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Basic

Research Article

A mutation within the SH2 domain of slp-76 regulates the tissue distribution and cytokine production of iNKT cells in mice

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TCR ligation is critical for the selection, activation, and integrin expression of T lymphocytes. Here, we explored the role of the TCR adaptor protein slp-76 on iNKT-cell biology. Compared to B6 controls, *slp-76$^{ace/ace}$* mice carrying a missense mutation (Thr428Ile) within the SH2-domain of slp-76 showed an increase in iNKT cells in the thymus and lymph nodes, but a decrease in iNKT cells in spleens and livers, along with reduced ADAP expression and cytokine response. A comparable reduction in iNKT cells was observed in the livers and spleens of ADAP-deficient mice. Like ADAP$^{-/-}$ iNKT cells, *slp-76$^{ace/ace}$* iNKT cells were characterized by enhanced CD11b expression, correlating with an impaired induction of the TCR immediate-early gene *Nur77* and a decreased adhesion to ICAM-1. Furthermore, CD11b-intrinsic effects inhibited cytokine release, concanavalin A-mediated inflammation, and iNKT-cell accumulation in the liver. Unlike B6 and ADAP$^{-/-}$ mice, the expression of the transcription factors Id3 and PLZF was reduced, whereas NP-1-expression was enhanced in *slp-76$^{ace/ace}$* mice. Blockade of NP-1 decreased the recovery of iNKT cells from peripheral lymph nodes, identifying NP-1 as an iNKT-cell-specific adhesion factor. Thus, slp-76 contributes to the regulation of the tissue distribution, PLZF, and cytokine expression of iNKT cells via ADAP-dependent and -independent mechanisms.

Keywords: ADAP · Cytokine · iNKT cell · Integrin · slp-76

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Introduction

iNKT cells express a panoply of NK-cell receptors [1] and a canonical TCR through which they recognize (glyco-)lipid antigens [2]. iNKT cells activate similar signaling cascades after TCR ligation like other T lymphocytes [3], but utilize unique transcription factors for their development such as the promyelocytic leukemia zinc finger (PLZF) [4–6].

According to two different developmental models PLZF characterizes distinct maturation stages and polarized subsets. The sequential lineage model suggests a gradual decrease of PLZF-expression following selection of iNKT cells which show a Th2-dominated cytokine profile during earlier and a Th1-dominated cytokine profile during later stages of intrathymic maturation [1, 7]. The second model describes lineage diversification and simultaneous differentiation into Th1-, Th2-, or Th17-polarized subsets that are defined by the level of PLZF-expression [8]. Although many iNKT cells release both Th1 and Th2 cytokines on a single cell level [9], the production of IL-17 and IFN-γ is mutually exclusive within $NK1.1^{-}$ cells [10–12]. Several transcription factors, such as Egr2, T-bet, ThPOK, Id2, Id3, and the Tec kinases Itk and Rlk have been implicated in the differentiation of iNKT cell subsets [6, 8, 13–17] which home to distinct tissues. Specifically, liver and spleen constitute the main source for the Th1-polarized sublineage which is $PLZF^{low}$. Th2- or Th-17-polarized iNKT cells maintain their PLZF-expression at high or intermediate levels, respectively, and are primarily recovered from the lungs and peripheral lymph nodes [8, 12, 15]. Both subsets frequently express IL-17RB [18] and NP-1 which characterizes iNKT cells that recently emigrated from the thymus [19]. However, the role of NP-1 in iNKT cell biology has remained undefined.

Although iNKT cells exhibit diverse functions, they still share molecules for tissue migration and adhesion. The chemokine receptor CXCR6 and the integrin LFA-1, for example, promote the accumulation of iNKT cells in the livers and lungs [20, 21]. While CXCR6 interacts with CXCL16, the heterodimeric integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) interact with ICAM-1 [22–24]. Although an immune-suppressive role of $CD11b^{+}$ iNKT cells has been reported [25], the regulation of CD11b-expression on iNKT cells is poorly understood. Furthermore, the mechanisms mediating the accumulation of iNKT cells at organ sites other than the liver and lung are unknown.

TCR-specific signals influence the phenotype of iNKT cells [26]. One of the pivotal molecules engaged in TCR signaling is the adaptor protein Src homology (SH)2-domain-containing leukocyte phosphoprotein of 76 kDa (slp-76) [27, 28]. Due to impaired signals from the pre-TCR, T-cell development is completely blocked in slp-$76^{-/-}$ mice [29–31].

