-/-} mice rendered deficient in *Rag1*, and therefore lacking in adaptive lymphocytes but retaining innate immunity, had levels of plasma IFN- γ equivalent to control mice (Fig. 3A). This background level reflects IFN- γ produced by innate immune cells, including NK cells, and independently confirms that *Tgfb1*^{-/-} NK cells and other innate immune cells do not appreciably contribute to IFN- γ production in *Tgfb1*^{-/-} mice. To eliminate individual T cell subsets specifically, *Tgfb1*^{-/-} mice were treated with subset-specific mAbs before the onset of tissue damage. Depletion of CD4⁺ T cells completely prevented the elevation in plasma IFN- γ , whereas depletion of CD8⁺ T cells did not (Fig. 3A). In addition, CD8⁺ T cell depletion did not prevent tissue damage in *Tgfb1*^{-/-} mice, whereas elimination either of all adaptive lymphocytes (*Rag1*^{-/-}*Tgfb1*^{-/-} mice) or specifically of CD4⁺ T cells (anti-CD4) did prevent tissue damage (Fig. 3B). Together with the intracellular IFN- γ analysis, these results directly implicate CD4⁺ cells as the important source of pathologic IFN- γ in the *Tgfb1*^{-/-} mouse. Because plasma levels of IFN- γ and AST correlate closely (Fig. 1B), we propose that CD4⁺ T cell production of IFN- γ is the key determinant of tissue damage in this mouse model of fulminant hepatitis.

Class II overexpression in *Tgfb1*^{-/-} mice is *Ifng* dependent

We explored potential mechanisms by which IFN- γ might contribute to tissue damage in the liver. We examined the involvement of IFN- γ in CD4⁺ T cell responses. We have previously shown that *Tgfb1*^{-/-} liver CD4⁺ T cells are activated (26) and have a skewed TCR repertoire (23). It has been shown that *Tgfb1*^{-/-} mice strongly overexpress MHC class II genes in multiple organs (30). Because IFN- γ potently activates class II MHC expression in APC, we hypothesized that IFN- γ -mediated class II up-regulation in turn drives CD4⁺ T cell activation and TCR repertoire deviation. In this way, IFN- γ might contribute to tissue damage through a positive feedback loop involving augmentation of Ag presentation and CD4⁺ T cell responses. In cells propagated in vitro, class II expression is positively regulated by IFN- γ and negatively regulated by TGF- β 1, through antagonistic effects on the gene encoding CIITA (31). Indeed, both I-E α and I-E β were strongly overexpressed in *Tgfb1*^{-/-} livers, and overexpression of each was nearly completely dependent upon *Ifng* (Fig. 4A). Similar patterns were observed for the expressions of I-A α and I-A β (data not shown). Expression patterns for class II genes correlated well with that for CIITA (Fig. 4A), consistent with a model in which TGF- β 1 and IFN- γ counter-regulate class II expression in vivo through this key class II gene coactivator. Expression of the housekeeping gene β -actin was equivalent between samples. At the protein level, analyses of isolated *Tgfb1*^{-/-} liver NPC indicated that class II protein overexpression was observed on CD11b⁺CD11c⁻ and CD11b⁺CD11c⁺ cell populations (Fig. 4B), and that overexpression was *Ifng* dependent.

CD4⁺ T cell activation, TCR repertoire deviation, and T cell accumulation in liver are independent of IFN- γ

Despite effects of *Ifng* genotype on class II expression, *Ifng* genotype had no discernible impact on expression of markers indicating CD4⁺ T cell activation, that is liver *Tgfb1*^{-/-} CD4⁺ T cells

were CD69^{high}CD62L^{low}CD44^{high}, whether on an *Ifng*^{+/+} or *Ifng*^{-/-} background (Fig. 5A). TCR repertoire skewing, as assessed by TCR spectratype, was also independent of *Ifng*. Spectratype analyses of control liver *Ifng*^{-/-}*Tgfb1*^{+/+} CD4⁺ T cells yielded largely Gaussian distributions, indicating that the population is polyclonal, whereas spectratype analyses of liver *Ifng*^{-/-}*Tgfb1*^{-/-} CD4⁺ T cells yielded non-Gaussian spectratype distributions, indicative of strong oligoclonal T cell responses (Fig. 5B, top). *Ifng*^{-/-}*Tgfb1*^{-/-} CD4SP thymocytes yielded polyclonal profiles, similar to littermate controls (Fig. 5B, bottom). Thus, skewing of the CD4⁺ TCR repertoire in *Ifng*^{-/-}*Tgfb1*^{-/-} mice occurs in the periphery, not in the thymus, similar to what we have observed for *Ifng*^{+/+}*Tgfb1*^{-/-} mice (24). Finally, CD4⁺ T cells accumulated in *Ifng*^{-/-} *Tgfb1*^{-/-} liver at d 11 in numbers similar to those found in *Ifng*^{+/+} *Tgfb1*^{-/-} liver (Fig. 5C). Thus, although liver disease in *Tgfb1*^{-/-} mice requires *Ifng*, we find no evidence to support a role for *Ifng* in the activation, oligoclonal expansion, or accumulation in liver of CD4⁺ T cells.

