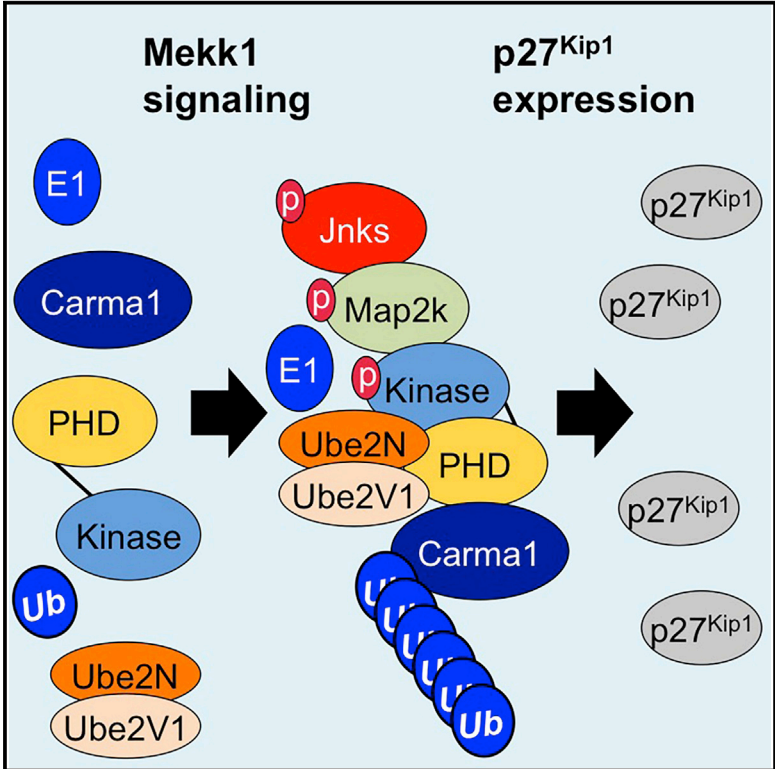


# Cell Reports

## T-Cell-Specific Deletion of *Map3k1* Reveals the Critical Role for Mekk1 and Jnks in *Cdkn1b*-Dependent Proliferative Expansion

### Graphical Abstract



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### In Brief

Suddason et al. use a T-cell-specific deletion of *Map3k1* to show that Mekk1 regulates TCR-dependent Jnk activation and *Cdkn1b* expression to drive proliferation in response to antigen.

### Highlights

- iNKT cell expansion is aberrant in *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice
- *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice have enhanced liver damage in response to glycolipids
- Mekk1 regulates TCR-dependent Jnk activation
- Mekk1 regulates p27<sup>Kip1</sup> expression to regulate proliferation

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# T-Cell-Specific Deletion of *Map3k1* Reveals the Critical Role for Mekk1 and Jnks in *Cdkn1b*-Dependent Proliferative Expansion

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## SUMMARY

MAPK signaling is important for T lymphocyte development, homeostasis, and effector responses. To better understand the role of Mekk1 (encoded by *Map3k1*) in T cells, we conditionally deleted *Map3k1* in *Lck<sup>Crel/+</sup> Map3k1<sup>fl/fl</sup>* mice, and these display larger iNKT cell populations within the liver, spleen, and bone marrow. Mekk1 signaling controls splenic and liver iNKT cell expansion in response to glycolipid antigen. *Lck<sup>Crel/+</sup> Map3k1<sup>fl/fl</sup>* mice have enhanced liver damage in response to glycolipid antigen. Mekk1 regulates Jnk activation in iNKT cells and binds and transfers Lys63-linked poly-ubiquitin onto Carma1. *Map3k1* is critical for the regulation of p27<sup>Kip1</sup> (encoded by *Cdkn1b*).