The divergent functions of slp-76 are mediated by its distinct signaling domains. The N-terminal acidic domain contains three tyrosine phosphorylation sites [32] that are activated by ZAP-70 [33, 34] and subsequently bind to the guanine nucleotide exchange factor Vav [34–36], the adaptor protein Nck [37, 38], and the Tec-family kinase Itk [39, 40]. The central proline-rich domain of slp-76 interacts with PLCγ-1 [41] and the adaptor molecule GADS [42]. The C-terminal SH2 domain of slp-76 binds to the hematopoietic progenitor kinase HPK-1 [43] and to the adhesion and degranulation-promoting adaptor protein ADAP [44, 45]. While HPK-1 inhibits TCR signals [46], ADAP is required for thymocyte selection and TCR-mediated integrin-activation/-expression [47–50].

Using *slp-76*$^{ace/ace}$ mice that exhibit a threonine to isoleucine amino acid change at position 428 within the SH2 domain of slp-76 [51, 52], we characterize slp-76 as critical for the regulation of PLZF- and NP-1-expression, cytokine release and the tissue distribution of iNKT cells. While the accumulation of iNKT cells in the thymus and the peripheral lymph nodes is ADAP-independent, interactions of slp-76 with ADAP disrupt the distribution of iNKT cells to the spleens and livers. The inverse expression of LFA-1 and CD11b by *slp-76*$^{ace/ace}$ iNKT cells compared to B6 controls inhibits thereby the entry of iNKT cells into the livers of *slp-76*$^{ace/ace}$ mice. While the regulation of iNKT cell adhesion in the spleens remained unknown, we identify NP-1 as critical adhesion factor for iNKT cells within peripheral lymph nodes.

Results

iNKT cells from *slp-76*$^{ace/ace}$ mice express less slp-76- and ADAP-protein

Whereas the N-terminal region of slp-76 is critical for T-cell selection [31], the role of its C-terminal SH2 domain is less well understood. Therefore, we evaluated the phenotype of *slp-76*$^{ace/ace}$ mice carrying a threonine to isoleucine amino acid change at position 428 within the SH2-domain of slp-76 (Thr428Ile) (52, 53). In contrast to previously described mutations [33, 53, 54], *slp-76*$^{ace/ace}$ mice exhibited a reduced slp-76 protein expression in splenocyte lysates which was not rescued upon TCR-ligation (Fig. 1A). Similar to NK and $CD8^{+}$ T cells [52], purified iNKT and $CD4^{+}$ T cells from *slp-76*$^{ace/ace}$ mice expressed significantly less slp-76 protein than their B6 counterparts (Fig. 1B, C).

In order to define the molecular pathways affected by the slp-76 mutation, we evaluated the expression of signaling molecules known to interact with slp-76 [27] in lysates of iNKT cells or $CD4^{+}$ T cells purified from α-GalCer- or anti-CD3-expanded splenocyte cultures of *slp-76*$^{ace/ace}$ and B6 mice. While the slp-76 mutation neither affected ADAP- nor HPK-1-expression in $CD4^{+}$ T cells, strikingly less ADAP was recovered from *slp-76*$^{ace/ace}$ iNKT cells compared to B6 controls (Fig. 1D, E). Other slp-76-associated signaling molecules were not significantly altered (data not shown).

Slp-76$^{ace/ace}$ and $ADAP^{-/-}$ mice harbor lower numbers of iNKT cells in liver and spleen than B6 mice

Slp-$76^{-/-}$ mice and mouse strains with mutations in the N-terminal acidic domain lack mature T cells in the periphery [31],

