

## **CD1d-restricted pathways in hepatocytes control local natural killer T cell homeostasis and hepatic inflammation**

Sebastian Zeissig<sup>a, b, c</sup>, Kenneth Peuker<sup>c</sup>, Shankar Iyer<sup>a</sup>, Thomas Gensollen<sup>a</sup>, Stephanie K. Dougan<sup>a, 2</sup>, Torsten Olszak<sup>a, 3</sup>, Arthur Kaser<sup>a, 1</sup>, Richard S. Blumberg<sup>a</sup>

<sup>a</sup>Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

<sup>b</sup>Department of Medicine I, University Medical Center Dresden, Technical University Dresden, 01307 Dresden, Germany

<sup>c</sup>Center for Regenerative Therapies Dresden (CRTD), Technical University Dresden, 01307 Dresden, Germany

<sup>1</sup>Current address: Division of Gastroenterology, Addenbrooke Hospital, University of Cambridge, Cambridge CB20QQ, UK

<sup>2</sup>Current address: Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

<sup>3</sup>Current address: Department of Medicine II, Grosshadern, Ludwig-Maximilians-University (LMU), 81377 Munich, Germany.

Correspondence:

Richard S. Blumberg, MD

Division of Gastroenterology, Hepatology, and Endoscopy

Department of Medicine

Brigham and Women's Hospital

Harvard Medical School

75 Francis Street

Boston, MA 02115

Tel (617) 732 6917

Fax (617) 264 5185

e-mail: [rblumberg@partners.org](mailto:rblumberg@partners.org)

Short title: Hepatocyte CD1d in liver iNKT cell homeostasis

Character count: 51,280

**Classification:** Biological Sciences, Immunology and Inflammation

**Keywords:** CD1d, NKT cells, hepatocyte

**Invariant natural killer T (iNKT) cells recognize lipid antigens presented by CD1d and play a central role in regulating immunity and inflammation in peripheral tissues. However, the mechanisms which govern iNKT cell homeostasis after thymic emigration are incompletely understood. Here we demonstrate that microsomal triglyceride transfer protein (MTP), a protein involved in the transfer of lipids onto CD1d, regulates liver iNKT cell homeostasis in a manner dependent on hepatocyte CD1d. Mice with hepatocyte-specific loss of MTP exhibit defects in the function of CD1d and show increased hepatic iNKT cell numbers as a consequence of altered iNKT cell apoptosis. Similar findings were made in mice with hepatocyte-specific loss of CD1d confirming a critical role of CD1d in this process. Moreover, increased hepatic iNKT cell abundance in the absence of MTP is associated with susceptibility to severe iNKT cell-mediated hepatitis thus demonstrating the importance of CD1d-dependent control of liver iNKT cells in maintaining immunological homeostasis in the liver. Together, these data demonstrate an unanticipated role of parenchymal cells, as shown here for hepatocytes, in tissue-specific regulation of CD1d-restricted immunity and further suggest that alterations in lipid metabolism may affect iNKT cell homeostasis through effects on CD1d-associated lipid antigens.**

## **SIGNIFICANCE STATEMENT**

Invariant natural killer T (iNKT) cells recognize lipid antigens in the context of CD1d and have potent effects on immunity. Control of iNKT cells is critical to prevent inflammation, but the mechanisms that maintain homeostasis of iNKT cells in peripheral tissues are incompletely understood. Here, we demonstrate that hepatocytes promote the apoptosis of local, liver-resident iNKT cells in a manner dependent on CD1d. In the absence of hepatocyte CD1d or microsomal triglyceride transfer protein, a protein responsible for lipid transfer onto CD1d, hepatic iNKT cell numbers are increased, which is associated with susceptibility to iNKT cell-dependent hepatitis. This work thus reveals a novel pathway of peripheral induction of immune tolerance, which limits susceptibility to iNKT cell-dependent inflammation in the liver. (120/120 words)

\body

## **INTRODUCTION**

NKT cells recognize lipid antigens presented by CD1d and are distinguished into invariant (i) and non-invariant NKT cells (1). NKT cells demonstrate autoreactivity towards endogenous lipid antigens, show enrichment in tissues such as the liver and adipose tissue, and exhibit potent effector functions in immunity (2, 3). Since iNKT cells represent long-lived tissue resident cells in most organs (4), tight regulation of iNKT cell homeostasis is required to prevent inappropriate inflammation. Studies of intestinal iNKT cells suggest that this tissue residency may be established during narrow time frames of early life through microbially regulated homing and expansion that results in a finely tuned steady-state level of local iNKT cells (5, 6). Further, as iNKT cells exhibit limited proliferation and carefully coordinated tissue trafficking under non-inflammatory conditions (4, 5, 7-9), it is likely that strict control of iNKT cell levels and their activation is important to maintain tissue homeostasis. Consistent with this, triggering iNKT cell activation in the context of their numeric increase in germ-free mice leads to profound inflammation of the colon which is prevented by blockade of CD1d (5, 6).

These peripheral, extrathymic mechanisms of control of iNKT cells are poorly understood but likely include the levels of cytokines and hormones, for example, which can directly regulate iNKT cell responses as well as alter the threshold to iNKT cell activation by endogenous lipid autoantigens (10-12). This further suggests that the activation of iNKT cells, and potentially their homeostasis, may be regulated by the balance between activating and non-activating lipids, and thus by lipid presentation by CD1d (13, 14). However, iNKT cells exhibit similar proliferation and survival after transfer into wild-type (WT) and CD1d-deficient mice, which has suggested that CD1d expression in the periphery is dispensable for the regulation of iNKT

cell homeostasis (7, 8). Whether tissue- and cell-type specific effects of CD1d-dependent regulation of iNKT cell homeostasis exist remains to be addressed, however.

Here we demonstrate, using mouse models with hepatocyte-specific deletion of CD1d and microsomal triglyceride transfer protein (MTP), an intracellular lipid transfer protein responsible for CD1d lipidation and biogenesis (15-19), that hepatocytes control liver iNKT cell homeostasis through MTP- and CD1d-dependent transcriptional regulation of iNKT cell apoptosis. Furthermore, we show that these regulatory mechanisms are critical for the prevention of uncontrolled hepatic inflammation upon iNKT cell activation.

## RESULTS

### **Hepatocyte-specific deletion of *Mttp* is associated with structural and functional alterations in CD1d**

To delete *Mttp*, the gene encoding MTP, in a hepatocyte-specific manner, *Alb-Cre* mice expressing Cre recombinase under control of the albumin promoter (20) were crossed with *Mttp*<sup>fl/fl</sup> mice harboring loxP sites which flank exon 1A of the *Mttp* gene (14, 21, 22). Two of the three known *Mttp* transcripts (*Mttp*-A, *Mttp*-C) include exon 1A, while the *Mttp*-B transcript uses an alternative exon 1B that would escape targeting in the conditional knockout mice described above (16, 23). However, quantitative PCR (qPCR) with exon-specific primers revealed that *Mttp* transcripts in hepatocytes almost exclusively contained exon 1A (Fig. 1A). Accordingly, primary hepatocytes from *Alb-Cre;Mttp*<sup>fl/fl</sup> mice (hereafter, hepatocyte (H)-*Mttp*<sup>-/-</sup> mice) exhibited a dramatic decrease in *Mttp* transcripts containing exon 1A without a compensatory increase in the expression of transcripts containing exon 1B (Fig. 1B). This is consistent with strongly reduced hepatocyte MTP protein expression in these mice (14).

MTP can directly transfer phospholipids onto CD1d (16, 17). Accordingly, *Mttp* mutations in patients with abetalipoproteinemia (ABL) are associated with severe defects in lipid antigen presentation by group 1 (CD1a, CD1b, CD1c) and group 2 (CD1d) CD1 (18). In dendritic cells of ABL patients, group 1 CD1 undergoes proteasomal degradation, while CD1d shows unimpaired expression but is unable to load exogenous antigens (18). Consistent with these findings, hepatocytes of H-*Mttp*<sup>-/-</sup> mice demonstrated unimpaired cell surface and intracellular expression of CD1d as measured by the 1B1 monoclonal antibody (Fig. 1C). However, analysis of CD1d expression using an extended panel of monoclonal and polyclonal antibodies showed that a subset of antibodies exhibited impaired recognition of CD1d at the cell-surface of H-*Mttp*<sup>-/-</sup> hepatocytes (Fig. 1D). This subset of antibodies included the monoclonal antibodies 19G11, 15C6, and 20H2, which potently blocked CD1d-restricted antigen presentation by hepatocytes (Fig. 1E) and were previously demonstrated to bind to adjacent clusters of epitopes in the vicinity of the CD1d antigen binding groove (24). Quantitative PCR showed a minor increase in *Cd1d1* expression by H-*Mttp*<sup>-/-</sup> hepatocytes indicating that impaired CD1d recognition by a subset of monoclonal anti-CD1d antibodies was not the consequence of decreased CD1d expression (Fig. 1F). Together, these results show that deletion of hepatocyte *Mttp* and associated defects in lipid transfer onto CD1d lead to impaired recognition of CD1d by a subset of monoclonal antibodies, thus suggesting structural alterations in CD1d in the absence of this lipid transfer protein.