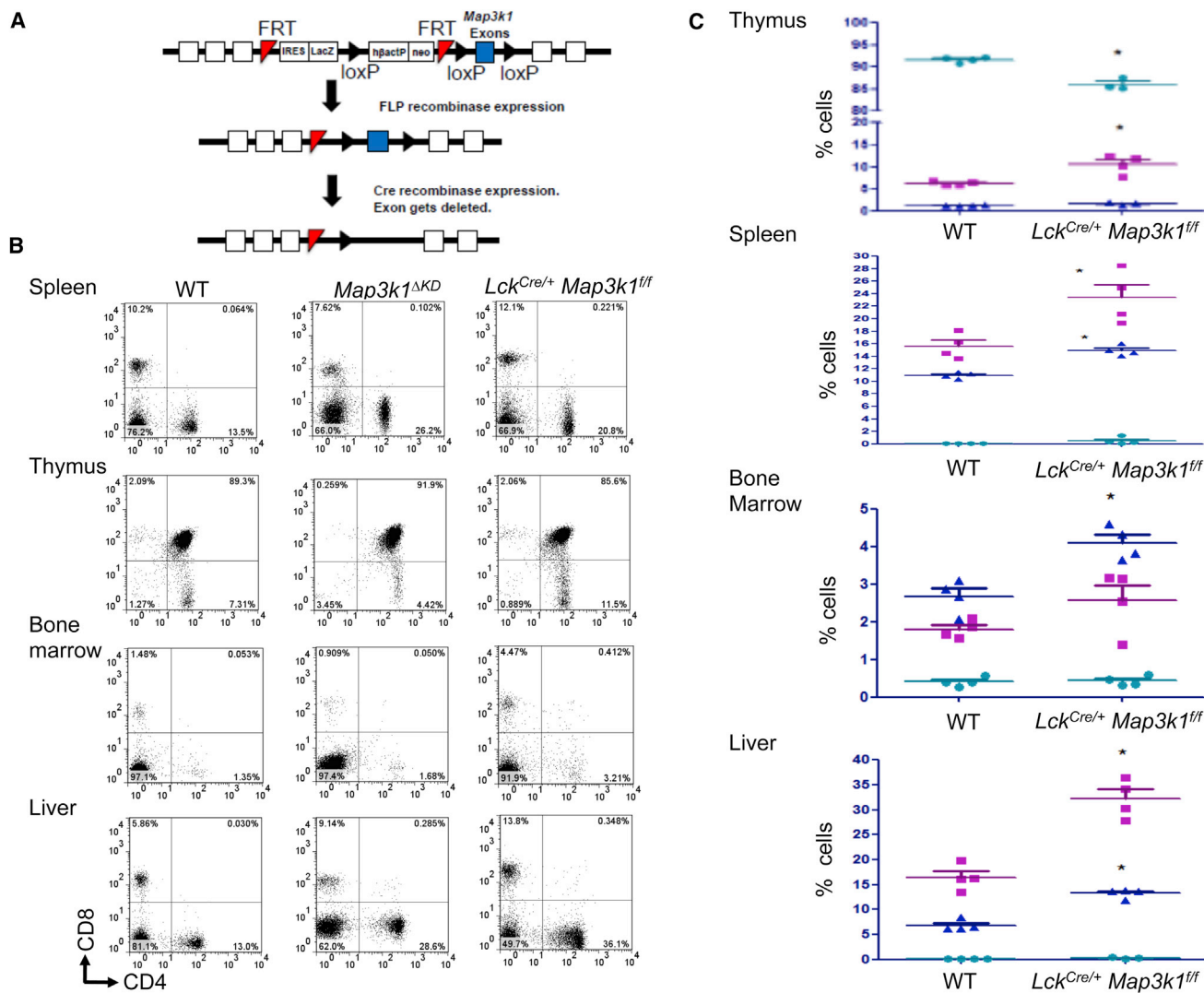
## INTRODUCTION

Mitogen-activated protein kinase (MAPK) kinase (MAP2K) kinases (MAP3Ks) are important regulators of I $\kappa$ B kinases (IKKs) and MAP2Ks (Dong et al., 2002; Ghosh and Karin, 2002; Kyriakis and Avruch, 2001; Raman et al., 2007; Suddason and Gallagher, 2015). Nineteen MAP3Ks are present in mammals, though their precise roles in regulating the immune system are not fully understood (Karin and Gallagher, 2005; Suddason and Gallagher, 2015). Mek kinase 1 (Mekk1) is unique in containing both a kinase domain and a plant homeodomain (PHD), that can bind transforming growth factor (Tgf)- $\beta$ -activated kinase 1 (Tak1)-binding protein 1 (Tab1) and act as an E3 ubiquitin (Ub) ligase (Suddason and Gallagher, 2015; Charlaftis et al., 2014). *Map3k1<sup>ΔKD</sup>* B cells regulate Jnk and p38 signaling from tumor necrosis factor (TNF) receptor family members (TNFRs) (Matsuzawa et al., 2008; Gallagher et al., 2007; Karin and Gallagher, 2009). Analysis of *Map3k1<sup>ΔKD</sup>* T cells demonstrated that Mekk1 is an important regulator of T helper 2 (Th2) cytokine production by the Jnk-dependent activation of Itch (Enzler et al., 2009; Gallagher et al., 2006; Gao et al., 2004; Fang et al., 2002; Venuprasad et al., 2006). Moreover, an intact Mekk1 PHD motif is required for Itch phosphorylation following T cell receptor (TCR) signaling (Suddason and Gallagher, 2015; Charlaftis et al., 2014), though the means by which Mekk1 is recruited to the TCR remain to

be clarified. *Map3k1<sup>ΔKD</sup>* CD8<sup>+</sup> T cells display enhanced expansion in response to viruses, but the mechanism remains uncertain (Labuda et al., 2006). The analysis of the precise role of Mekk1 in T cells using *Map3k1<sup>ΔKD</sup>* mice has been complicated by both B lymphocyte defects and also the partial lethality of *Map3k1<sup>ΔKD</sup>* mice on the C57BL/6 background (Bonnesen et al., 2005; Gallagher et al., 2007).

T lymphocytes form a critical cellular component of the adaptive immune response and can be broadly subdivided into conventional and unconventional subtypes (Kronenberg and Gopin, 2002; von Boehmer, 1990). Of these, natural killer T (NKT) cells constitute a unique unconventional T cell population of the immune system (Kronenberg and Gopin, 2007). By contrast to conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which are reactive to major histocompatibility complex (MHC) class I- or II-associated peptides, NKT cells can recognize lipids in the context of CD1d molecules (Bendelac et al., 1995; Spada et al., 1998; Brossay et al., 1998). NKT cells may express a skewed range of TCR variable region genes and the natural killer (NK) cell marker NK1.1 (Sköld et al., 2000). NKT cells can be subdivided into three categories based on their reactivity to the glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), TCR  $\alpha$  chain diversity, and CD1d dependency. Type I invariant NKT (iNKT) cells have invariant V $\alpha$ 14-J $\alpha$ 18 TCR  $\alpha$  chains and react to  $\alpha$ -GalCer in a CD1d-dependent manner. Type II nonclassical NKT cells are unreactive to  $\alpha$ -GalCer and have TCR  $\alpha$  chain diversity but are CD1d dependent. NKT-like (or type III) cells are CD1d independent, unresponsive to  $\alpha$ -GalCer, and possess diverse TCR  $\alpha$  chains (Bendelac et al., 2007). Following TCR engagement by glycolipid presented by CD1d, iNKT cells undergo proliferative expansion and secrete cytokines (Kawano et al., 1997; Crowe et al., 2003; Parekh et al., 2005; Godfrey et al., 2010). Type I iNKT cells are abundant within the liver, where they are important regulators of inflammation and liver damage (Van Kaer et al., 2013).

Here, we investigate *Map3k1* by T-cell-specific and germline ablation in mice. *Map3k1* regulates iNKT cell proliferative expansion in response to glycolipid antigen. CARD-containing MAGUK protein 1 (Carma1), a TCR-associated scaffold protein, is a target for the Mekk1 PHD motif and provides a mechanism for Mekk1 recruitment to the TCR (Blonska and Lin, 2009; Rincón and Davis, 2007). Microarray gene profiling of *Map3k1*-deficient iNKT cells undergoing their clonal burst in response to glycolipid antigen identified *Cdkn1b* as a cell-cycle gene that is aberrantly expressed in *Map3k1*-deficient mice (Kiyokawa et al., 1996). The



**Figure 1. T Cell Development and Homeostasis with *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* Mice**

(A) A schematic diagram representing the construction of the *Map3k1<sup>fl/fl</sup>* allele. (B) Thymocytes, splenocytes, bone marrow, and liver cells from WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice (all on the C57BL/6 background) were isolated, stained with anti-CD4 and anti-CD8 antibodies, and analyzed by flow cytometry as indicated. Data are representative of three independent experiments. Numbers in the profiles indicate the percentages of the gated populations. (C) The average percentage ( $\pm$ SEM) of three cell populations CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> cells from *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* and WT mice from six independent experiments was statistically analyzed (green circle, CD4<sup>+</sup>CD8<sup>+</sup>; purple square, CD4<sup>+</sup>CD8<sup>-</sup>; blue triangle, CD4<sup>-</sup>CD8<sup>+</sup>), where appropriate, by two-tailed Student's t test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