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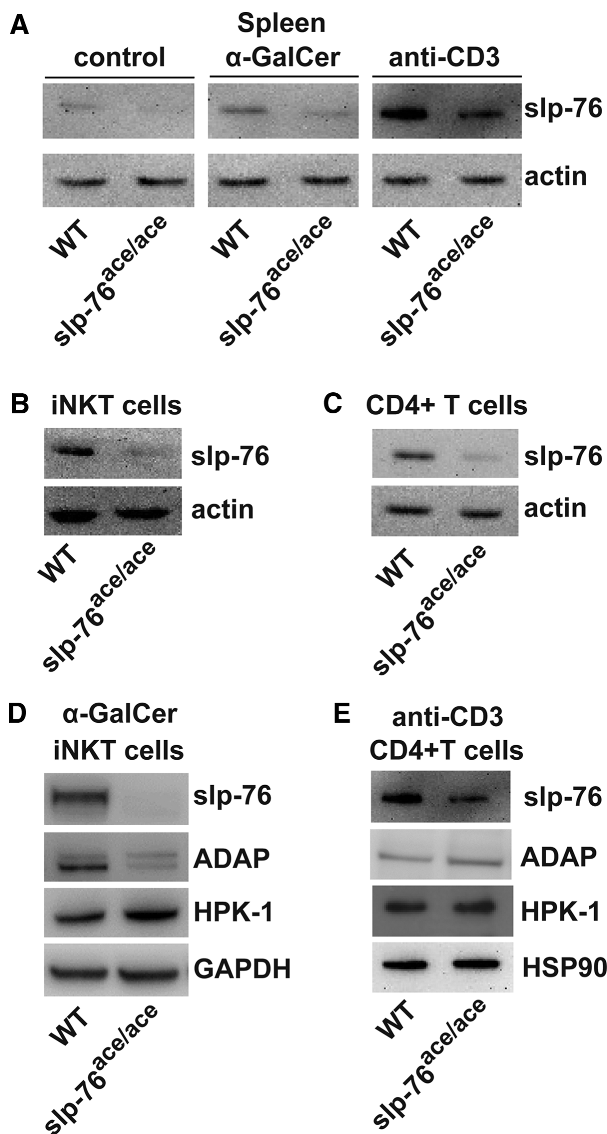

Figure 1. Reduced slp-76 and ADAP protein expression in *slp-76$^{ace/ace}$* **iNKT cells.** (A–C) Spleens were harvested from B6 and *slp-76$^{ace/ace}$* mice and the expression of slp-76 and actin in protein lysates of (A) splenocytes, (B) iNKT cells (α-GalCer-tet$^+$ TCRβ$^+$) and (C) CD4$^+$ TCRβ$^+$ T lymphocytes was analyzed by western blotting. (D, E) Splenocytes from B6 and *slp-76$^{ace/ace}$* mice were expanded for 3 days with (D) α-GalCer (E) or anti-CD3. iNKT cells (D) or CD4$^+$ TCRβ$^+$ T cells (E) were purified from these cultures by cell-sorting and the expression of slp-76, ADAP and HPK-1 was determined in protein lysates from these purified cell populations by western blot analysis. The expression of actin, GAPDH and HSP-90 was assessed as loading control. Representative western blot signals from three independent experiments and —two to three individual mice each are displayed.

but exhibit no numeric alterations of myeloid cells. While the distribution of TCRβ$^+$, γδTCR$^+$, and FoxP3$^+$ T cells was comparable between B6 and *slp-76*$^{ace/ace}$ mice (Supporting Information Fig. 1A–C), we observed an accumulation of iNKT cells in the thymi and peripheral lymph nodes and reduced iNKT cell numbers in the livers and spleens of *slp-76*$^{ace/ace}$ compared to B6 mice (Fig. 2A). This phenotype was *slp-76*$^{ace/ace}$-specific and associated with a reduced slp-76 protein expression (Supporting Information Fig. 1D) compared to slp-76$^{+/-}$ mice which exhibited a comparable iNKT cell distribution (Fig. 2B). In contrast to recent studies, which reported an expansion of NKT cells expressing a γ/δ TCR in mice with mutations within proximal TCR signaling molecules or a lack of the transcription factor Id3 [55–61], we did not observe an accumulation of this rare NKT cell subset in *slp-76*$^{ace/ace}$ mice (data not shown). Thus, besides NK cells [53] the slp-76-mutation specifically affects α/β-TCR$^+$ iNKT cells.

As the phenotype in *slp-76$^{ace/ace}$* mice might be mediated via hampered interactions with ADAP (Fig. 1D) [47–50], we analyzed ADAP$^{-/-}$ and littermate control mice. Similar to *slp-76$^{ace/ace}$* mice, iNKT cells in the spleens and livers of ADAP$^{-/-}$ mice were reduced (Fig. 2C). In contrast to *slp-76$^{ace/ace}$* mice, there was no accumulation of iNKT cells in the thymi and peripheral lymph nodes of ADAP$^{-/-}$ mice (Fig. 2C). Thus, while being dispensable for the iNKT cell phenotype in the thymi and lymph nodes of *slp-76$^{ace/ace}$* mice, slp-76-ADAP interactions control the population of the spleens and livers with iNKT cells.