To address whether deletion of *Mttp* affects CD1d antigen binding and presentation, we studied the presentation of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), an iNKT cell-activating glycosphingolipid, by wildtype and *Mttp*-deficient hepatocytes. Alpha-GalCer presentation by *Mttp*-deficient hepatocytes was associated with reduced iNKT cell activation, which could not be compensated by loading with higher concentrations of  $\alpha$ -GalCer (Fig. 1G, left). Indeed,

iNKT cell-dependent IL-2 release reached a plateau at considerably lower concentrations of  $\alpha$ -GalCer upon presentation by H-*Mttp*<sup>-/-</sup> compared to WT hepatocytes (Fig. 1G, left). Impaired  $\alpha$ -GalCer presentation by H-*Mttp*<sup>-/-</sup> hepatocytes was not the consequence of altered CD1d trafficking as similar observations were made upon surface loading of  $\alpha$ -GalCer onto fixed hepatocytes (Fig. 1G, right). In addition, defects in antigen presentation were specific to CD1d since H-2K<sup>b</sup>-restricted presentation of SIINFEKL did not differ between H-*Mttp*<sup>-/-</sup> and WT hepatocytes (Fig. 1H). Together, these results are in accordance with structural defects of CD1d in the absence of hepatocyte *Mttp*, which lead to impaired antibody-mediated recognition and defects in antigen loading and/or presentation.

Cognate lipids stabilize heterodimers of CD1d and  $\beta_2$ -microglobulin thus contributing to the structural integrity of CD1d (25). This raised the question of whether *Mttp* deficiency and associated structural defects in CD1d affect the stability of interactions between CD1d and cognate lipid antigens. To address this question,  $\alpha$ -GalCer-loaded hepatocytes were maintained in the absence of exogenous lipids to allow for the dissociation of CD1d-bound lipids, after which hepatocytes were co-cultured with iNKT cells. In accordance with the reported stability of  $\alpha$ -GalCer-CD1d complexes,  $\alpha$ -GalCer presentation by WT hepatocytes was not affected by extended culture of hepatocytes in the absence of exogenous antigen (Fig. 1I left;  $P=0.52$  for IL-2 in two-tailed *t* test of 0h vs. 12h). In contrast, H-*Mttp*<sup>-/-</sup> hepatocytes exhibited a time-dependent decrease in  $\alpha$ -GalCer presentation with a 39.5 % decrease in NKT cell-derived IL-2 after 12 hours of hepatocyte culture in the absence of  $\alpha$ -GalCer (Fig. 1I left,  $P=0.03$  for IL-2 in two-tailed *t* test of 0h vs. 12h). Importantly, differences in antigen presentation between WT and H-*Mttp*<sup>-/-</sup> hepatocytes were not the consequence of altered CD1d trafficking as similar observations were made with fixed hepatocytes (Fig. 1I right, IL-2 in two-tailed *t* test of 0h vs. 12h  $P=0.33$  for WT and  $P=0.005$  for H-*Mttp*<sup>-/-</sup>).



Together, these results demonstrate that MTP is critically involved in the regulation of CD1d-restricted antigen presentation by hepatocytes, while its deletion is associated with structural alterations in hepatocyte CD1d, impaired loading of CD1d with exogenous antigens and decreased stability of CD1d-lipid complexes.

### **Hepatocyte MTP controls liver iNKT cell homeostasis through regulation of apoptosis**

We next investigated whether hepatocyte-specific deletion of *Mttp* and associated defects in CD1d-restricted antigen presentation affect the homeostasis of hepatic iNKT cells. Flow cytometry-based analysis revealed no alterations in the expression of activation, memory, and NK cell markers expressed by hepatic iNKT cells obtained from H-*Mttp*<sup>-/-</sup> mice (Fig. 2A). Moreover, T cell receptor (TCR) V $\beta$  usage by hepatic iNKT cells and the ratio of CD4<sup>+</sup> to double-negative iNKT cells was not altered in H-*Mttp*<sup>-/-</sup> mice (Fig. 2B-C). While the phenotype of iNKT cells was not altered, H-*Mttp*<sup>-/-</sup> mice consistently showed an increase in relative and absolute numbers of hepatic iNKT cells, whereas B cells, NK cells, and  $\alpha$ -GalCer/CD1d-tetramer-negative hepatic T cells, which mainly consist of non-CD1d-restricted classical T cells (hereafter termed conventional T cells), were not altered in abundance (Fig. 2D-E). Moreover, the increase in iNKT cells was specific for the liver and not observed in the spleen or lymph nodes of H-*Mttp*<sup>-/-</sup> mice (Fig. 2F).

To provide insight into the mechanistic basis of increased hepatic iNKT cell numbers in H-*Mttp*<sup>-/-</sup> mice, we first investigated the proliferation of iNKT cells *in vivo*. In accordance with previous observations (7, 8), incorporation of bromodeoxyuridine (BrdU) by hepatic iNKT cells required continuous BrdU exposure for several days. Importantly, BrdU incorporation by hepatic iNKT cells and conventional T cells did not differ between WT and H-*Mttp*<sup>-/-</sup> mice (Fig. 2G). Further, analysis of chemokine receptors critical for T cell homing to the liver, such as CXCR6 and CXCR3 (9), revealed no difference in expression by iNKT cells obtained from

WT and H-*Mttp*<sup>-/-</sup> mice (Fig. 2H). We therefore investigated whether iNKT cell death contributes to MTP-dependent regulation of hepatic iNKT cells. Indeed, H-*Mttp*<sup>-/-</sup> mice, compared to WT littermates, showed a decrease in the percentage of annexin V<sup>+</sup> hepatic iNKT cells (Fig. 2I). In contrast, hepatic conventional T cells as well as splenic iNKT cells and conventional T cells did not exhibit differences in annexin V<sup>+</sup> staining between WT and H-*Mttp*<sup>-/-</sup> mice (Fig. 2I-J). Similar findings were made when analyzing the subG0/G1 fraction of apoptotic hepatic and splenic iNKT and conventional T cells (Fig. S1). Together, these results suggest that hepatocyte MTP regulates liver iNKT cell homeostasis through effects on iNKT cell apoptosis.

### **Control of liver iNKT cell homeostasis is mediated by hepatocyte CD1d**

MTP not only facilitates the loading of lipids onto CD1d but also transfers triglycerides and phospholipids onto apolipoprotein B thus contributing to the secretion of chylomicrons and very low density lipoproteins (VLDL). Consequently, H-*Mttp*<sup>-/-</sup> mice showed an increase in hepatic triglyceride, cholesterol and phospholipid levels (Fig. 3A and (22)). These results raised the question of whether defects in hepatocyte CD1d observed in H-*Mttp*<sup>-/-</sup> mice are a direct consequence of altered MTP-mediated lipid transfer onto CD1d or represent an indirect consequence of hepatic steatosis. To delineate these possibilities, we first investigated WT mice on a high fat (HF) and high fat/sucrose (HS) diet to induce hepatic steatosis. WT mice on HF and HS diet exhibited increased weight gain compared to mice on a standard diet (Fig. 3B) and showed significant hepatic steatosis, which was comparable to that found in H-*Mttp*<sup>-/-</sup> mice on a control diet (Fig. 3A, C-D). Genetically induced obesity and hepatic steatosis are associated with reduced hepatocyte CD1d expression (26, 27). In accordance with these observations, mice on a HS diet exhibited a moderate reduction in cell surface CD1d expression by primary hepatocytes, while a similar but non-significant trend was observed for mice on HF diet (Fig. 3E). In contrast to observations in H-*Mttp*<sup>-/-</sup> mice, however, this