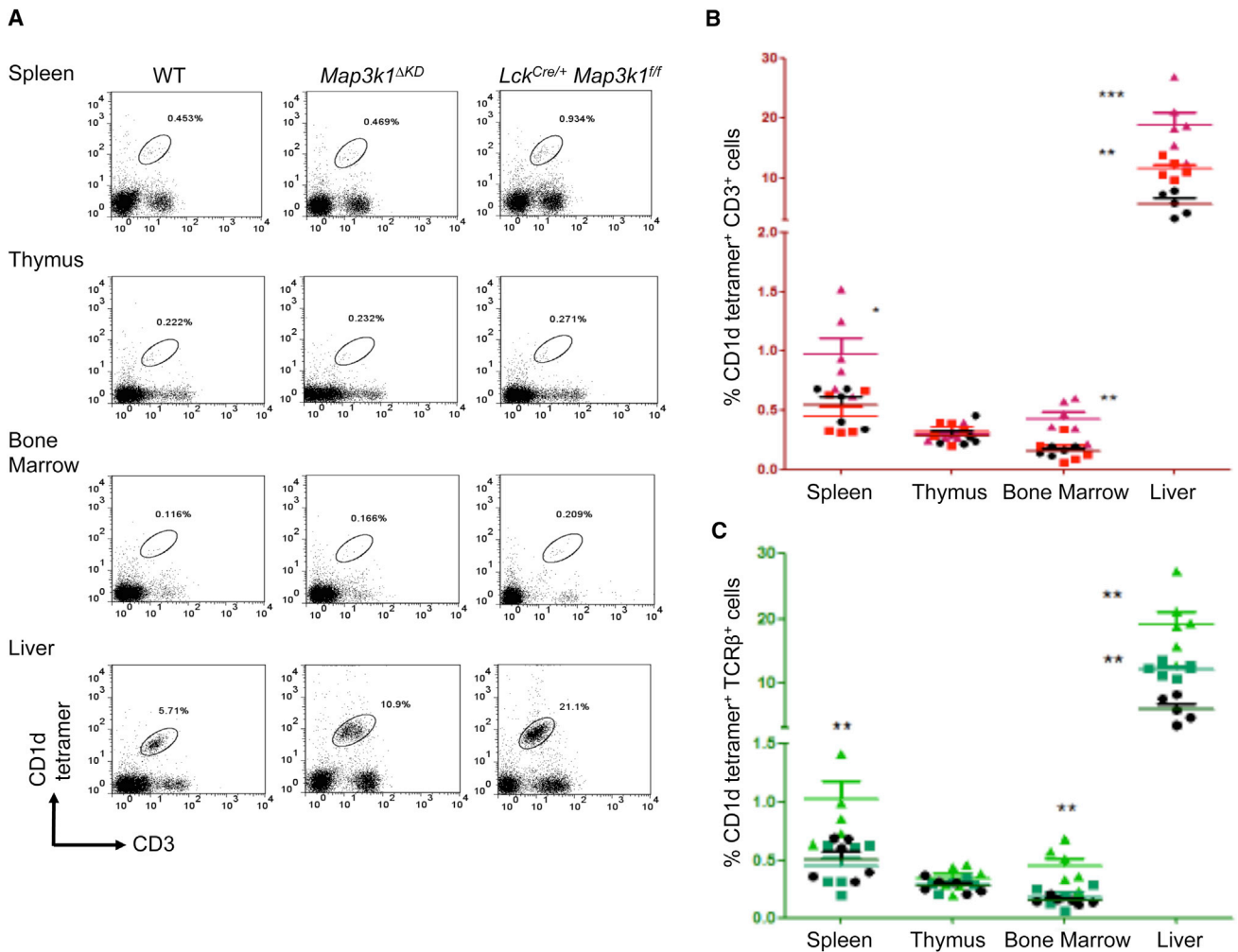
regulation of p27<sup>Kip1</sup> by Mekk1 signaling provides a cell intrinsic molecular explanation for the altered proliferative expansion observed in both *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* iNKT cells.

## RESULTS

### *Map3k1* Regulates Conventional T Cells

Because *Map3k1<sup>ΔKD</sup>* mice have both B cell defects and partial lethality on the C57BL/6 background, we generated *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice (Figures 1A, S1A, and S1B) to better understand the roles of *Map3k1* in T cells (Gallagher et al., 2007; Bonnesen

et al., 2005). Within the thymus of *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice, there is a minor development defect with significantly fewer CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes than WT but a significantly larger than WT population of CD4<sup>+</sup> single-positive thymocytes (Figures 1B and 1C; Chang et al., 2011; Charlaftis et al., 2014). However, the total number of thymocytes in *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice is similar to WT (Table S1; Gao et al., 2004; Venuprasad et al., 2006; Labuda et al., 2006). Splenic CD4<sup>+</sup> T cells isolated from *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice display an enhanced production of *Il4* following TCR crosslinking with anti-CD3 and anti-CD28 antibodies (Figure S1C), the



**Figure 2. iNKT Cell Development and Homeostasis in *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* Mice**

(A) Cell suspensions were isolated from the spleen, thymus, bone marrow, and liver from WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice, stained with CD1d tetramer and anti-CD3 antibody, and analyzed by flow cytometry as indicated. Data are representative of five independent experiments. Numbers in the profiles indicate the percentages of the gated populations.

(B) Statistical analysis of iNKT populations (CD1d tetramer<sup>+</sup>CD3<sup>+</sup>) within the spleen, thymus, bone marrow, and liver from WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice. The average percentage ( $\pm$ SEM) of CD1d-tetramer and CD3-positive cells from five independent experiments is shown (black circle, WT; red square, *Map3k1<sup>ΔKD</sup>*; purple triangle, *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice). Statistical differences were analyzed by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