PLZF^{low} and Id3^{low} iNKT cells accumulate in the thymi of *slp-76$^{ace/ace}$* mice

iNKT cells can be divided into Th1-, Th2-, and Th17-polarized subsets [62]. To evaluate whether the slp-76 mutation affects the polarization of iNKT cells and thus leads to their striking tissue redistribution, we analyzed the iNKT cell subsets at different organ sites from B6 and *slp-76$^{ace/ace}$* mice using PLZF and RORγt as distinctive transcription factors [8]. We observed a significantly reduced expression of PLZF by iNKT cells in the thymus, but not the periphery of *slp-76$^{ace/ace}$* as compared to B6 mice (Fig. 3A). Furthermore, the expression level of the transcription factor Id3 [63], which was reported to regulate PLZF-expression [17], was reduced in thymic *slp-76$^{ace/ace}$* iNKT cells (Fig. 3B). In contrast to *slp-76$^{ace/ace}$* iNKT cells, ADAP$^{-/-}$ iNKT cells revealed no differences in PLZF- or Id3-expression compared to control iNKT cells (Fig. 3C, D). Thus, slp-76 does not require ADAP for the expression of PLZF and Id3 by iNKT cells in the thymus. Furthermore, altered polarization mechanisms do not underlie the striking tissue redistribution of iNKT cells in the periphery of *slp-76$^{ace/ace}$* mice.

Slp-76$^{ace/ace}$ mice exhibit defects in the cytokine production by iNKT cells

To elucidate the mechanisms underlying their redistribution in *slp-76$^{ace/ace}$* mice, we further analyzed the iNKT cell phenotype. Similar to NK cells [52], *slp-76$^{ace/ace}$* iNKT cells more frequently expressed NK-cell receptors (especially NK1.1, NKG2D, and Ly49G2) and CD122 than their B6 counterparts (Supporting Information Table 1). In contrast, IL-17RB, which is predominantly expressed on NKT2 and NKT17 cells [18], was selectively reduced (Supporting Information Table 1). iNKT cells from

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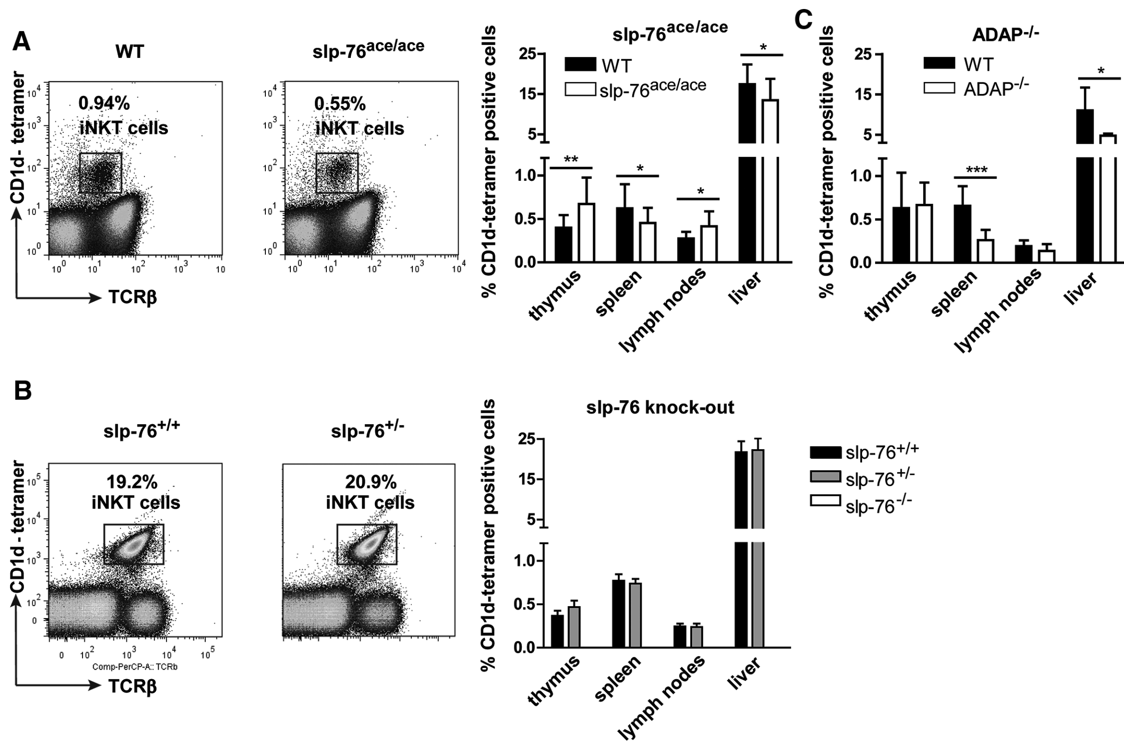