Liver T cell activation is not due to an absence of PD-L1 expression

The B7-like molecule PD-L1 inhibits T cell activation and function through interaction with PD-1, expressed on T cells. PD-L1 is expressed by a variety of liver parenchymal and nonparenchymal cells and is important for the down-regulation of intrahepatic T cell responses (32–34). We therefore considered the possibility that the high degree of T cell activation observed in *Tgfb1*^{-/-} liver T cells is due to a deficiency of PD-L1 expression. PD-L1 mRNA expression was not suppressed in *Tgfb1*^{-/-} liver, but, conversely, was strongly up-regulated (Fig. 6A). In addition, PD-L1 protein was overexpressed on some populations of *Tgfb1*^{-/-} liver mononuclear cells, CD11b⁺ cells in particular (Fig. 6B). Consistent with previous studies (35–37), PD-L1 expression was largely dependent on *Ifng*.

Tissue damage is Fas independent

We examined whether liver injury requires the FasL/Fas death receptor pathway, as we have previously shown that FasL mRNA is overexpressed in *Tgfb1*^{-/-} livers in an *Ifng*-dependent fashion (26). Indeed, highly purified *Tgfb1*^{-/-} liver CD4⁺ T cells overexpressed FasL mRNA (Fig. 7A). Fas is constitutively expressed on hepatocytes and its engagement rapidly results in hepatocyte apoptosis in vivo (38). Moreover, the direct apoptotic effects of IFN- γ on murine hepatocytes may require FasL/Fas interactions (39). With these considerations in mind, we hypothesized that liver injury in *Tgfb1*^{-/-} mice is Fas dependent. To test this, we rendered *Tgfb1*^{-/-} mice Fas-deficient by crossing them with *Fas*^{lpr/lpr} mice (27). *Fas*^{lpr/lpr}*Tgfb1*^{-/-} mice and *Fas*^{+/+}*Tgfb1*^{-/-} mice developed equivalent elevations in AST (Fig. 7B) and ALT (not shown) as well as the characteristic necroinflammatory hepatic lesions at histologic examination (not shown), indicating that hepatocellular damage is Fas independent.

Discussion

We have previously shown that IFN- γ is necessary for the development of hepatocellular damage in the *Tgfb1*^{-/-} mouse. In the current work, we make several observations that advance the understanding of pathogenesis in this mouse model of inflammatory liver disease. First, the levels of circulating IFN- γ correlate with AST, indicating that IFN- γ production predicts the extent of tissue damage. Second, the most important, and perhaps only relevant, source of IFN- γ is the CD4⁺ T cell, with no significant contributions from either CD8⁺ T cells or NK cells. Third, CD8⁺ T cells are dispensable for liver disease in *Tgfb1*^{-/-} mice. Fourth, while IFN- γ is important for overexpression of class II molecules in *Tgfb1*^{-/-} liver, it has no detectable role in the activation, expansion, or accumulation of hepatic CD4⁺ T cells. Fifth, the relative expression level of the T cell inhibitor molecule PD-L1, while strongly dependent on IFN- γ , is not a determining factor in T cell activation in the *Tgfb1*^{-/-} liver. Finally, although IFN- γ is

required for the overexpression of FasL in *Tgfb1*^{-/-} liver (26), tissue damage in this model system is Fas independent.

AIH is believed to be caused by the failure of normal immune tolerance mechanisms in the liver, resulting in an immune attack upon hepatocytes (40). Studies of biopsy material show that AIH is characterized by the expansion of predominantly CD4⁺ Th cells and chronic viral hepatitis by the expansion of CD8⁺ T cells (1). To classify AIH as a CD4⁺ T cell disease and chronic viral hepatitis as a CD8⁺ T cell disease is, however, undoubtedly an over-simplification of the pathologies, which are more complex. CD4⁺ T cells are important for the immune response to hepatitis C virus, as shown in studies in primates (41). In addition, recent in vitro studies of T cell clones derived from AIH patients strongly suggest that CD8⁺ T cells participate in disease pathogenesis (6). These findings notwithstanding, the studies presented here demonstrate that, at least in this model system, CD4⁺ T cells, through the production of IFN- γ , can elicit tissue damage without the apparent participation of other lymphocytes of the cellular immune system. Like CD4⁺ T cells, CD8⁺ T cells are expanded in *Tgfb1*^{-/-} livers, but mAb-mediated depletion of the CD8⁺ T cell subset had no discernible effect on the development of hepatocellular damage. Although we did not directly test the requirement for NK cells, they are not sufficient for the pathology. NKT cells are abundant in normal murine liver (42), but evidence suggests that they are significantly underrepresented in *Tgfb1*^{-/-} livers (23,24). As such, their contribution to pathology is probably minimal; however, we cannot formally rule out the possibility that part of the effectiveness of anti-CD4 treatment is via depletion of NKT cells.