decrease in cell surface CD1d expression was observed with all anti-CD1d antibodies investigated including 1B1 (Fig. 3E) and associated with a decrease in *Cd1d1* mRNA expression (Fig. 3F). Moreover, the decrease in cell surface CD1d expression by primary hepatocytes of mice on a HS diet was not associated with defects in CD1d-restricted presentation of  $\alpha$ -GalCer (Fig. 3G), which is consistent with limited correlation between cell surface CD1d expression and the ability of antigen presenting cells to activate iNKT cells (28). In addition, while H-*Mtpp*<sup>-/-</sup> mice exhibited an increase in hepatic iNKT cells (Fig. 2D-E), WT mice on a HF or HS diet exhibited unaltered levels of hepatic iNKT cells (Fig. 3H). Furthermore, several recent studies on diet- and genetically induced obesity and steatosis showed reduced rather than increased levels of hepatic iNKT cells in steatotic mice (26, 27, 29, 30). This is in accordance with an increase in annexin V<sup>+</sup> apoptotic iNKT cells in mice on a HF diet (27), while H-*Mtpp*<sup>-/-</sup> mice exhibited a decrease in the percentage of annexin V<sup>+</sup> hepatic iNKT cells (Fig. 2I). Together, these data suggest that CD1d defects observed in H-*Mtpp*<sup>-/-</sup> mice are a direct consequence of altered MTP-mediated lipid transfer onto CD1d and not an indirect result of hepatic steatosis.

To further extend these findings, we generated mice with hepatocyte-specific deletion of *Cd1d1* (hereafter, H-*Cd1d1*<sup>-/-</sup> mice) by crossing Alb-*Cre* mice with *Cd1d1*<sup>fl/fl</sup> mice (28). *Cd1d1*<sup>fl/fl</sup> mice were generated and maintained on the C57BL/6J background, which harbors an inactivating frameshift mutation in *Cd1d2* (31). H-*Cd1d1*<sup>-/-</sup> mice, similar to H-*Mtpp*<sup>-/-</sup> mice, showed an increase in the abundance of iNKT cells but not conventional T cells among liver mononuclear cells (Fig. 4A). Consistent with results in H-*Mtpp*<sup>-/-</sup> mice, these alterations in iNKT cell abundance in H-*Cd1d1*<sup>-/-</sup> mice were specific to the liver and not observed in the spleen (Fig. 4B). Moreover, hepatic iNKT cells but not conventional T cells obtained from H-*Cd1d1*<sup>-/-</sup> mice exhibited a reduction in the percentage of annexin V<sup>+</sup> cells (Fig. 4C). These results show that hepatocyte-specific deletion of CD1d is associated with alterations in iNKT

homeostasis that are similar to those observed in mice with deletion of MTP thus demonstrating that hepatocyte CD1d regulates liver iNKT cell homeostasis under constitutive conditions.

Decreased hepatic iNKT cell apoptosis in H-*Mttp*<sup>-/-</sup> and H-*Cd1d1*<sup>-/-</sup> mice raised the question of whether CD1d-restricted antigen presentation by hepatocytes directly regulates liver iNKT cell apoptosis. To test this hypothesis, we turned to a reductionist *in vitro* approach, in which hepatic iNKT cells obtained from WT mice were co-cultured with primary WT, MTP- and CD1d-deficient hepatocytes. Invariant NKT cells co-cultured with MTP- and CD1d-deficient hepatocytes exhibited considerably less apoptosis compared to iNKT cells cocultured with WT hepatocytes (Fig. 4D). These findings suggest that hepatocytes can directly induce iNKT cell apoptosis in a manner dependent on CD1d and MTP.

### **Hepatocyte MTP controls liver NKT cell homeostasis through transcriptional regulation of apoptosis**

We next investigated the mechanisms underlying the regulation of liver NKT cell apoptosis by hepatocytes. To sort hepatic NKT cells and to avoid  $\alpha$ -GalCer/CD1d tetramer-induced activation of NKT cells during sorting, we crossed H-*Mttp*<sup>-/-</sup> mice with IL-4/GFP-enhanced transcript (4Get) mice, which express GFP via an internal ribosome entry site in the *Il4* transcript. 4Get mice allow for sensitive and specific detection of iNKT cells due to iNKT cell-specific transcription of *Il4* under constitutive conditions (32). Indeed, more than 90 % of GFP<sup>+</sup> CD3<sup>+</sup> liver mononuclear cells (LMNCs) stained with  $\alpha$ -GalCer/CD1d tetramers thus representing iNKT cells (Fig. 5A). In addition, similar to observations on  $\alpha$ -GalCer/CD1d tetramer<sup>+</sup> iNKT cells in H-*Mttp*<sup>-/-</sup> mice, GFP<sup>+</sup> CD3<sup>+</sup> liver NKT cells obtained from H-*Mttp*<sup>-/-</sup>;4Get mice exhibited decreased cell death compared to Alb-*Cre*-negative 4Get littermates (Fig. 5B). Of note, percentages of apoptotic cells, both among NKT cells and conventional T

cells, were higher in H-*Mtpp*<sup>-/-</sup>;4Get mice (Fig. 5B) compared to H-*Mtpp*<sup>-/-</sup> mice (Fig. 2I), presumably as a consequence of a mixed genetic background of H-*Mtpp*<sup>-/-</sup>;4Get mice (see Materials and Methods). Using the 4Get model, GFP<sup>+</sup> CD3<sup>+</sup> NKT cells and GFP<sup>-</sup> CD3<sup>+</sup> conventional T cells were sorted from LMNCs of H-*Mtpp*<sup>-/-</sup>;4Get mice and Alb-*Cre*-negative 4Get littermates. Sorted NKT and conventional T cells were then subjected to qPCR-based expression analysis of a broad panel of pro- and anti-apoptotic mediators. As shown in Fig. 5C, GFP<sup>+</sup> CD3<sup>+</sup> NKT cells obtained from H-*Mtpp*<sup>-/-</sup>;4Get mice, as compared to NKT cells from Alb-*Cre*-negative *Mtpp*<sup>fl/fl</sup>;4Get littermates, exhibited increased expression of selected negative regulators of apoptosis including *Bcl2*, a potent inhibitor of iNKT cell apoptosis (33). In contrast, expression of the proapoptotic gene *FasL*, a critical mediator of iNKT cell apoptosis (34), was decreased in NKT cells obtained from H-*Mtpp*<sup>-/-</sup>;4Get mice compared to Alb-*Cre*-negative *Mtpp*<sup>fl/fl</sup>;4Get mice (Fig. 5C). Importantly, altered expression of these regulators of apoptosis in H-*Mtpp*<sup>-/-</sup> mice was significantly more pronounced for GFP<sup>+</sup> CD3<sup>+</sup> NKT cells compared to GFP<sup>-</sup> CD3<sup>+</sup> conventional T cells (Fig. 5D). These results were confirmed in sorted iNKT cells and conventional T cells of H-*Mtpp*<sup>-/-</sup> mice and wildtype littermates (Fig. S2). Thus, hepatocyte-specific deletion of *Mtpp* is associated with increased transcription of inhibitors of apoptosis as well as decreased transcription of pro-apoptotic genes in hepatic NKT cells, both of which likely contributes to decreased apoptosis of liver iNKT cells in H-*Mtpp*<sup>-/-</sup> mice.

### **MTP-dependent control of liver iNKT cells regulates the susceptibility to hepatic inflammation**

NKT cells represent a major subset of liver mononuclear cells with critical roles in the pathogenesis of hepatic inflammation. We therefore investigated whether alterations in hepatic iNKT cell homeostasis with a numerically increased level of iNKT cells as observed in H-*Mtpp*<sup>-/-</sup> mice influences the severity of concanavalin A (ConA) hepatitis, a mouse model

of NKT cell-dependent autoimmune hepatitis that is elicited by direct, CD1d-independent NKT cell activation by the lectin ConA (34). *H-Mttp*<sup>-/-</sup> mice, compared to WT littermates, exhibited significantly increased severity of ConA hepatitis as determined by serum alanine aminotransferase (ALT) levels (Fig. 5E). Moreover, while ConA administration induced substantial activation-induced cell death in iNKT cells and conventional T cells, *H-Mttp*<sup>-/-</sup> mice showed reduced hepatic iNKT cell but not conventional T cell apoptosis compared to WT littermates (Fig. 5F), which was not the consequence of alterations in ConA-induced iNKT cell activation (Fig. 5G). Together, these results suggest that increased levels of hepatic iNKT cells as well as resistance of iNKT cells to activation-induced apoptosis, as observed in *H-Mttp*<sup>-/-</sup> mice, are associated with increased susceptibility to iNKT cell-mediated hepatic inflammation.