Figure 2. Slp-76 and ADAP control the tissue distribution of iNKT cells. (A-C) The percentages of iNKT cells were compared between the organs of (A) B6 and *slp-76*$^{ace/ace}$, (B) slp-76$^{+/+}$, slp-76$^{+/-}$ and slp-76$^{-/-}$ as well as (C) ADAP$^{-/-}$ and littermate control mice by flow cytometry. Representative FACS dot plots for of the spleens of B6 and *slp-76*$^{ace/ace}$ mice (A, left) and of the livers of slp-76$^{+/+}$ and slp-76$^{+/-}$ littermates (C, left) as well as the summaries for eight individual B6 and *slp-76*$^{ace/ace}$ mice (A, right), five individual slp-76$^{+/+}$, slp-76$^{+/-}$ and slp-76$^{-/-}$ littermates (B), or seven individual ADAP$^{-/-}$ and littermate control mice (C) are displayed as mean ±SD and are representative of four independent experiments. Statistical significance of differences was determined using Student's t-test. A *p*-value <0.5 (*), <0.01 (**) or <0.001 (***) was considered significant.

ADAP$^{-/-}$ mice exhibited no significant differences in the expression of any of these markers compared to control mice (data not shown). Although based on these observations the *slp-76*$^{ace/ace}$ mutation was expected to favor a Th1-polarization, iNKT cells from *slp-76*$^{ace/ace}$ mice released less IL-4, and IFN-γ upon stimulation with α-GalCer compared to WT cells, i.e. did not show a cytokine shift (Fig. 4A, B). A similar reduction of IL-4- and IFN-γ-responses was observed in ADAP$^{-/-}$ mice when compared to littermate controls (Fig. 4C, D). However, this was likely due to the reduced iNKT cell numbers in *slp-76*$^{ace/ace}$ and ADAP$^{-/-}$ mice rather than to a cell-intrinsic defect in *slp-76*$^{ace/ace}$ and ADAP$^{-/-}$ iNKT cells, as no significant differences in the number of IFN-γ^{+}, IL-4^{+}, or IL-17^{+} iNKT cells were observed between *slp-76*$^{ace/ace}$, ADAP$^{-/-}$ and control mice (Fig. 4E–H; data not shown). Thus, the slp-76 mutation restricts rather than polarizes cytokine responses of iNKT cells. This restriction is more likely to reflect the reduced iNKT cell numbers in *slp-76*$^{ace/ace}$ mice than cell-intrinsic defects.

Slp-76 requires ADAP for the regulation of LFA-1- and CD11b-, but not NP-1- expression