How might CD4⁺ T cells elicit tissue damage here? IFN- γ is required for the development of hepatocellular damage, and the concentration of plasma IFN- γ quantitatively reflects the degree of tissue damage, as measured by AST. Moreover, the CD4⁺ T cell appears to be the only relevant source of IFN- γ here. Together, the data strongly support a model in which CD4⁺ T cells cause tissue damage through the production of IFN- γ . These results mirror those reported by Ma et al. (43). PBMCs isolated from type 2 AIH patients were stimulated in vitro with peptides derived from the microsomal liver enzyme cytochrome P450IID6, the principal target of autoimmunity in type 2 AIH. The level of IFN- γ produced in response strongly correlated with the degree of tissue damage, as assessed by serum AST. In the second part of this work, we considered hypotheses to explain how CD4⁺ T cells producing IFN- γ might lead to hepatocellular damage.

One hypothesis is that the critical role of IFN- γ is in the activation and expansion of CD4⁺ T cells themselves. IFN- γ can modulate Ag presentation to CD4⁺ T cells by inducing APC expression of class II proteins. Indeed, we found that class II genes are strongly overexpressed in *Tgfb1*^{-/-} livers, and that overexpression is almost entirely IFN- γ dependent. In vitro, IFN- γ and *Tgfb1* modulate class II levels through regulated expression of the critical coactivator protein CIITA (31). Consistent with this model, CIITA mRNA is overexpressed in *Tgfb1*^{-/-} livers, but not in *Ifng*^{-/-}*Tgfb1*^{-/-} livers. Following this line of reasoning, IFN- γ could, through enhanced CIITA expression, induce the overexpression of class II proteins, which would then be responsible for the aberrant activation, oligoclonal expansion, and accumulation of CD4⁺ T cells in the *Tgfb1*^{-/-} liver. However, we find no role for IFN- γ in the early stages of the CD4⁺ T cell response.

Another possibility is that the lack of TGF- β 1 leads to a failure to express the T cell inhibitor PD-L1, a cell surface molecule that inhibits T cell mediated liver inflammation in other model systems. However, there was no failure in PD-L1 expression; indeed, in the absence of TGF- β 1, PD-L1 was strongly overexpressed. As PD-L1's effects in the liver may be more selective for CD8⁺ T cells than for CD4⁺ T cells (34), we cannot rule out the possibility that high PD-L1 expression may be responsible for the (relatively) lower expression of IFN- γ from

Tgfb1^{-/-} CD8⁺ T cells than from *Tgfb1*^{-/-} CD4⁺ T cells. That notwithstanding, the relative expression level of the T cell inhibitor molecule PD-L1 is not a determining factor in T cell activation in the *Tgfb1*^{-/-} liver.

A third possibility is that the critical role of IFN- γ is to regulate the effector function of hepatotoxic CD4⁺ T cells. The ligand FasL is expressed on activated Th1 cells (44,45). The Fas receptor is constitutively expressed on hepatocytes (46), and its triggering rapidly leads to apoptosis (47). We hypothesized that IFN- γ 's pathologic role is to drive FasL expression on CD4⁺ T cells, and that CD4⁺ T cell engagement of hepatocellular Fas is the final effector pathway in the *Tgfb1*^{-/-} liver. The predominant use of FasL by CD4⁺ CTLs in the killing of target tissue has precedence in a mouse model of graft-vs-host disease (48). Consistent with this hypothesis, FasL mRNA expression is up-regulated in *Tgfb1*^{-/-} liver in an IFN- γ -dependent fashion (26). Moreover, as we have shown, FasL is indeed overexpressed in purified liver *Tgfb1*^{-/-} CD4⁺ T cells. However, experimental elimination of functional Fas from the system through breeding in the *lpr* Fas allele had no effect on the development of liver damage. Liver disease is Fas independent, so IFN- γ must drive pathology in this mouse model system through a distinct pathway.

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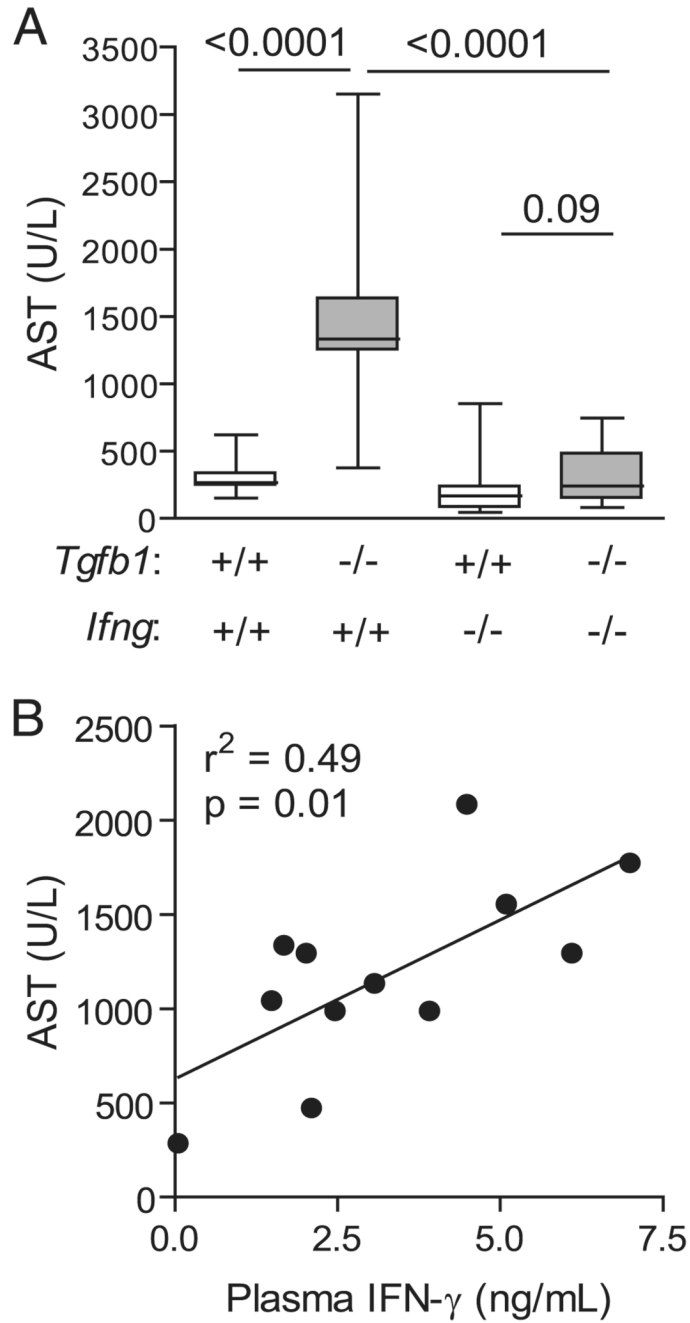