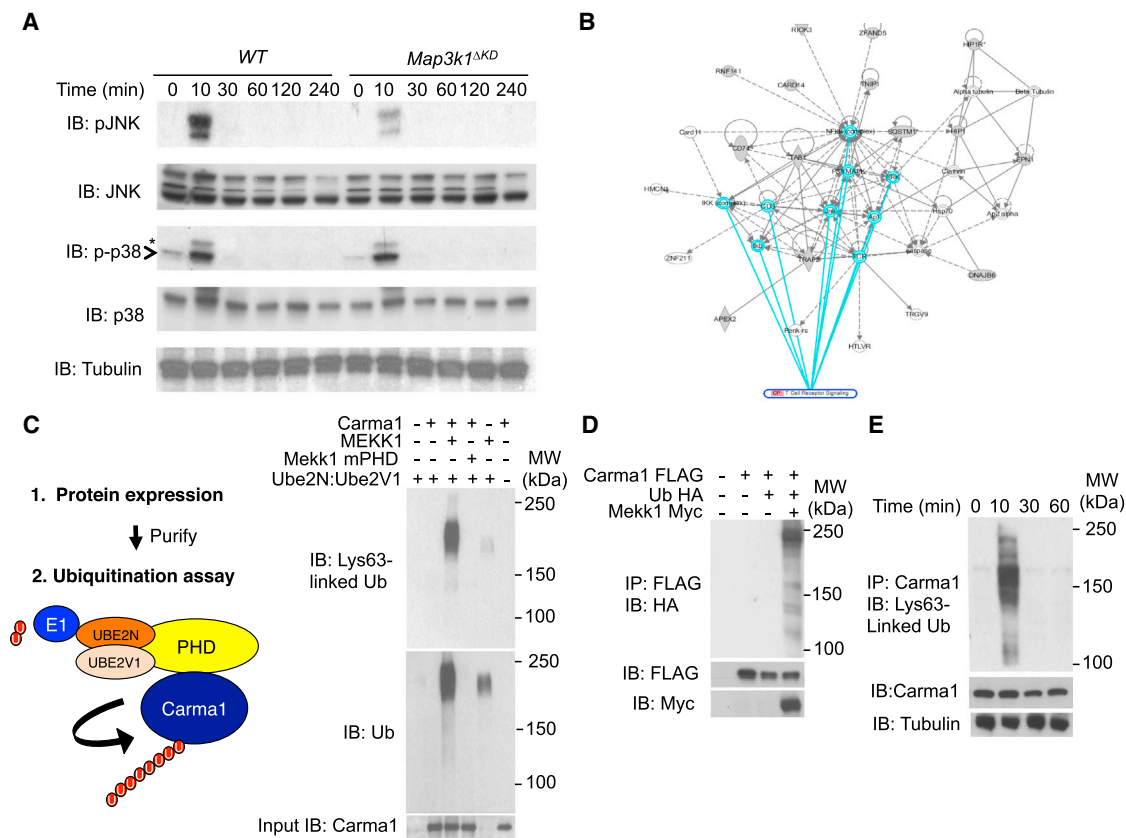
(C) Statistical analysis of iNKT populations (CD1d tetramer<sup>+</sup>TCRβ<sup>+</sup>) within the spleen, thymus, bone marrow, and liver from WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice. The average percentage ( $\pm$ SEM) of CD1d-tetramer and TCRβ-positive cells from five independent experiments is shown (black circle, WT; green square, *Map3k1<sup>ΔKD</sup>*; green triangle, *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice). Statistical differences were analyzed by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

same Itch activation-dependent Th2 phenotype observed in *Map3k1<sup>ΔKD</sup>* CD4<sup>+</sup> T cells (Gao et al., 2004; Venuprasad et al., 2006). By contrast,  $\gamma\delta$  T cells isolated from *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* or WT mice are not significantly different (Figure S1D; Maki et al., 1996). *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice display significantly more CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the spleen and liver and a significantly larger CD8<sup>+</sup> T cell population within the bone marrow (Figures 1B and 1C; Chang et al., 2011; Charlaftis et al., 2014). No significant difference was detected in T cells isolated from the thymus, spleen, liver, or bone marrow between WT and *Lck<sup>Cre/+</sup>* mice (Figure S1E; data not shown).

### Map3k1 Regulates iNKT Cells

*Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice have significantly higher numbers of iNKT cells (CD1d tetramer<sup>+</sup>CD3<sup>+</sup>, CD1d tetramer<sup>+</sup>TCRβ<sup>+</sup>, or CD1d tetramer<sup>+</sup>NK1.1<sup>+</sup>) within the liver, spleen, and bone marrow relative to WT or *Lck<sup>Cre/+</sup>* mice (Figures 2A–2C, S2A, and S2B; data not shown; Ansari et al., 2010). *Map3k1<sup>ΔKD</sup>* mice, which display a germline deletion of the *Map3k1* exons encoding the Mekk1 kinase domain (Gao et al., 2004), similarly displayed significantly higher numbers of iNKT cells (CD1d tetramer<sup>+</sup>CD3<sup>+</sup>, CD1d tetramer<sup>+</sup>TCRβ<sup>+</sup>, or CD1d tetramer<sup>+</sup>NK1.1<sup>+</sup>) in the liver (Figures 2B and 2C; data not shown). However,





**Figure 3. *Map3k1* Regulates Jnk Activation in iNKT Cells**

(A) iNKT cells were isolated (four mice per experiment) and stimulated by TCR crosslinking with antibodies over a 240-min time course as indicated. Cell lysates were made and analyzed by IB with the indicated antibodies. Arrowhead indicates phospho-p38 and asterisk a non-specific band.  
 (B) IPA network diagram of TCR signaling to show the presence of the Meck1 PHD substrate Carma within this pathway.  
 (C) In vitro ubiquitination assays using Meck1 PHD, Meck1 mPHD, Ube2N:Ube2V1, E1, Ub, and Carma1. Reactions were performed as indicated and analyzed by IB as indicated. A fraction of the ubiquitination reactions was taken pre-incubation, boiled, analyzed by IB as shown, and indicated as input.  
 (D) HEK293 cells were transfected with the indicated constructs. To detect in vivo ubiquitination, lysates were made under denaturing conditions for IP (Gallagher et al., 2007) and IB performed as indicated. Lysates were also made under non-denaturing conditions as a loading control and IB performed with the indicated antibodies.  
 (E) iNKT cells were isolated (four mice per experiment) and stimulated by TCR crosslinking with antibodies over a 60-min time course. To detect in vivo ubiquitination, lysates were made under denaturing conditions for IP (Gallagher et al., 2007) and IB performed as indicated. Lysates were also made under non-denaturing conditions as a loading control and IB performed with the indicated antibodies.

iNKT cell development in the thymus is normal for both *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice (Figure S2C).