Although individual iNKT cell subsets exhibit diverse functions, they still share molecules for tissue migration and adhesion [20, 21, 64]. Thus, the iNKT cell phenotype in *slp-76*$^{ace/ace}$ mice might result from an altered trafficking and/or homing rather than a developmental selection and/or polarization process. The distribution and level of CXCR6-, CXCR3-, CCL21-, and CCR7-expression were similar for B6 and *slp-76*$^{ace/ace}$ iNKT cells (data not shown). In contrast, there was an organ-specific reduction of LFA-1-expression on *slp-76*$^{ace/ace}$ iNKT cells (Fig. 5A), particularly in the peripheral lymph nodes, but not in the liver, where LFA-1 had been implicated in the recruitment of iNKT cells [21, 64]. Thus, additional molecules might be involved in the organ distribution of iNKT cells. As both LFA-1 and CD11b bind to ICAM-1 [22, 24] and CD11b/ICAM-1 interactions promote the tissue adhesion of different cell populations [65–68], we also analyzed the expression of CD11b on iNKT cells. In contrast to conventional T cells [69], peripheral iNKT cells were CD11b^{+} already under steady-state conditions. CD11b-expression was significantly increased on iNKT cells from *slp-76*$^{ace/ace}$ mice with the majority of CD11b^{+} iNKT cells accumulating in the peripheral lymph nodes (Fig. 5B). As ADAP interferes with different adhesion molecules [48] and as the expression of ADAP was reduced in *slp-76*$^{ace/ace}$ iNKT cells (Fig. 1D), we evaluated the distribution of integrins on iNKT cells from ADAP$^{-/-}$ mice. Similar to *slp-76*$^{ace/ace}$ mice, ADAP$^{-/-}$ iNKT cells expressed less LFA-1 (Fig. 5C) and more CD11b (Fig. 5D) compared to littermate controls. As the expression of ICAM-1 and CD1d was comparable on antigen-presenting cells in B6, *slp-76*$^{ace/ace}$ and ADAP$^{-/-}$ mice (Supporting Information Fig. 2), these

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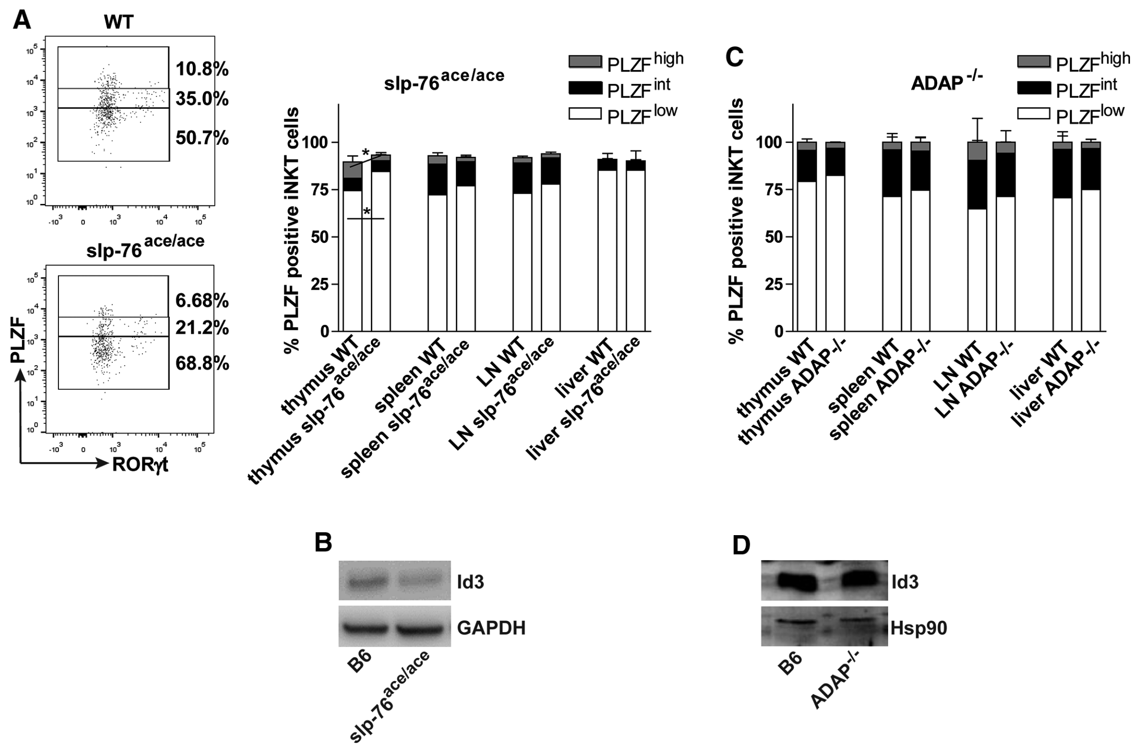