## DISCUSSION

NKT cells are potent effector T cells, which play protective roles in antimicrobial immunity but can also promote autoimmunity (2, 3, 5, 6, 14, 28). Tight control of NKT cell homeostasis is therefore required to prevent uncontrolled inflammation. However, little is known about how this occurs and in particular whether CD1d-restricted presentation of tissue- or cell type-specific antigens contributes to this process. Here, we describe a novel and unanticipated pathway of peripheral induction of immune tolerance, in which hepatocytes govern control of iNKT cells in the liver and protect from hepatic inflammation through CD1d- and MTP-dependent transcriptional regulation of iNKT cell apoptosis.

The finding of CD1d-dependent regulation of hepatic iNKT cells is reminiscent of recent observations in the intestine. There, neonatal exposure to the microbiota was found to limit local recruitment and proliferation of iNKT cells in a CD1d-dependent manner (5, 6). Interference with these pathways was associated with increased iNKT cell numbers and

susceptibility to severe inflammation upon activation of iNKT cells (5, 6). Similar to these findings, we observed here that the deletion of CD1d or MTP in hepatocytes is associated with an increase in liver iNKT cells, which promotes susceptibility to iNKT cell-mediated hepatic inflammation. While the underlying mechanisms of CD1d-dependent regulation of iNKT cells differ between the intestine (proliferation, recruitment) and the liver (apoptosis), both tissues thus harbor pathways which promote a regulatory environment and protect from inflammation through numeric control of iNKT cells.

MTP- and CD1d-mediated control of hepatic iNKT cells suggests that the presentation of lipid antigens by CD1d and thus the balance between iNKT cell-activating and -non-activating lipids acts as a rheostat in the control of NKT cell-dependent immunity. Since the balance between activating and -non-activating lipids is tightly connected to lipid metabolism, our results further indicate a central role of hepatocellular metabolism in this process. Moreover, given the recent demonstration of iNKT cell-dependent regulation of hepatic metabolism (27, 29, 30), such pathways of interaction between iNKT cells and lipid metabolism are likely bidirectional and consistent with cross talk between immunity and metabolism in the liver. These observations have far-ranging implications for metabolic disorders associated with NKT cell-dependent hepatic inflammation, such as non-alcoholic fatty liver disease (35-37), in which alterations in lipid metabolism may act as triggers of NKT cell-dependent hepatic inflammation. Enzymes involved in the generation or degradation of disease-associated lipids and lipid antigens as well as proteins involved in the transfer of these lipids onto CD1d may thus provide suitable targets for pharmacologic intervention with disease progression.

The cell-specific nature of the *Mttp* deletion in H-*Mttp*<sup>-/-</sup> mice as well as the observation of CD1d-dependent regulation of iNKT cell death by hepatocytes *in vitro* suggest that direct

interactions between hepatocytes and liver iNKT cells regulate the homeostasis of iNKT cells in the liver. This is in accordance with previous findings by us and others, which demonstrated that hepatocytes can directly present antigens to hepatic NKT cells and conventional T cells *in vivo* (14, 38, 39). While we did not investigate the structural basis of such interactions in the present study, previous ultrastructural work revealed that T cells interact with hepatocytes through cytoplasmic extensions, which penetrate sinusoidal endothelial fenestrations (40). As such, it is anticipated that similar interactions provide the basis for interactions between NKT cells and hepatocytes within liver sinusoids.

Previous studies which investigated the homeostasis of iNKT cells upon transfer into CD1d-deficient as compared to WT hosts did not reveal CD1d-dependent effects on iNKT cell proliferation and expansion (7, 8). While we were also unable to detect alterations in iNKT cell proliferation upon deletion of CD1d, we observed an increase in the expansion of iNKT cells in hosts with hepatocyte-specific deletion of CD1d and MTP. These results differ from those obtained in previous studies, which is likely due to differences in the methodological approach. As such, while previous work analyzed the homeostasis of iNKT cells within transferred suspensions of thymocytes, splenocytes and liver mononuclear cells and focused on hosts globally deficient in CD1d (7, 8), we investigated the proliferation and apoptosis of endogenous iNKT cells in the context of cell-specific deletion of CD1d. The investigation of endogenous iNKT cells is of particular relevance given the long-lived and tissue-resident nature of liver iNKT cells, which limits the ability to study these cells in experiments based on cell transfer (4). In addition, recent observations of opposing, cell-type specific roles of CD1d in the intestine (28) suggest that similar principles may be operative in other tissues such as the liver and may require mice with conditional deletion of CD1d, as used here, for their identification.



Together, our findings reveal a novel pathway of local, tissue-specific control of immunity in the liver, which has important implications for autoimmune and metabolic disorders as well as their associated inflammatory and malignant complications.

## **MATERIALS AND METHODS**

### **Mice**

Experiments were performed with the approval of authorities at the Christian-Albrechts-University Kiel, the Technical University Dresden, and Harvard Medical School. Mice were housed in a specific pathogen-free (SPF) barrier facility. *Cd1d1<sup>-/-</sup>;Cd1d2<sup>-/-</sup>* (CD1d<sup>-/-</sup>), *H-Mttp<sup>-/-</sup>*, and *H-Mttp<sup>-/-</sup>;4Get* mice were described before (14, 22, 41). *H-Cd1d1<sup>-/-</sup>* mice were generated by crossing of *Cd1d1<sup>fl/fl</sup>* mice (28) with *Alb-Cre* mice (20). *H-Mttp<sup>-/-</sup>;4Get* mice were on a mixed C57BL/6J, BALB/cJ background, all other mice on a C57BL/6J background. Both male and female mice were used. Co-housed littermates were used as controls in all experiments. For further information on mouse treatment, please refer to SI.

### **Extraction of primary hepatocytes**

Primary mouse hepatocytes were isolated as described before (14).

### **Flow cytometry**

Flow cytometry was performed as previously described (14). For further information, please refer to SI.

### **Antigen presentation and ELISAs**

Antigen presentation assays were described before (14). For further information, please refer to SI.

### **RNA extraction and quantitative PCR**

Real-time PCR was performed as described before (14). For further information, please refer to SI.

### **Hepatic triglycerides**

Hepatic triglyceride levels were determined as described before (22).

### **Statistical analysis**

Datasets of skewed distribution were analyzed using the Mann-Whitney U test and those of Gaussian distribution using the two-tailed Student's *t* test or, in case of multiple testing, one-way ANOVA followed by the Dunnett's test.

## ACKNOWLEDGEMENTS

We thank Steven A. Porcelli (Bronx, NY, USA) and Randy R. Brutkiewicz (Indianapolis, IN, USA) for CD1d antibodies; Stephen G. Young (San Francisco, CA, USA) for *Mttp*<sup>fl/fl</sup> mice; Irani Kathun and Mahmood M. Hussain (Brooklyn, NY, USA) for advice on lipid measurements; and Erez Scapa and David E. Cohen for advice on primary hepatocyte isolation. Work was supported by: The Deutsche Forschungsgemeinschaft (DFG) (ZE814/5-1), the European Research Council (ERC Starting Grant agreement n°336528), the Crohn's and Colitis Foundation of America (Postdoctoral Fellowship Award), the European Commission (Marie Curie International Reintegration Grant n°256363), and the DFG Excellence Clusters "Inflammation at Interfaces" and "Center for Regenerative Therapies" (S.Z.) and NIH grants DK044319, DK051362, DK053056, DK088199 and the Harvard Digestive Diseases Center (HDDC) (DK0034854) (R.S.B.).

**Author Contributions:** S.Z., K.P., A.K., S.K.D. analyzed H-*Mttp*<sup>-/-</sup> mice. S.I., T.G. and T.O. analyzed H-*Cd1d*<sup>-/-</sup> mice. S.Z. and R.S.B. designed the study, coordinated the experimental work and wrote the manuscript with input from co-authors.