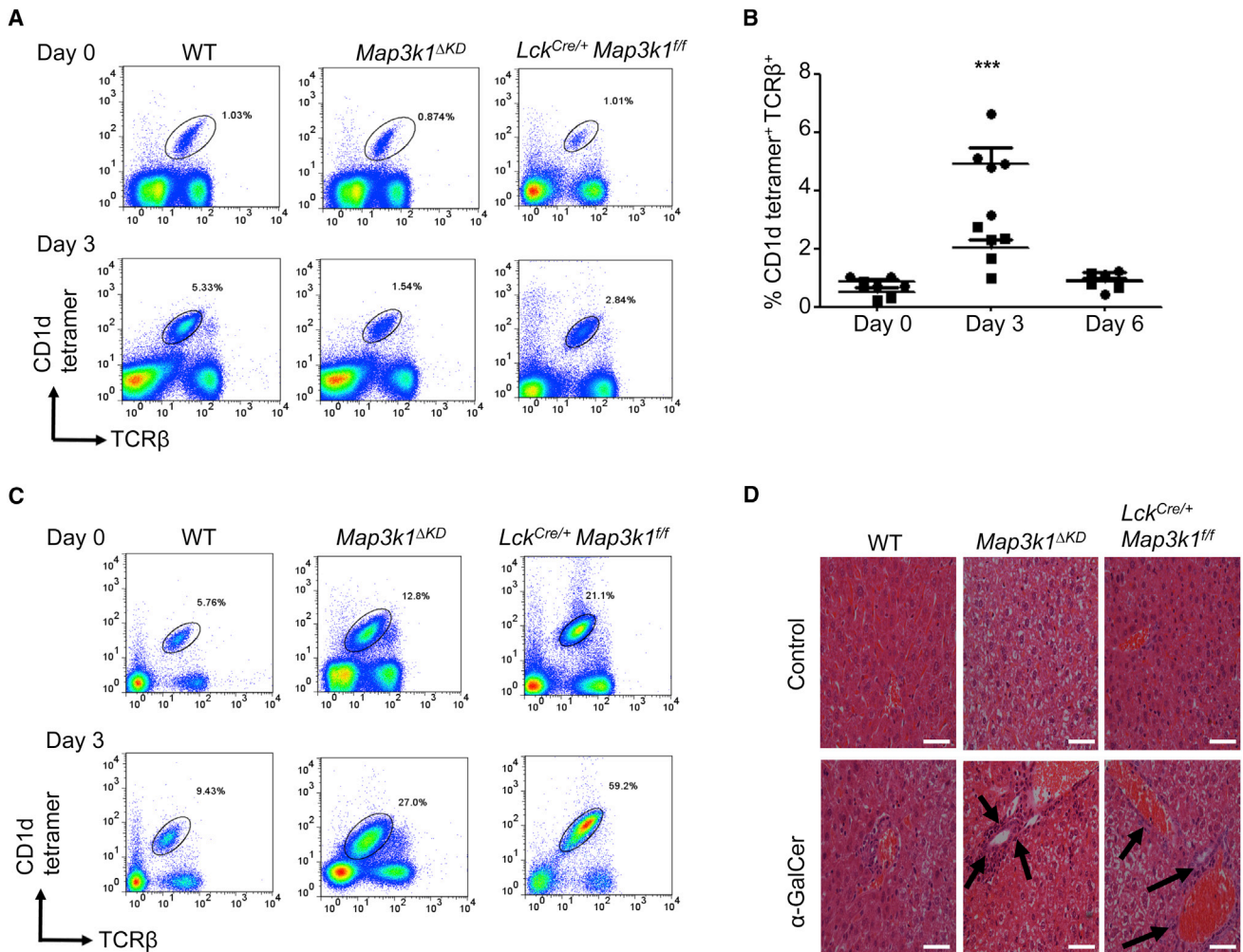
### Mekk1 Regulates TCR-Dependent Jnk Activation in iNKT Cells

*Map3k1<sup>ΔKD</sup>* iNKT cells were isolated and stimulated in vitro by TCR crosslinking with antibodies (Figure 3A; Gao et al., 2004; Nagaleekar et al., 2011). WT iNKT cells display a transient Jnk activation at 10 min that is significantly reduced in *Map3k1<sup>ΔKD</sup>* iNKT cells following TCR crosslinking with antibodies (Figures 3A and S3A). Phosphorylation of c-Jun is similarly reduced in *Map3k1<sup>ΔKD</sup>* iNKT and conventional cells following TCR crosslinking with antibodies (data not shown), but there is no significant defect in p38 activation (Figure S3B; Gao et al., 2004). Because Meck1 binds and ubiquitinates proteins by its PHD motif, we analyzed a comprehensive Meck1 PHD protein array

screen by ingenuity pathway analysis (IPA) bioinformatics to identify hits that are important for TCR signaling and identified Carma1 as a possible Meck1 PHD substrate (Figure 3B; Suddason and Gallagher, 2015; Charlaftis et al., 2014). The Meck1 PHD binds and, in association with Ub-conjugating enzyme E2N (Ube2N), transfers Lys63-linked Ub chains onto Carma1 (Figures 3C and 3D). Meck1 and Carma1 transiently co-purify from iNKT cells 10 min following TCR crosslinking (Figure S3C), and endogenous Carma1 is transiently ubiquitinated following TCR crosslinking with antibodies (Figures 3E and S3D).

### *Map3k1* Regulates Splenic and Liver iNKT Cell Expansion

To assess the role of Meck1 signaling in iNKT cell responses to antigen, WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice were immunized with the iNKT cell TCR agonist  $\alpha$ -GalCer (Figures 4



**Figure 4. iNKT Cell Expansion in *Map3k1*-Deficient Mice**

(A) WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice were i.p. injected with  $\alpha$ -GalCer for 3 days. Splenocytes were harvested at days 0 and 3, stained with anti-TCR $\beta$  antibody and CD1d tetramer, and analyzed by flow cytometry as indicated. Data are representative of three independent experiments. Numbers in the profiles indicate the percentages of the gated populations.

(B) Statistical analysis of  $\alpha$ -GalCer-dependent iNKT expansion at days 0, 3, and 6 in *Map3k1<sup>ΔKD</sup>* mice. The average percentage ( $\pm$ SEM) of PBS-57-loaded CD1d tetramer<sup>+</sup> TCR $\beta$ <sup>+</sup> cells from five independent experiments is shown (black circle, WT; black square, *Map3k1<sup>ΔKD</sup>* mice). Differences were analyzed by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

(C) Liver cells were harvested at days 0 and 3 from WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice following i.p. immunization with  $\alpha$ -GalCer. Liver cells were stained with CD1d tetramer and anti-TCR $\beta$  antibodies and analyzed by flow cytometry as indicated. Data are representative of three independent experiments. Numbers in the profiles indicate the percentages of the gated populations.