Figure 3. Reduced expression of PLZF and Id3 in thymic iNKT cells of *slp-76$^{ace/ace}$* mice. (A, C) iNKT cells from spleens were identified as TCRβ^{+} αGalCer-tetramer^{+} cells (see Fig. 2A) and divided into PLZF^{high}-, PLZF^{int} - and PLZF^{low}-expressing subtypes (A, left). The distribution of the respective iNKT-cell subsets at the indicated organ sites was evaluated in (A) 6 individual B6 and *slp-76$^{ace/ace}$* mice (A, right) or (C) seven individual ADAP$^{-/-}$ and littermate control mice. Data are shown as mean ±SD and are representative of three independent experiments. Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*) was considered significant. (B, D) The expression of Id3 was determined by western blot analysis in protein lysates of iNKT cells purified from the thymi of (B)9 B6 and *slp-76$^{ace/ace}$* mice as well as (D) ADAP$^{-/-}$ mice and littermate controls by cell sorting. The expression of GAPDH or Hsp90 was assessed as loading control (representative western blots from four individually analyzed mice out of two independent experiments are displayed).

data suggest that slp-76 requires ADAP for the regulation of LFA-1- und CD11b-expression on iNKT cells.

CD11b was most frequently expressed in the peripheral lymph nodes (Fig. 5B), suggesting that iNKT cells with limited access to spleens and livers might be primarily redirected to this tissue site. Therefore, we assessed the distribution of additional markers on CD11b^{+} iNKT cells. While there was no difference in the expression of CXCR6, CXCR3, CCL21, LFA-1, and CCR7, significantly more iNKT cells were NP-1^{+} in the peripheral lymph nodes of *slp-76$^{ace/ace}$* compared to B6 mice (Fig. 5E, F) suggesting an accumulation of recent iNKT cell thymic emigrants at this organ site [19]. As no differences in the expression of NP-1 were observed between ADAP$^{-/-}$ and control mice (data not shown), these data suggested that slp-76 is sufficient for the regulation of NP-1 on iNKT cells.

The reduced TCR signal strength is associated with an enhanced CD11b-expression

The *slp-76$^{ace/ace}$* mutation promotes the accumulation of PLZF^{low} iNKT cells in the thymus (Fig. 3A) and of CD11b^{+} iNKT cells in the periphery (Fig. 5B). As altered TCR signals [31, 70–72] might contribute to this phenotype, we explored the regulation of the TCR-specific immediate early gene *Nr4a1/Nur77* [31, 73], which has been used as readout for TCR signaling strength [74]. To this end, we crossed *slp-76$^{ace/ace}$* with *Nur77^{GFP}* mice, in which GFP is expressed in T cells after TCR-specific engagement [74]. In accordance with both developmental models (see introduction), the brightest expression of *Nur77* was reported in PLZF^{high} iNKT cells in the thymus, whereas GFP-expression was lost upon thymic emigration so that iNKT cells remained Nur77-GFP^{-} in the periphery [8, 74]. Compared to their B6 counterparts iNKT cells from the thymi of *slp76$^{ace/ace}$* mice exhibited an overall reduced GFP-expression (data not shown). This difference was most prominent within the PLZF^{high} and PLZF^{int} populations (Supporting Information Fig. 3A). Accordingly, the GFP signal in splenic iNKT cells on the *Nur77^{GFP}* x *slp-76$^{ace/ace}$* background was attenuated upon stimulation with α-GalCer (Fig. 5G). Furthermore, the expression of PD-1 that transduces inhibitory signals [75] and correlates with the strength of TCR signaling [74], was significantly reduced on iNKT cells within all tissue sites of *slp-76$^{ace/ace}$* mice (Supporting Information Fig. 3B).

To test, whether the TCR signal strength affects the expression of integrins, we compared the distribution of CD11b, LFA-1, and NP-1 on iNKT cells with respect to their Nur77-expres-sion. TCR ligation enhanced the expression of CD11b, LFA-1, and NP-1