FIGURE 1. IFN- γ determines organ damage in *Tgfb1*^{-/-} mice. *A*, Damage is *Ifng* dependent. Mice of the indicated genotypes were euthanized on day 11–12 after birth. AST was measured from plasma. Data are shown as box and whiskers plots indicating means, 75% confidence intervals, and 95% confidence intervals. Numbers above lines show *p* values for group comparisons by Mann-Whitney *U* test ($n \geq 12$ mice per group). *B*, Plasma concentrations of IFN- γ correlate with end-organ damage (AST). Data points show individual *Tgfb1*^{-/-} mice ($n = 12$; $r^2 = 0.49$; $p = 0.01$).

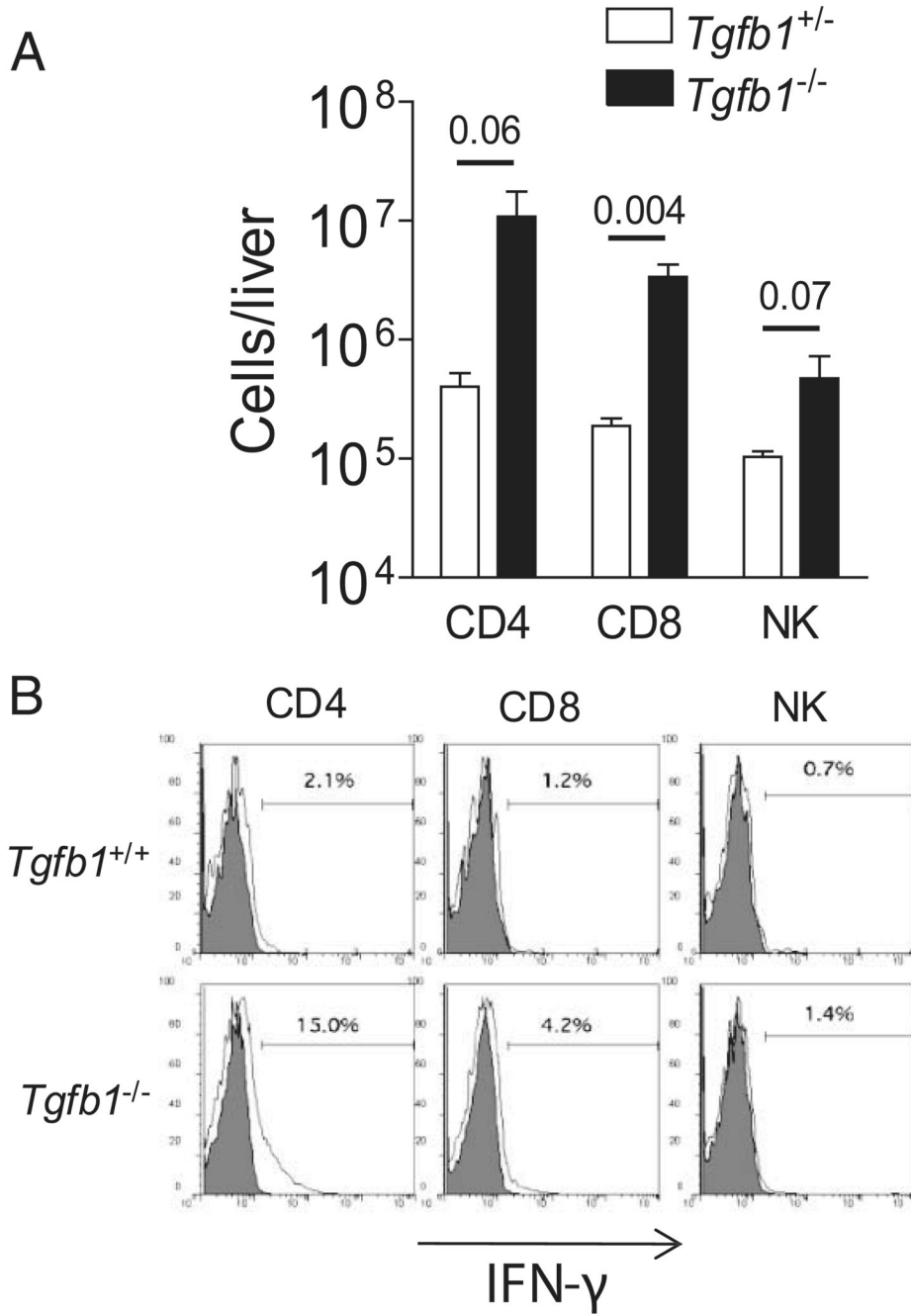


FIGURE 2. *Tgfb1*^{-/-} liver CD4⁺ T cells produce copious IFN-γ. **A**, Liver NPC isolated from 11-day-old *Tgfb1*^{-/-} mice, and littermate control *Tgfb1*^{+/-} mice were counted and stained with Abs to identify CD4⁺ T cells (CD3⁺CD4⁺), CD8⁺ T cells (CD3⁺CD8⁺), and NK cells (CD3⁻DX5⁺). Data show mean + SD of calculated cell numbers (*n* = 3 mice per genotype); *p* values are shown. **B**, Freshly isolated liver NPC were surface stained to classify cell subset, and stained intracellularly to determine IFN-γ production. Unshaded profiles, anti-IFN-γ mAb; shaded profiles, isotype mAb. Similar data were acquired from three independent experiments.

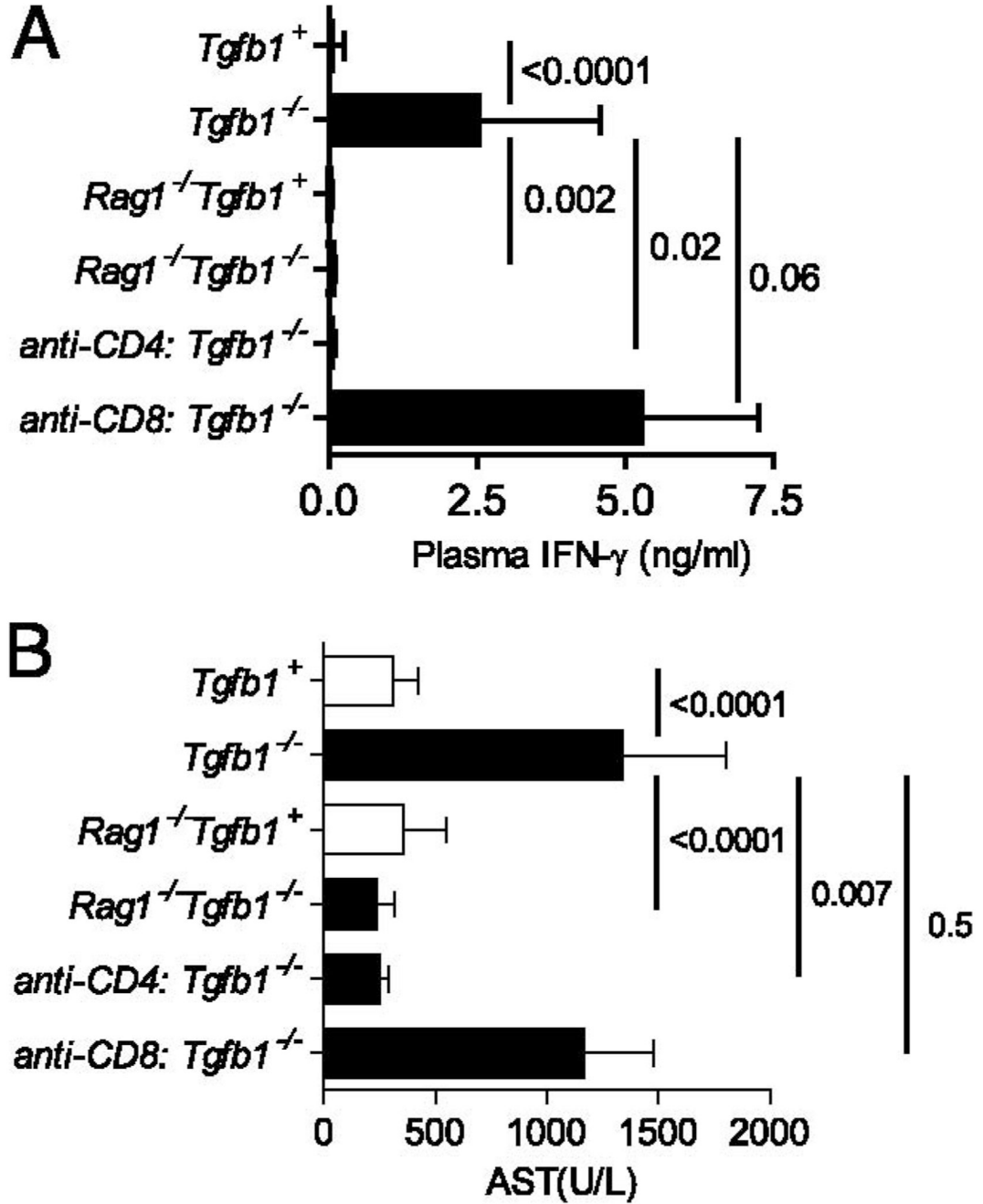


FIGURE 3. CD4⁺ T cells but not CD8⁺ T cells are required for both high plasma IFN- γ and end-organ damage. Plasma from mice of the indicated genotypes and/or treatments was obtained at day 11–12, and IFN- γ (A) and AST (B) were measured. Data show mean + SD ($n \geq 3$ mice per group); p values are shown. Black bars, data from *Tgfb1*^{-/-} mice; white bars, data from *Tgfb1*^{+/+} mice and *Tgfb1*^{+/-} mice combined.