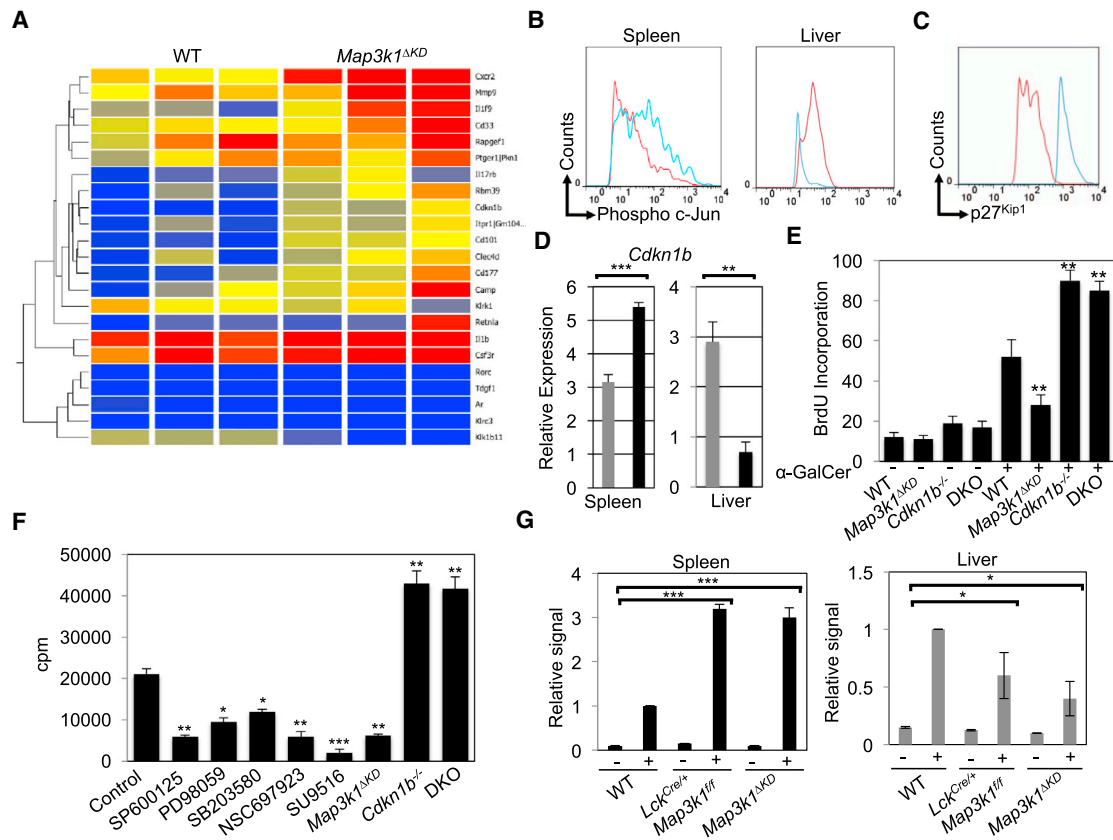
(D) Representative H&E-stained liver sections were prepared from unstimulated (upper panels) and 3-day  $\alpha$ -GalCer stimulated (lower panels) WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice (original magnification  $\times 40$ ; scale bar, 10  $\mu$ m). Arrows indicate lymphocyte infiltration. Data are representative of three independent experiments (two mice per experiment).

and S4). Short-term stimulation of *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice with  $\alpha$ -GalCer lead to normal iNKT activation and cytokine production (Figure S4A; data not shown). By contrast, splenic iNKT cells from *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice display significantly reduced long-term proliferative expansion following immunization with  $\alpha$ -GalCer (Figures 4A, 4B, and S4B; data not shown). Conversely, liver iNKT cells from *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice showed significantly enhanced long-term proliferative expansion following immunization with  $\alpha$ -GalCer (Figures 4C, S4B, and S4C). Analysis

of the livers from *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice revealed significantly enhanced lymphocyte infiltration and liver damage following long-term immunization with  $\alpha$ -GalCer relative to control mice (Figures 4D and S4D).

#### Mekk1 Controls iNKT Cell Proliferative Expansion by the Regulation of Jnk-Dependent p27<sup>Kip1</sup> Expression

To understand the molecular basis underpinning the aberrant proliferative expansion of both *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* and *Map3k1<sup>ΔKD</sup>* iNKT cells, we analyzed global gene expression



**Figure 5. Mekk1 Signaling Controls p27<sup>Kip1</sup> Expression to Regulate iNKT Cell Proliferation**

(A) WT and *Map3k1<sup>ΔKD</sup>* mice were i.p. injected with  $\alpha$ -GalCer for 3 days. RNA was isolated from WT and *Map3k1<sup>ΔKD</sup>* splenic iNKT cells, processed, and hybridized onto Affymetrix arrays. Bioinformatics analysis was performed, and a heatmap comparing gene hits between WT and *Map3k1<sup>ΔKD</sup>* iNKT cell microarray screens was constructed. The data are from three independent experiments (four mice per experiment).

(B) Splenic and liver iNKT cells were isolated from 3-day  $\alpha$ -GalCer-immunized *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>*, WT mice stained with anti-phospho c-Jun antibody, and flow cytometry performed as indicated (red line, WT; blue line, *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>*). Data were representative of three independent experiments. Histograms show the phospho c-Jun present in the gated iNKT cell population.

(C) Splenic iNKT cells from 3-day  $\alpha$ -GalCer-immunized *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* and WT mice were isolated and stained with anti-p27<sup>Kip1</sup> antibody and flow cytometry performed as indicated (red line, WT; blue line, *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>*). Data were representative of three independent experiments. Histogram shows the p27<sup>Kip1</sup> present in the gated iNKT cell population.

(D) iNKT cells from the spleen and liver of WT and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice were isolated 3 days post-i.p. injection with  $\alpha$ -GalCer and their RNA analyzed by real-time PCR as indicated (gray square, WT; black square, *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>*). The average relative expression ( $\pm$ SEM) of genes from three independent experiments was statistically analyzed, where appropriate, by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

(E) WT, *Map3k1<sup>ΔKD</sup>*, *Cdkn1b<sup>-/-</sup>*, or *Map3k1<sup>ΔKD</sup>/Cdkn1b<sup>-/-</sup>* (DKO) mice were treated with water containing BrdU and i.p. immunized with  $\alpha$ -GalCer (day 3) or left unstimulated (day 0). Splenocytes were extracted and analyzed as indicated. Representative results ( $\pm$ SEM) from three quantitated iNKT proliferation experiments were statistically analyzed, where appropriate, by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

(F) WT, *Cdkn1b<sup>-/-</sup>*, *Map3k1<sup>ΔKD</sup>*, or *Map3k1<sup>ΔKD</sup>/Cdkn1b<sup>-/-</sup>* (DKO) iNKT cells were isolated and incubated in [<sup>3</sup>H] thymidine containing media for 24 hr in the presence of DMSO (control), SP600125, PD98059, SB203580, NSC697923, or SU9516. The average cpm ( $\pm$ SEM) from three independent experiments was statistically analyzed, where appropriate, by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