## References

1. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, & Moody DB (2015) The burgeoning family of unconventional T cells. *Nat Immunol* 16(11):1114-1123.
2. Gapin L, Godfrey DI, & Rossjohn J (2013) Natural Killer T cell obsession with self-antigens. *Curr Opin Immunol* 25(2):168-173.
3. Brennan PJ, Brigl M, & Brenner MB (2013) Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* 13(2):101-117.
4. Thomas SY, *et al.* (2011) PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *J Exp Med* 208(6):1179-1188.
5. Olszak T, *et al.* (2012) Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 336(6080):489-493.
6. An D, *et al.* (2014) Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* 156(1-2):123-133.
7. Matsuda JL, *et al.* (2002) Homeostasis of V alpha 14i NKT cells. *Nat Immunol* 3(10):966-974.
8. McNab FW, *et al.* (2005) The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol* 175(6):3762-3768.
9. Geissmann F, *et al.* (2005) Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol* 3(4):e113.
10. Brigl M, Bry L, Kent SC, Gumperz JE, & Brenner MB (2003) Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* 4(12):1230-1237.
11. Wong CH, Jenne CN, Lee WY, Leger C, & Kubes P (2011) Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science* 334(6052):101-105.

12. Dowds CM, Kornell SC, Blumberg RS, & Zeissig S (2014) Lipid antigens in immunity. *Biol Chem* 395(1):61-81.
13. Fox LM, *et al.* (2009) Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* 7(10):e1000228.
14. Zeissig S, *et al.* (2012) Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* 18(7):1060-1068.
15. Brozovic S, *et al.* (2004) CD1d function is regulated by microsomal triglyceride transfer protein. *Nat Med* 10(5):535-539.
16. Dougan SK, Rava P, Hussain MM, & Blumberg RS (2007) MTP regulated by an alternate promoter is essential for NKT cell development. *J Exp Med* 204(3):533-545.
17. Dougan SK, *et al.* (2005) Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells. *J Exp Med* 202(4):529-539.
18. Zeissig S, *et al.* (2010) Primary deficiency of microsomal triglyceride transfer protein in human abetalipoproteinemia is associated with loss of CD1 function. *J Clin Invest* 120(8):2889-2899.
19. Sagiv Y, *et al.* (2007) A distal effect of microsomal triglyceride transfer protein deficiency on the lysosomal recycling of CD1d. *J Exp Med* 204(4):921-928.
20. Postic C, *et al.* (1999) Dual Roles for Glucokinase in Glucose Homeostasis as Determined by Liver and Pancreatic  $\beta$  Cell-specific Gene Knock-outs Using Cre Recombinase. *Journal of Biological Chemistry* 274(1):305-315.
21. Raabe M, *et al.* (1999) Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J Clin Invest* 103(9):1287-1298.
22. Khatun I, *et al.* (2012) Phospholipid transfer activity of microsomal triglyceride transfer protein produces apolipoprotein B and reduces hepatosteatosis while maintaining low plasma lipids in mice. *Hepatology* 55(5):1356-1368.

23. Suzuki T, Brown JJ, & Swift LL (2016) Identification of a Novel Transcript and Regulatory Mechanism for Microsomal Triglyceride Transfer Protein. *PLoS One* 11(1):e0147252.
24. Roark JH, *et al.* (1998) CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160(7):3121-3127.
25. Odyniec AN, *et al.* (2010) Regulation of CD1 antigen-presenting complex stability. *J Biol Chem* 285(16):11937-11947.
26. Yang L, Jhaveri R, Huang J, Qi Y, & Diehl AM (2007) Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. *Lab Invest* 87(9):927-937.
27. Kotas ME, *et al.* (2011) Impact of CD1d deficiency on metabolism. *PLoS One* 6(9):e25478.
28. Olszak T, *et al.* (2014) Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* 509(7501):497-502.
29. Lynch L, *et al.* (2012) Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* 37(3):574-587.
30. Wu L, *et al.* (2012) Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proc Natl Acad Sci U S A* 109(19):E1143-1152.
31. Park SH, Roark JH, & Bendelac A (1998) Tissue-specific recognition of mouse CD1 molecules. *J Immunol* 160(7):3128-3134.
32. Stetson DB, *et al.* (2003) Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* 198(7):1069-1076.

33. Yao Z, Liu Y, Jones J, & Strober S (2009) Differences in Bcl-2 expression by T-cell subsets alter their balance after in vivo irradiation to favor CD4+Bcl-2hi NKT cells. *European Journal of Immunology* 39(3):763-775.
34. Takeda K, *et al.* (2000) Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci U S A* 97(10):5498-5503.
35. Syn WK, *et al.* (2012) NKT-associated hedgehog and osteopontin drive fibrogenesis in non-alcoholic fatty liver disease. *Gut* 61(9):1323-1329.
36. Wehr A, *et al.* (2013) Chemokine Receptor CXCR6-Dependent Hepatic NK T Cell Accumulation Promotes Inflammation and Liver Fibrosis. *J Immunol* 190(10):5226-5236.
37. Wolf MJ, *et al.* (2014) Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell* 26(4):549-564.
38. Bertolino P, Bowen DG, McCaughan GW, & Fazekas de St Groth B (2001) Antigen-specific primary activation of CD8+ T cells within the liver. *J Immunol* 166(9):5430-5438.
39. Bowen DG, *et al.* (2004) The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest* 114(5):701-712.
40. Warren A, *et al.* (2006) T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* 44(5):1182-1190.
41. Smiley ST, Kaplan MH, & Grusby MJ (1997) Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 275(5302):977-979.

## FIGURE LEGENDS

**Fig. 1. *Mttp*-deficient hepatocytes show defects in CD1d.** (A-B) Transcription of *Mttp* exons (qPCR) in primary hepatocytes of H-*Mttp*<sup>-/-</sup> (B) and WT mice (A). (C) Mean fluorescence intensity (MFI) of CD1d (1B1) on primary hepatocytes (flow cytometry). (D) Cell surface staining of CD1d on primary hepatocytes by the indicated antibodies (flow cytometry). Polycl., polyclonal. (E) Blocking of  $\alpha$ -GalCer presentation to the iNKT cell hybridoma 24.7 by the indicated antibodies. (F) *Cd1d1* mRNA (qPCR) in purified hepatocytes. (G-I) Presentation by primary hepatocytes of  $\alpha$ -GalCer to the iNKT cell hybridoma 24.7 (G, I) or of SIINFEKL to the T cell hybridoma RF33.70 (H). Results are representative of 2 independent experiments and based on 3 (A-B, F) or individual (C-D) mice per group. In E, G-I, results of triplicates are shown. Mean (A-I)  $\pm$  s.e.m. (A-B, E-I) are shown. For statistical analysis, the Mann-Whitney U test (A, B), the unpaired Student's *t* test (F, G, I), and ANOVA followed by Dunnett's multiple comparison test (E) were applied.

**Fig. 2. Hepatocyte MTP regulates liver iNKT cell homeostasis.** (A-C) Percentage of hepatic iNKT cells expressing the indicated cell surface markers (A left, B-C) or MFI of markers (A right panel) on hepatic iNKT cells. DN, double-negative. (D) Percentage of iNKT cells among hepatic TCR V $\beta$ <sup>+</sup> cells and of conventional T cells, NK cells, and B cells among liver mononuclear cells (LMNCs). (E) Absolute number of hepatic iNKT cells. Each dot represents one mouse. Bar indicates the median. (F) Percentage of iNKT cells among splenocytes, mesenteric lymph node (MLN) and peripheral lymph node (PLN) cells. (G) Representative plots (left) and quantification (right) of BrdU<sup>+</sup> cells among hepatic iNKT and conventional T cells. (H) Percentage of hepatic iNKT and conventional T cells expressing the indicated chemokine receptor. (I-J) Percentage of annexin V<sup>+</sup> iNKT and conventional T cells in the liver (I) and spleen (J). The histogram in (I, left) is gated on iNKT cells. Results are representative of 2 independent experiments and based on 3 (A-C), 10 (D), 8 (WT) and 7 (H-



*Mttp*<sup>-/-</sup>) (E), 4 (WT) and 6 (H-*Mttp*<sup>-/-</sup>) (F), 3 (WT) and 6 (H-*Mttp*<sup>-/-</sup>) (G), 5 (WT) and 7 (H-*Mttp*<sup>-/-</sup>) (H), and 4 (WT) and 5 (H-*Mttp*<sup>-/-</sup>) (I-J) mice per group. Mean ± s.e.m. are shown in (A-D, F-J). For statistical analysis, the Mann-Whitney U test (E) or the unpaired Student's *t* test (D, F-J) were applied. NS, not significant.