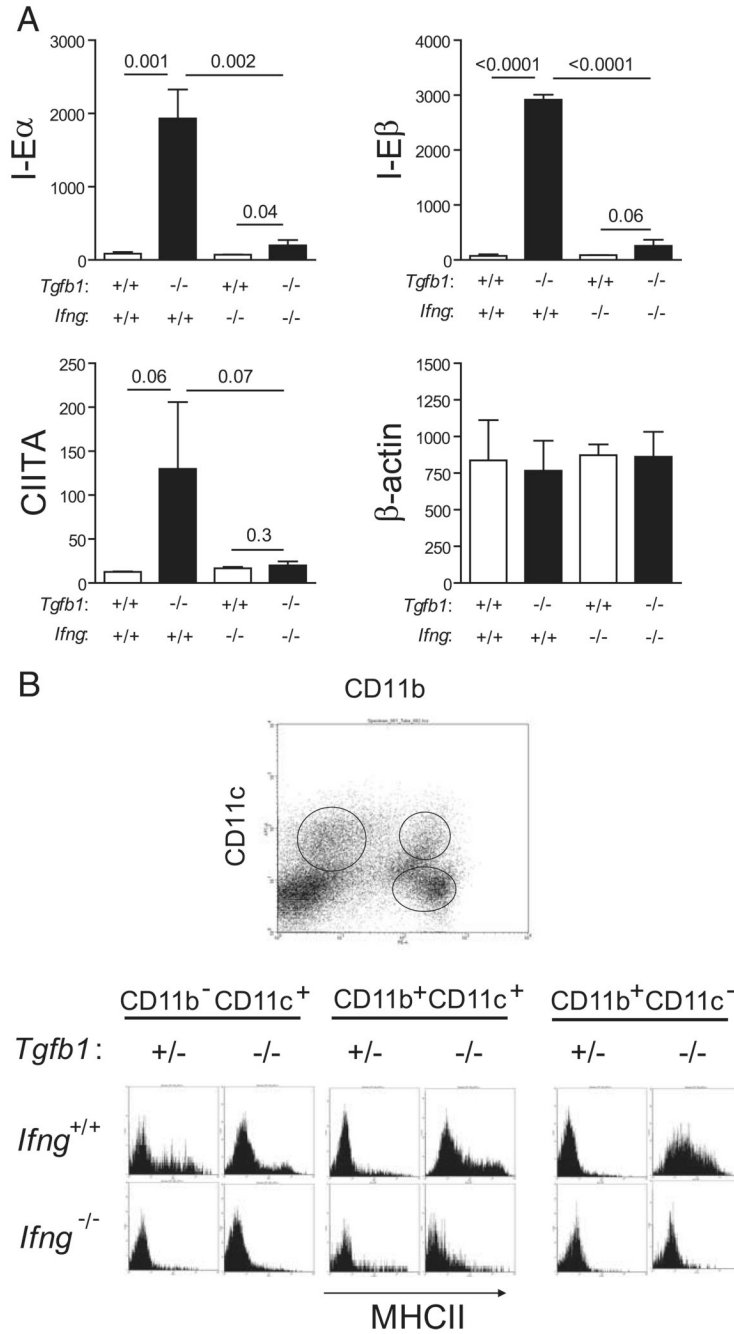


FIGURE 4.

IFN- γ is required for class II overexpression. *A*, Expression levels of mRNAs indicated were mined from microarray data from liver RNA isolated from mice of the indicated genotypes ($n = 3$ per group); p values are shown. *B*, Liver NPC were isolated from mice of the indicated genotypes and stained for CD11c, CD11b, and class II, gated as shown. Similar data were acquired from three independent experiments.