(G) WT, *Map3k1<sup>ΔKD</sup>*, and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice were immunized with  $\alpha$ -GalCer for 3 days, splenic or liver iNKT cells isolated, and *Cdkn1b* ChIP performed with anti-phospho c-Jun antibody as indicated. The average relative signal ( $\pm$ SEM) from three independent experiments was statistically analyzed, where appropriate, by two-tailed Student's t test (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

patterns following the long-term immunization of *Map3k1<sup>ΔKD</sup>* mice with  $\alpha$ -GalCer (Figure 5A; Table S2). Bioinformatics analysis of the screened hits identified *Cdkn1b* (encoding p27<sup>Kip1</sup>) as a *Map3k1*-dependent cell-cycle regulator (Figure S5A). *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice have enhanced long-term phospho-c-Jun in splenic iNKT cells but reduced phospho-c-Jun in liver iNKT cells, relative to WT following immunization with  $\alpha$ -GalCer

(Figure 5B). *Cdkn1b* and p27<sup>Kip1</sup> expression is significantly enhanced in splenic *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* iNKT cells, but *Cdkn1b* expression is significantly reduced in liver *Map3k1<sup>ΔKD</sup>* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* iNKT cells following long-term stimulation by  $\alpha$ -GalCer (Figures 5C and 5D; data not shown). Similarly, other screened hits (including *Rorc*, *Il1 $\beta$* , *Il1f9*, and *Cxcr2*) identified by global gene expression analysis

were verified by real-time PCR in splenic (Figure S5B; data not shown) and liver tissues (Figure S5C; data not shown). Integrin gene expression (*Itgb7*, *Itgb21*, and *Itgb1*) is equivalent between WT and *Map3k1 $\Delta$ KD* iNKT cells (Figure S5D). Whereas splenic iNKT cells from *Map3k1 $\Delta$ KD* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice hypoproliferate, splenic *Cdkn1b<sup>-/-</sup>* iNKT cells hyperproliferate following long-term stimulation by  $\alpha$ -GalCer (Figure 5E). iNKT cell proliferation in response to TCR crosslinking with antibodies is significantly reduced by chemical inhibition of Jnk, Ube2N, or cyclin-dependent kinases (CDKs) (Figure 5F). Splenic, in contrast to liver, *Map3k1 $\Delta$ KD* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* iNKT cells display greater phosphorylation of c-Jun at the *Cdkn1b* promoter activator protein-1 (AP-1)-binding site following long-term stimulation by  $\alpha$ -GalCer (Figure 5G; Khattar and Kumar, 2010).

## DISCUSSION

We have shown, using *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice, that Mekk1 has important roles in T cells. Thymic development is moderately skewed in *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice, with reduced numbers of CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes and enhanced numbers of CD4<sup>+</sup> single-positive thymocytes. Our findings differ from *Lck<sup>Cre/+</sup> Map3k7<sup>fl/fl</sup>* mice that display reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> single-positive thymocytes (Wan et al., 2006) and *Lck<sup>Cre/+</sup> Map3k2<sup>-/-</sup> Map3k3<sup>fl/fl</sup>* mice that display normal thymic development (Chang et al., 2011). The CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations are also larger within the spleen, bone marrow, and liver tissues of *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice. *Map3k1 $\Delta$ KD* mice have elevated numbers of T cells in the liver, and analysis of the iNKT cell population revealed that these are significantly expanded in the liver of both *Map3k1 $\Delta$ KD* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice. Two factors complicating the analysis of T cells using *Map3k1 $\Delta$ KD* mice have been the germline kinase domain mutation impacting B cells and partial embryonic lethality (Gao et al., 2004; Gallagher et al., 2007; Bonnesen et al., 2005). Bone marrow chimeras and in vitro assays using cells from *Map3k1 $\Delta$ KD* mice have previously demonstrated that B and T cell Mekk1 signaling defects are intrinsic (Gao et al., 2004; Venuprasad et al., 2006; Labuda et al., 2006; Gallagher et al., 2007). As such, conditional deletion of *Map3k1* in T cells using *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice represents a significant refinement of the analysis of *Map3k1* in T cells.

TCR signaling leads to the rapid activation of MAPK and the phosphorylation of its downstream targets (e.g., c-Jun), and these can then initiate T cell effector responses (Su et al., 1994; Dong et al., 2002). More recently, E3 Ub ligase Itch was identified as a downstream Mekk1-signaling target that is phosphorylated by Jnk1 to induce a conformational change within the protein leading to Itch activation and canonical ubiquitination of Jun transcription factors (Gallagher et al., 2006; Gao et al., 2004; Fang et al., 2002; Venuprasad et al., 2006). As with conventional CD4<sup>+</sup> T cells, TCR signal transduction rapidly activates Jnk in iNKT cells and this is significantly reduced in *Map3k1*-deficient iNKT cells (Gao et al., 2004). Mekk1 transiently binds and ubiquitinates Carma1, a scaffold known to regulate Jnk activation, following TCR engagement, and this provides a mechanism of recruitment for Mekk1 to the TCR that differs from TNFRs or

Tgf- $\beta$  receptors (Suddason and Gallagher, 2015; Charlaftis et al., 2014; Gaide et al., 2002).

Our work identifies a role for *Map3k1* in the regulation of the iNKT cell proliferative expansion in response to glycolipid antigen. In order to identify the mechanism underpinning *Map3k1*-dependent iNKT cell expansion, we analyzed their global gene expression profile to identify *Cdkn1b* as a regulated target. The modulation of p27<sup>Kip1</sup> expression in T cells by Mekk1 signaling represents a molecular mechanism that regulates T cell proliferation. We have identified increased iNKT cell infiltration into the liver and a higher degree of liver damage in *Map3k1 $\Delta$ KD* and *Lck<sup>Cre/+</sup> Map3k1<sup>fl/fl</sup>* mice. The aberrant iNKT cell expansion within the spleen and liver of *Map3k1*-deficient mice can be explained by altered c-Jun-dependent *Cdkn1b* expression. Our results reinforce the importance of Mekk1 signaling in T cells.

## EXPERIMENTAL PROCEDURES

### Gene Targeting of *Map3k1*

*Map3k1* was targeted by insertion of a *FRT* site followed by a *LacZ* sequence and a *loxP* site into chromosome 13 upstream of the exons of the *Map3k1* gene (to generate the *Map3k1<sup>f</sup>* allele; Skarnes et al., 2011). The first *loxP* site was followed by *neo* under the control of the human *B-actin* promoter, SV40 poly-A, a second *FRT* site, and a second *loxP* site. A third *loxP* site was inserted downstream of the *Map3k1* exons. *Map3k1<sup>+/f</sup>* ESCs (C57BL/6) were generated by standard procedures (Gossler et al., 1986) and genotyped by Southern blotting or genomic PCR (Ledermann, 2000; Gao et al., 2004; Charlaftis et al., 2014). Four independently generated *Map3k1<sup>+/f</sup>* ESC clones were injected into blastocysts, and the resulting transgenics were genotyped by PCR (Gao et al., 2004; Charlaftis et al., 2014). Genomic PCR was carried out on mice biopsies using primers to detect the *Map3k1*-specific WT allele, the shorter mutant allele, and the recombinant allele (5' to 3' primers: TCGTGGTATCGTTATGCGCC; AATAGGCCACACGTTGACTGG; and CAACCCACGAAAGGAGGTTTC; Charlaftis et al., 2014). *Map3k1<sup>+/f</sup>* mice were crossed with *ACTFLPe* mice (Jackson Laboratory) to initiate recombination at the *FRT* sites, deleting the *lacZ* and *neo* and resulting in offspring that contain a *Map3k1<sup>fl/fl</sup>* allele.