**Fig. 3. MTP-dependent regulation of hepatic iNKT cells is not the consequence of hepatic steatosis.** (A) Hepatic triglyceride (TG) content after 10 weeks on a regular control (CTR), high-fat (HF) or high sucrose (HS) diet. (B) Body weight of WT mice. (C-D) H&E (C, bar 50 μm) and macroscopic appearance (D, WT mice) of livers at week 10 of the respective diets. H-*Mttp*<sup>-/-</sup> mice were on control diet. (E) Histograms and MFI of CD1d (1B1) and H-2k<sup>b</sup> by primary hepatocytes of WT mice at week 10 of diets (flow cytometry). (F) *Cd1d1* mRNA (qPCR) in purified hepatocytes. (G) α-GalCer presentation by primary hepatocytes to the iNKT cell hybridoma 24.7. (H) Percentage of iNKT cells among hepatic TCR Vβ<sup>+</sup> cells and of conventional T cells among LMNCs at week 10 of the respective diet. Results are representative of 2 independent experiments and based on 6-8 (A-B), 3 (E-F), and 5-7 (H) mice per group. In (G), results of triplicates are shown. Mean ± s.e.m. are shown in (A-B, E-G). Statistical analysis was performed using ANOVA followed by the Dunnett's test (A-B, E-F).

**Fig. 4. Liver iNKT cell apoptosis is regulated by hepatocyte CD1d.** (A-B) Percentage of iNKT cells among TCR Vβ<sup>+</sup> cells and of conventional T cells among CD45<sup>+</sup> cells in the liver (A) and spleen (B). (C) Percentage of annexin V<sup>+</sup> cells among hepatic iNKT cells and conventional T cells. (D) Percentage of annexin V<sup>+</sup> iNKT cells after coculture of WT liver mononuclear cells with primary hepatocytes of the indicated genotype. Results are representative of 2 independent experiments and based on 4 (A-B) and 3 (C) mice per group.

In (D), results of triplicates are shown. Mean  $\pm$  s.e.m. are shown. Statistical analysis was performed using the Student's *t* test (A-C) or ANOVA followed by the Dunnett's test (D).

**Fig. 5. Hepatocyte MTP regulates NKT cell apoptosis and susceptibility to hepatitis.** (A) Representative plots of hepatic NKT cells in 4Get mice. (B) Percentage of Annexin V<sup>+</sup> cells among hepatic GFP<sup>+</sup> CD3<sup>+</sup> NKT and GFP<sup>-</sup> CD3<sup>+</sup> conventional T cells in the indicated mice. Representative histograms of GFP<sup>+</sup> CD3<sup>+</sup> NKT cells are shown on the left. (C-D). RNA expression of regulators of cell death in sorted hepatic GFP<sup>+</sup> CD3<sup>+</sup> NKT cells (C) and GFP<sup>-</sup> CD3<sup>+</sup> conventional T cells (D) from H-*Mtpt*<sup>-/-</sup>;4Get and Alb-*Cre*-negative *Mtpt*<sup>fl/fl</sup>;4Get (WT) mice. RNA expression is shown as fold of WT. (E) Serum ALT 24 hours after ConA. (F) Annexin V<sup>+</sup> staining of hepatic iNKT cells and conventional T cells 90 min after ConA. (G) Expression of CD25 (upper panel) and CD69 (lower panel) on iNKT cells 12 hours after ConA or vehicle (PBS). Results in C-D were obtained using cells pooled from 10 mice per group. Results in B, E-F, and G are based on 5 WT;4Get and 4 H-*Mtpt*<sup>-/-</sup>;4Get (B), 10 WT and 12 H-*Mtpt*<sup>-/-</sup> (E-F), and 5 ConA-treated H-*Mtpt*<sup>-/-</sup> and 6 ConA- and 6 PBS-treated WT (G) mice. Mean (B, C-E, G)  $\pm$  s.e.m. (B, E, G) is shown. Statistical analysis in (B, E, G) was performed using the Student's *t* test. NS, not significant.