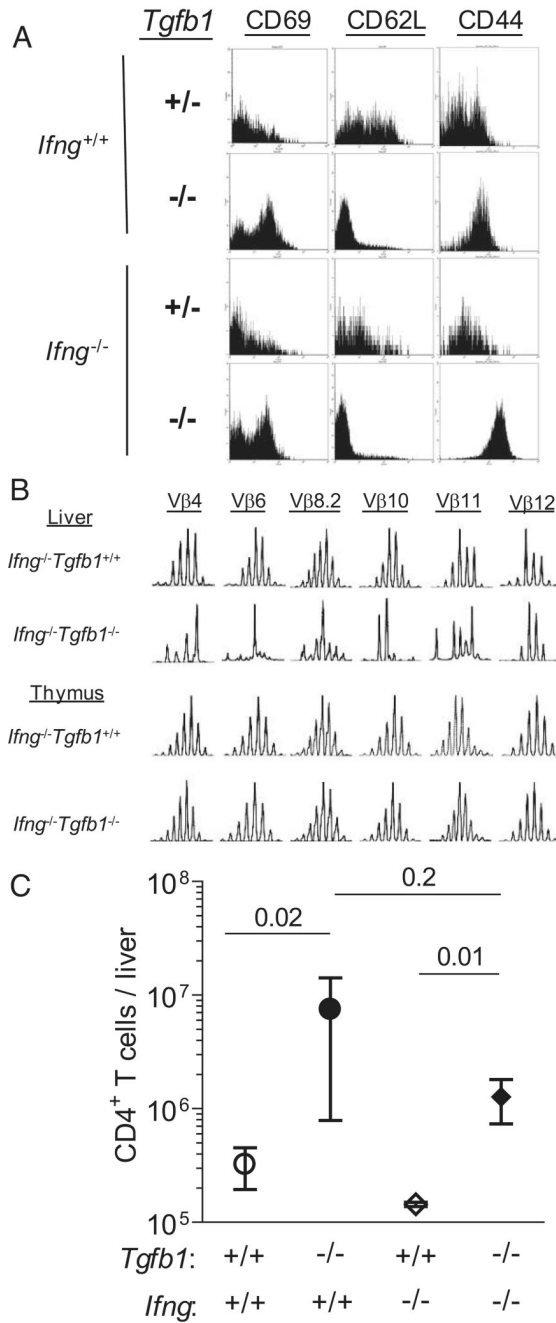


FIGURE 5.

IFN- γ is not required for liver *Tgfb1*^{-/-} CD4⁺ T cell activation, TCR repertoire deviation, or accumulation in liver. *A*, Liver CD4⁺ T cells were isolated from 11-day-old mice and stained with labeled Abs to CD69, CD62L, and CD44. Data are representative of at least three mice per genotype. *B*, Liver CD4⁺ T cells and CD4SP thymocytes were purified from 11-day-old mice. mRNA was isolated, converted to cDNA and CDR3 lengths assessed by the PCR-based spectratype technique for the V β segments depicted. Similar data were acquired from three independent experiments. *C*, Liver NPC isolated from 11-day-old mice were counted and stained for CD3⁺CD4⁺ T cells. Data show mean + SD of calculated cell numbers ($n \geq 3$ mice per genotype); p values are indicated.