### Cell Line and Cell Culture Conditions

HEK293 cells were maintained in DMEM (22320; Invitrogen) supplemented with 10% FBS (SH3007003; Thermo Scientific) and antibiotics in a humidified atmosphere at 37°C. Cells were passaged every 2 or 3 days when approaching full confluence (Charlaftis et al., 2014).

### Transfection

HEK293 cells were plated in 6-well plates at a density of  $1 \times 10^6$  cells per well. The following day, cells were transfected with Lipofectamine 2000 (11668-019; Invitrogen) or Jet Prime (114-07; Polyplus) transfection reagents according to the manufacturer's instructions. Cells were collected and lysed 48 hr later.

### Tissue Preparation

Spleen and perfused liver tissues were mashed through a 70- $\mu$ m and a 100- $\mu$ m strainer, respectively, and resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (ThermoScientific). Following low-speed centrifugation, the splenic pellet was treated with red blood cell (RBC) lysis buffer (Sigma), washed, and resuspended in medium. The liver cell pellet was resuspended in 38% Percoll (GE Healthcare) and then centrifuged at 500 g for 20 min at room temperature. Cell pellets were treated with RBC lysis buffer, washed, and resuspended in medium.

### Isolation of iNKT Cells

Mouse iNKT cells isolation from spleen or liver tissues was performed using PE-conjugated and  $\alpha$ -GalCer-loaded CD1d tetramer (Nagaleekar et al., 2011). Tetrameric CD1d: $\alpha$ -GalCer cell complexes were purified using anti-PE



MicroBeads (Miltenyi Biotec). Residual B cells were depleted prior to iNKT cell enrichment using a CD45R (B220) MicroBead kit (Miltenyi Biotec), and the final iNKT cell purity obtained was greater than 95%.

### Flow Cytometry

Cell staining with CD1d tetramer was followed by intracellular staining performed using a Fix/Perm kit (BD PharMingen). For intracellular staining, the cells were incubated with 50  $\mu\text{g}/\text{ml}$  PMA (Sigma-Aldrich), 1  $\mu\text{M}$  ionomycin (Sigma-Aldrich), and 10  $\mu\text{g}/\text{ml}$  brefeldin A (Sigma-Aldrich) for 2 hr before processing. For 5-bromo-2-deoxyuridine (BrdU) labeling, mice were fed with BrdU (0.8 mg/ml) in drinking water supplemented with 5% (weight/volume) glucose 1 day prior to  $\alpha$ -GalCer (2  $\mu\text{g}$ ) i.p. injection. Mice were treated with BrdU in drinking water for 3 days to study proliferation. Cells were surface stained, and BrdU staining was performed according to the manufacturer protocol (BD PharMingen BrdU Flow Kit). Cells were analyzed on a Cyan ADP (DakoCytomation) flow cytometer and further analyzed on a workstation using FlowJo software (TreeStar).

### Immunoblotting, Immunoprecipitation, Real-Time PCR, and Chromatin Immunoprecipitation

iNKT cells were isolated by magnetic selection using MACS LS columns according to the manufacturers protocols (Miltenyi Biotec). Immunoblotting (IB) and immunoprecipitation (IP) were carried out as previously described (Gao et al., 2004). iNKT cell RNA was prepared with the RNeasy kit (QIAGEN), and total RNA (500  $\mu\text{g}$ ) was converted to cDNA using the High Capacity cDNA RT-Kit (Applied Biosystems). Real-time PCR was performed in triplicate with the appropriate gene primers (Invitrogen; Table S3) using SYBR Green (Applied Biosystems) and an ABI Prism 7700 Sequence Detector (Applied Biosystems).  $\beta$ -actin was used for normalization of results. Chromatin IP (ChIP) was performed using an EpiTect ChIP qPCR primer assay for mouse *Cdkn1b* kit (QIAGEN) according to the manufacturer instructions.

### Ubiquitination Assays

Carma1 cDNA was overexpressed in HEK293 cells and IP performed with anti-FLAG antibody, washed extensively, and protein eluted. Subsequently, Carma1 was incubated for 1 hr at 37°C with the ubiquitination assay enzymes E1 (100 nM), Ube2N:Ub-conjugating enzyme E2 variant 1 (Ube2V1; 0.36  $\mu\text{M}$ ), Ub, and ATP, with or without WT Mekk1 PHD (100 ng) or Mekk1 mutant PHD (mPHD; 100 ng; Charlaftis et al., 2014). All ubiquitination assay reagents were from Boston Biochem.

### Microarray and Bioinformatics Analysis

Total RNA from iNKT cells was reverse transcribed into biotinylated cRNA with an RNA amplification kit according to the manufacturer's instructions (Ambion). RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies). Samples were hybridized to Mouse Gene 1.0 ST arrays (Affymetrix; Charlaftis et al., 2014). Partek software was used according to the vendor protocols for data analysis, quality control, and for creating gene lists and scatterplots. GeneSpringX software was used according to the vendor protocols to generate heatmaps. Probes with a fold change of less than two were discarded. Probes were quantile normalized among all microarray data. Gene lists were uploaded into the IPA program (Ingenuity Systems) to generate relevant signaling networks and gene wheels according to the vendor instructions.

### Liver Damage Assay

Mice were injected i.v. with 2  $\mu\text{g}$  KRN7000. After 3 days, the livers were harvested, fixed in 4% paraformaldehyde, processed, and paraffin embedded. H&E staining was carried out on liver sections (4  $\mu\text{m}$ ). Slides were analyzed using an Olympus light microscope, and pictures were taken using Image Pro-Software at 40 $\times$  magnification.

### Statistical Analysis

Data were expressed as SEM. Statistical significance was determined by two-tailed Student's *t* test. All analyses were performed using GraphPad Prism 5 software (GraphPad).

### ACCESSION NUMBERS

The accession number for the dataset reported in this paper is ArrayExpress: E-MTAB-1561.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.12.047>.

### AUTHOR CONTRIBUTIONS

T.S., S.A., N.C., and E.G. performed experiments, analyzed data, and wrote the manuscript.

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