Figure 1

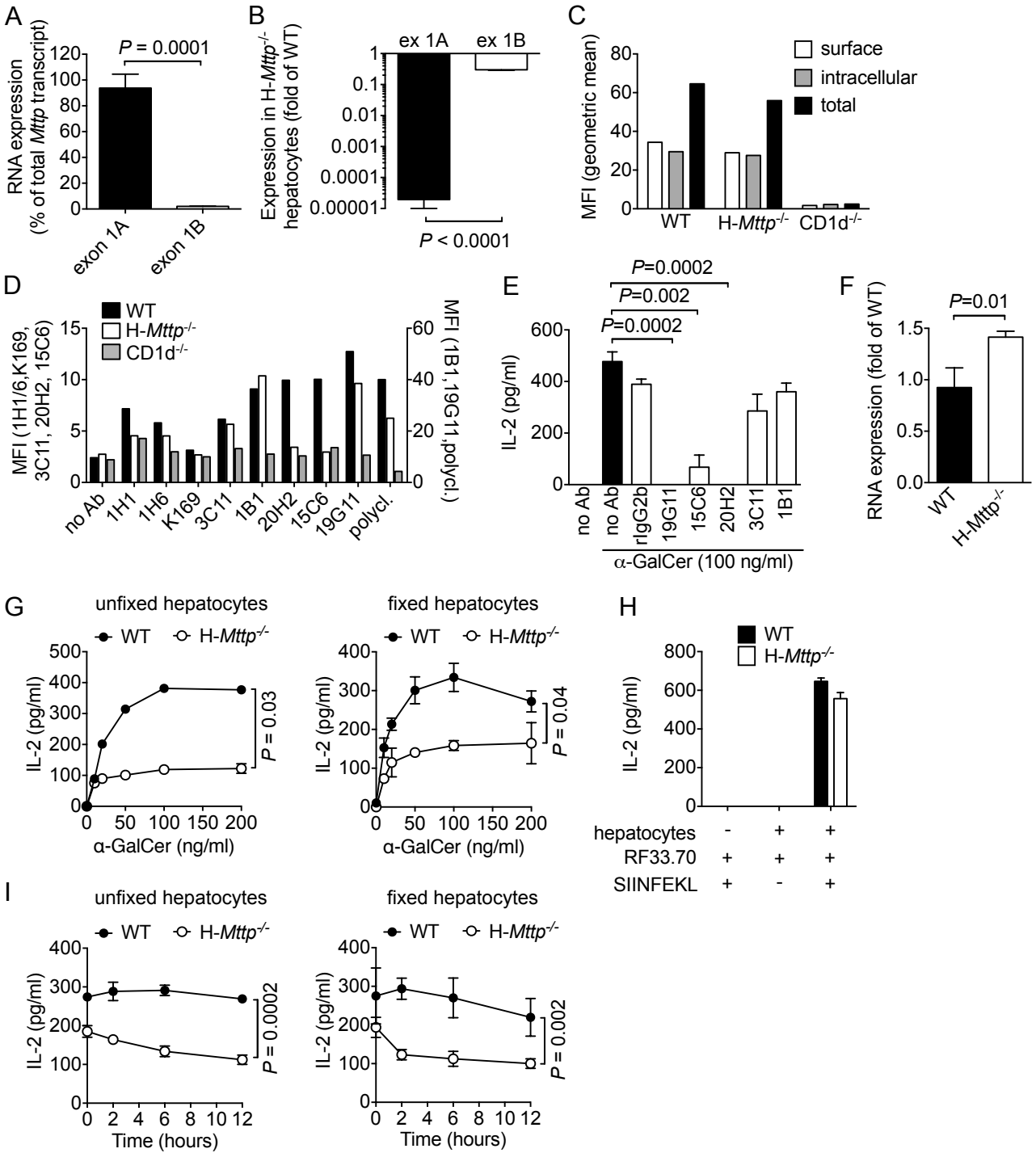


Figure 2

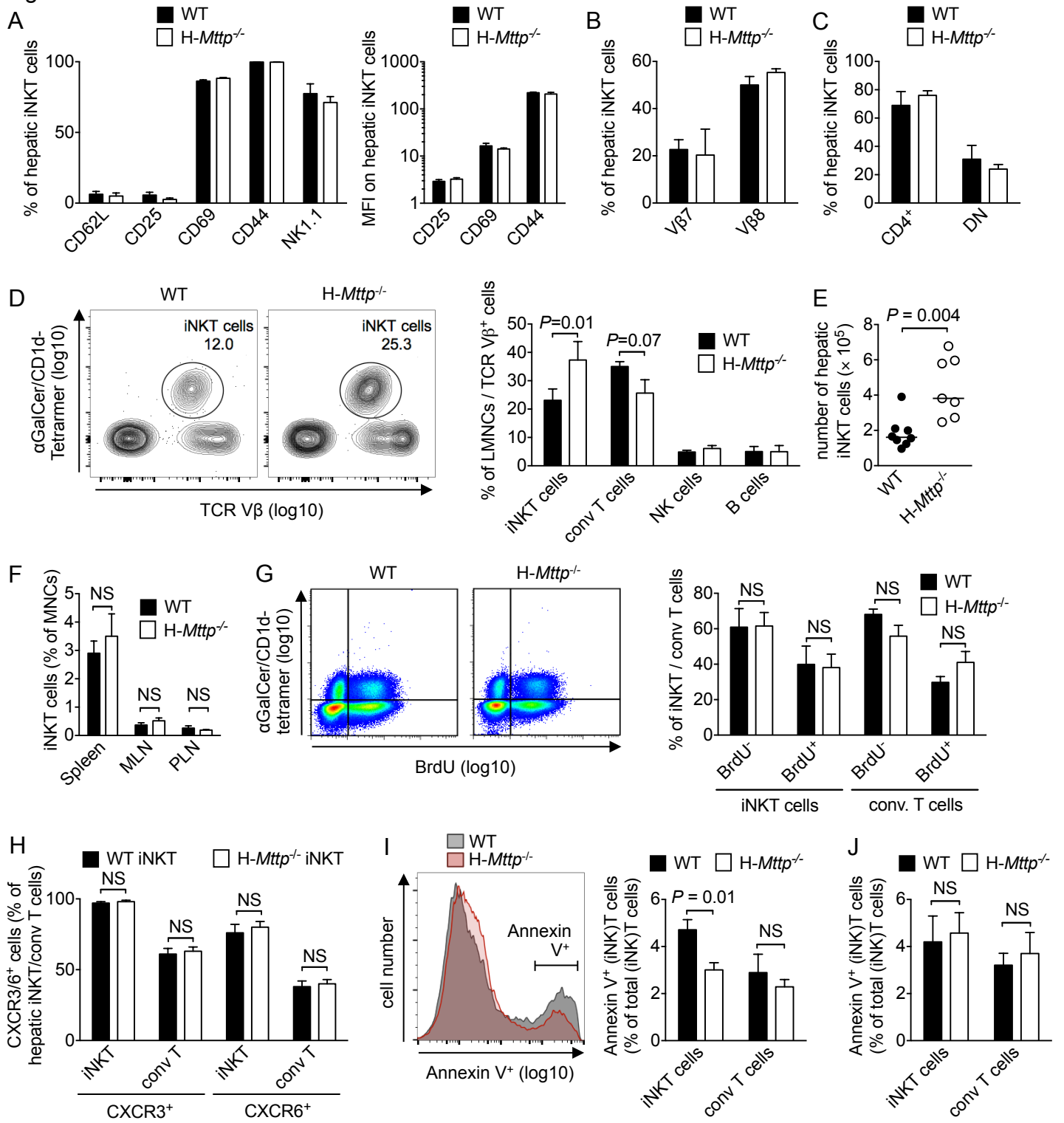


Figure 3

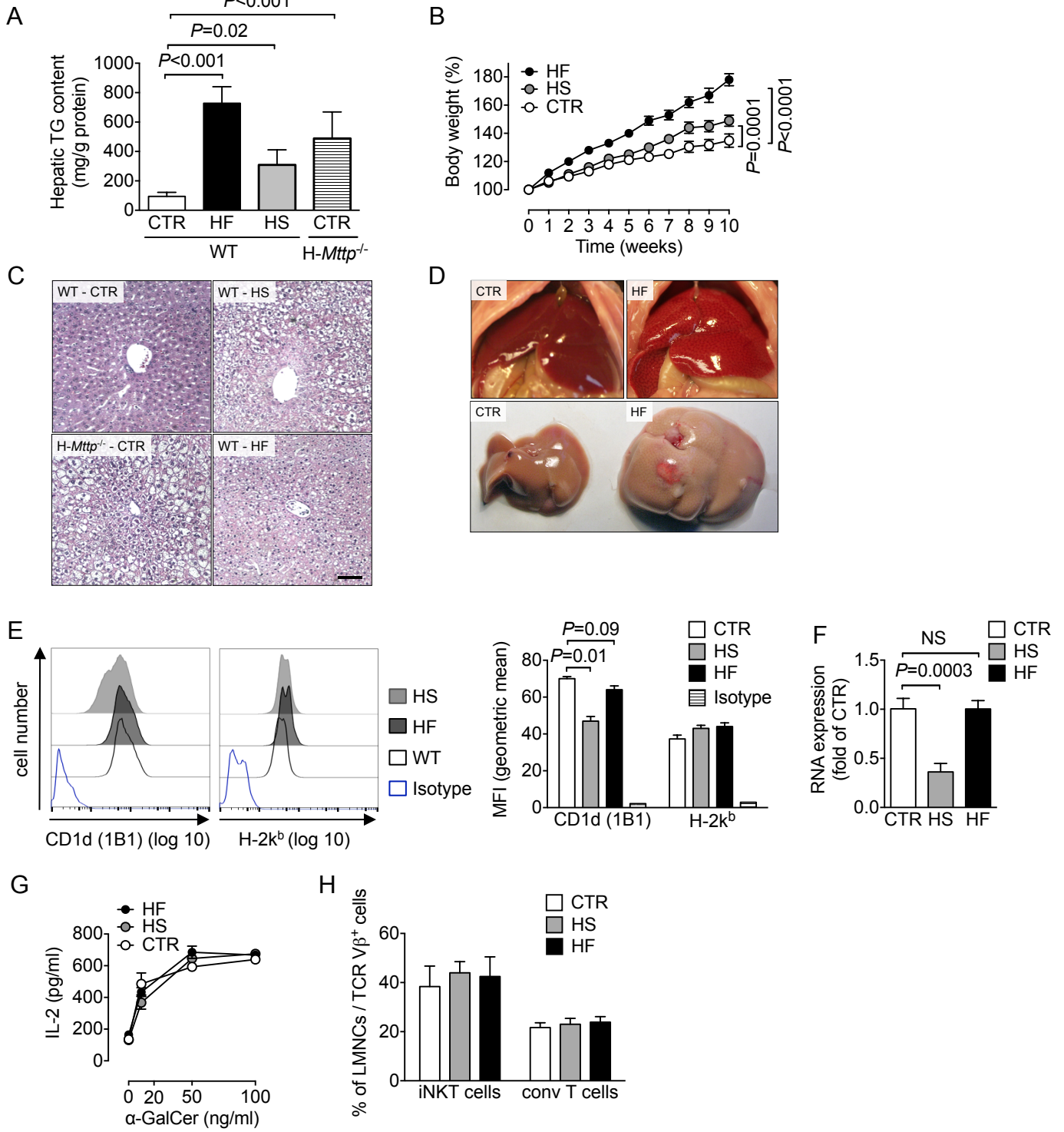


Figure 4

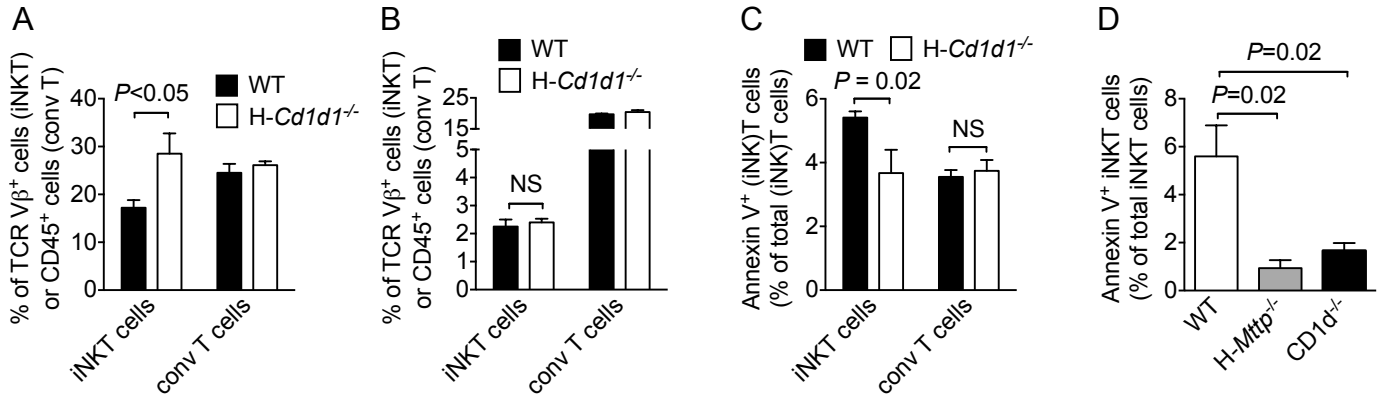


Figure 5

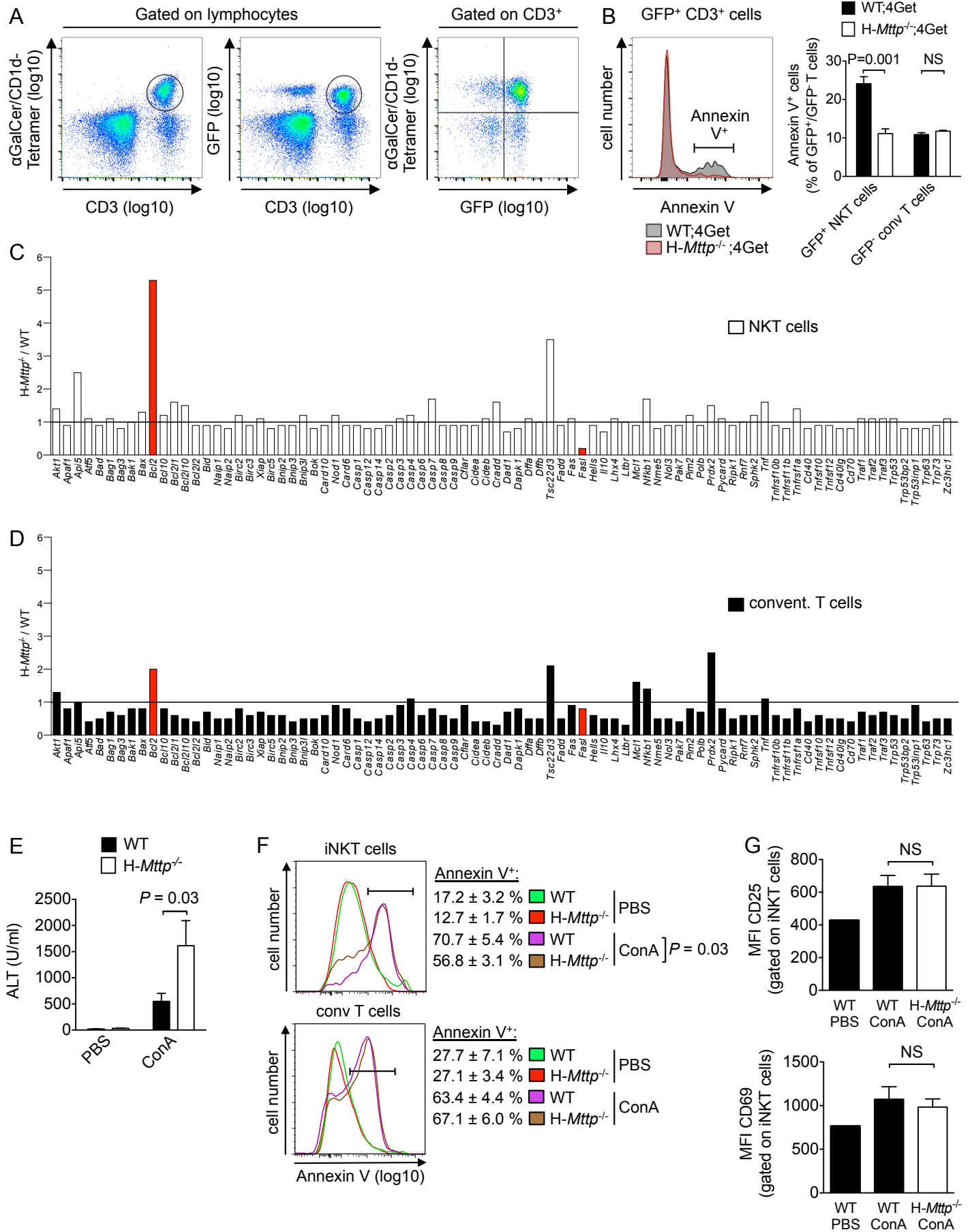
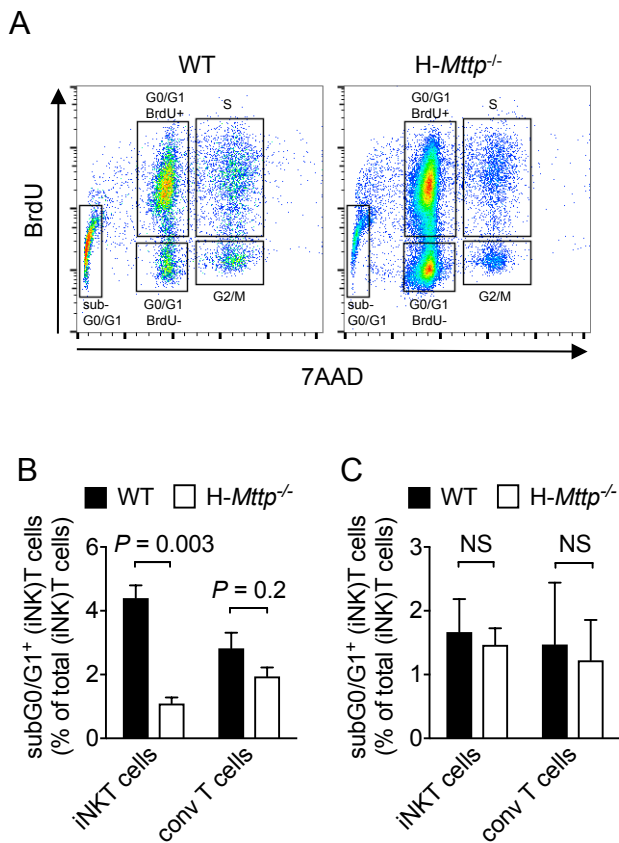
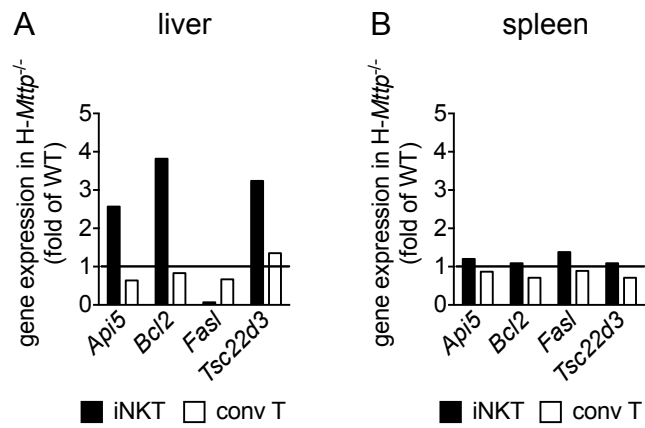


Fig. S1



**Fig. S1. Mice with hepatocyte-specific deletion of *Mtpt* exhibit decreased cell death of liver but not splenic iNKT cells.** (A) Representative pseudocolor plots demonstrating the gating of the apoptotic subG0/G1 fraction among gated  $\alpha$ -GalCer/CD1d-tetramer<sup>+</sup> CD3<sup>+</sup> iNKT cells of liver mononuclear cells in the indicated mice. (B-C) Quantification of apoptotic (subG0/G1 fraction)  $\alpha$ -GalCer/CD1d-tetramer<sup>+</sup> CD3<sup>+</sup> iNKT cells and  $\alpha$ -GalCer/CD1d-tetramer-negative CD3<sup>+</sup> conventional cells among liver (B) and spleen (C) mononuclear cells. Results are representative of two independent experiments and based on three mice per group. Mean  $\pm$  s.e.m. are shown. For statistical analysis, the unpaired Student's *t* test was applied. NS, not significant.





**Fig. S2. Expression of regulators of cell death in iNKT cells and conventional T cells of mice with hepatocyte-specific deletion of *Mttp*.** RNA expression of selected regulators of cell death in sorted hepatic (A) and splenic (B)  $\alpha$ GalCer/CD1d-tetramer<sup>+</sup> CD3<sup>+</sup> iNKT cells and  $\alpha$ GalCer/CD1d-tetramer<sup>-</sup> CD3<sup>+</sup> conventional T cells obtained from H-*Mttp*<sup>-/-</sup> mice and Alb-*Cre*-negative *Mttp*<sup>fl/fl</sup> (WT) littermates. RNA expression is shown as fold of WT cells. Results are based on four mice per group, whose liver or spleen mononuclear cells were pooled before FACS sorting and qPCR.