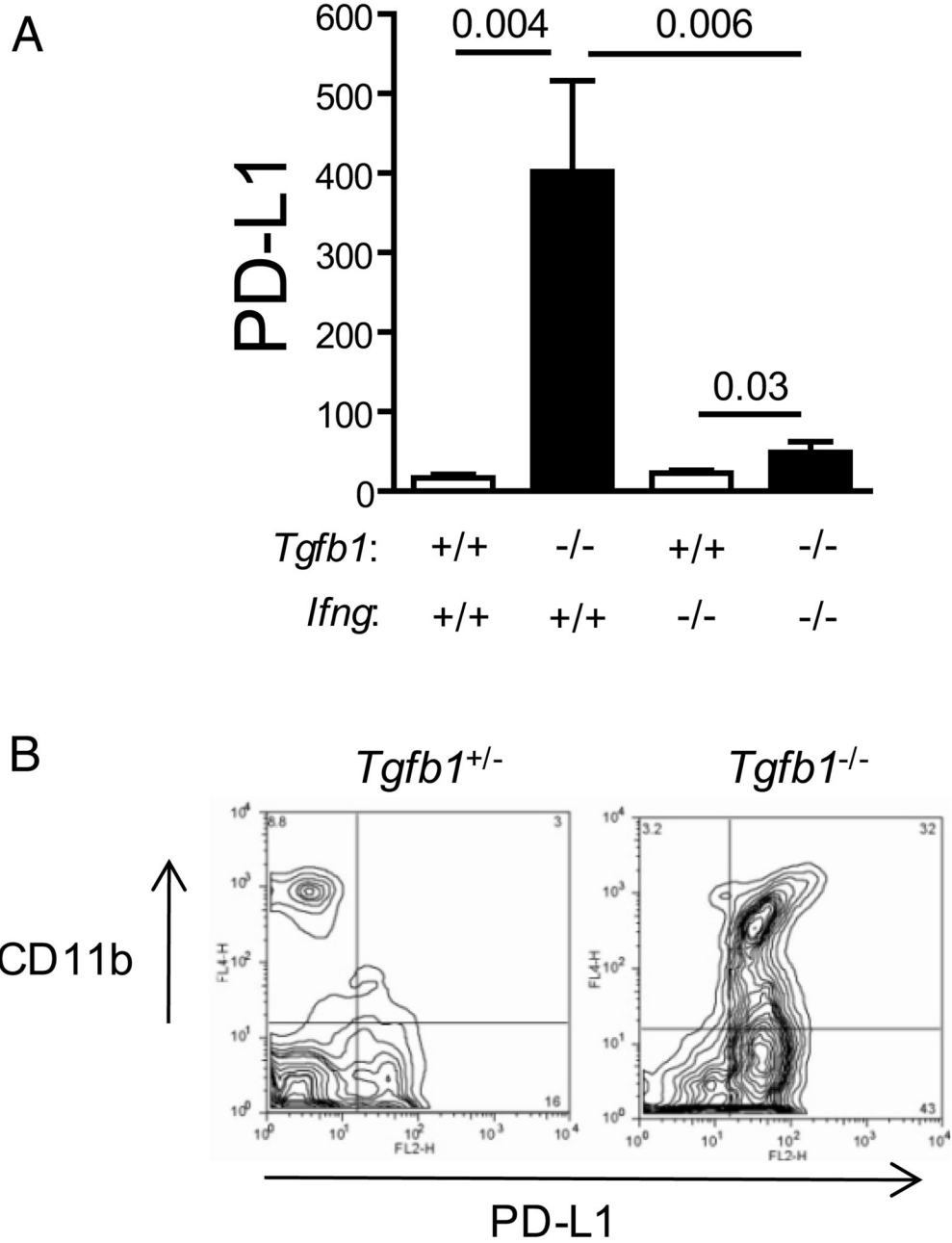


FIGURE 6. PD-L1 is overexpressed in *Tgfb1*^{-/-} liver. **A**, Expression levels of PD-L1 mRNA were mined from microarray data from liver RNA isolated from mice of the indicated genotypes (*n* = 3 per group). **B**, Liver NPC were isolated from mice of the indicated genotypes and stained for CD11b and PD-L1. Similar data were acquired from three independent experiments.

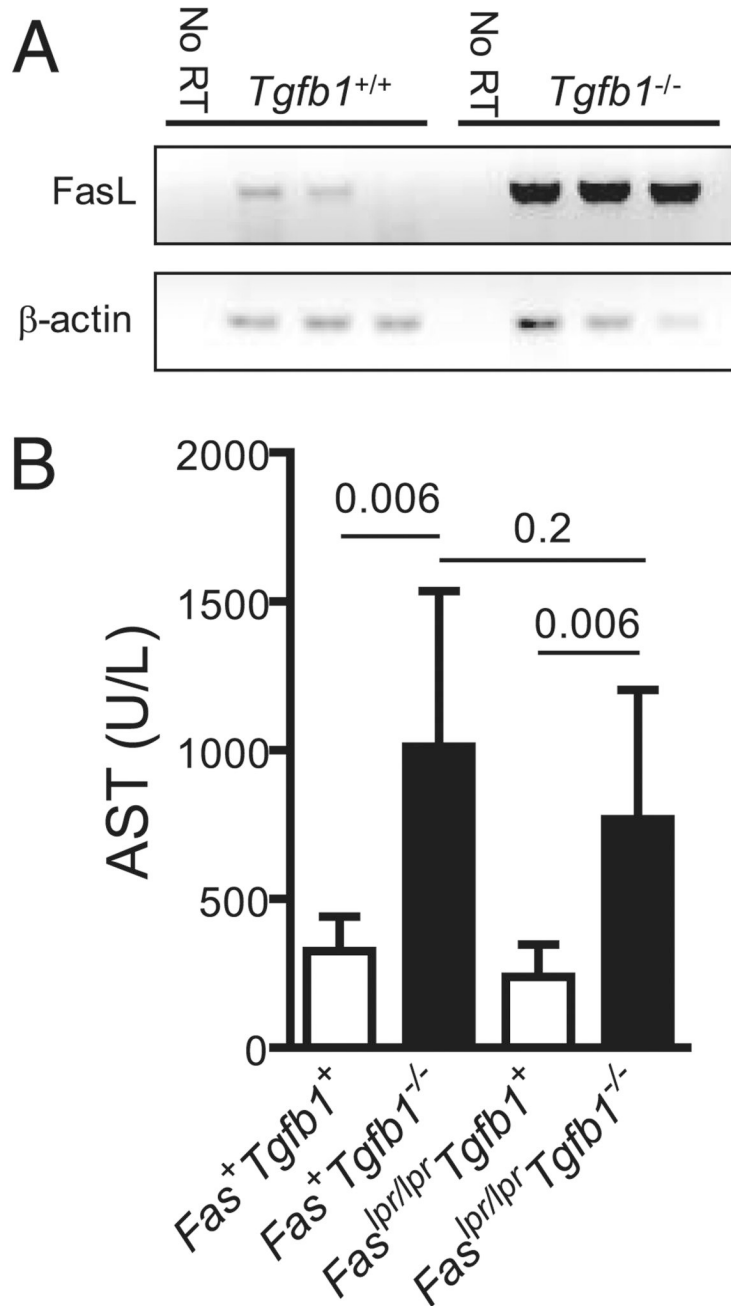


FIGURE 7.

Liver damage is Fas dependent. *A*, Liver CD4⁺ T cells were isolated from 11-day-old littermate *Tgfb1*^{+/+} mice (*n* = 3) and *Tgfb1*^{-/-} mice (*n* = 3). mRNA was purified, converted to cDNA, and FasL and β-actin were measured by RT-PCR. “No RT” controls indicate mRNA samples to which reverse transcriptase was not added, showing that the PCR amplicons were not templated by contaminating genomic DNA. *B*, Plasma AST was measured from 11- to 12-day-old mice. Bars indicate mean + SD. Values of *p* are shown (*n* = 4–7 mice per group).