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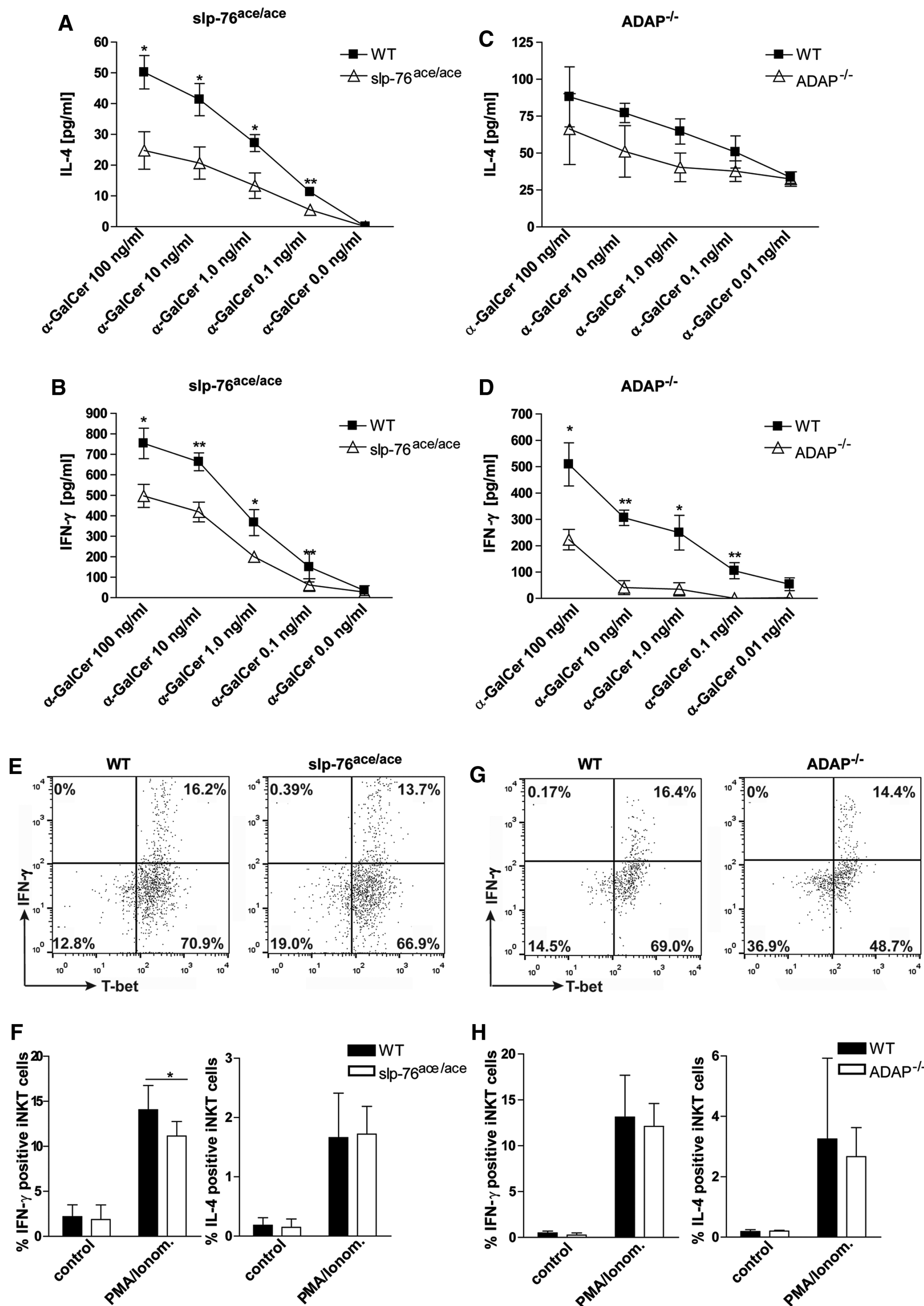

Figure 4. The slp-76-mutation affects the release of cytokines by iNKT cells. (A–D) Splenocytes of six individual (A, B) B6 and *slp-76$^{ace/ace}$* or (C, D) ADAP$^{-/-}$ and control mice each were stimulated with increasing concentrations of α-GalCer and the amounts of IFN-γ and IL-4 released into the cell culture supernatants were determined 72 h later by ELISA. Data are shown as mean ±SD and are representative of 3 independent experiments. (E–H) Accordingly, splenocytes of (E, F) six individual B6 and *slp-76$^{ace/ace}$*- or (G, H) ADAP$^{-/-}$ and control mice each were stimulated with a cell stimulation cocktail (eBioscience) according to the manufacturer's instructions and the numbers () of (E-H) IFN-γ^{+} and (F, H) IL-4^{+} iNKT cells were determined 4 h later by intracellular staining. Data are shown as mean ±SD and are representative of three experiments performed. iNKT cells were identified thereby as TCRβ^{+} αGalCer-tetramer^{+} cells (see Fig. 2A) in the spleens. Representative FACS plots for the release of IFN-γ by NKT cells are displayed (E, G). Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*) or <0.01 (**) was considered significant.

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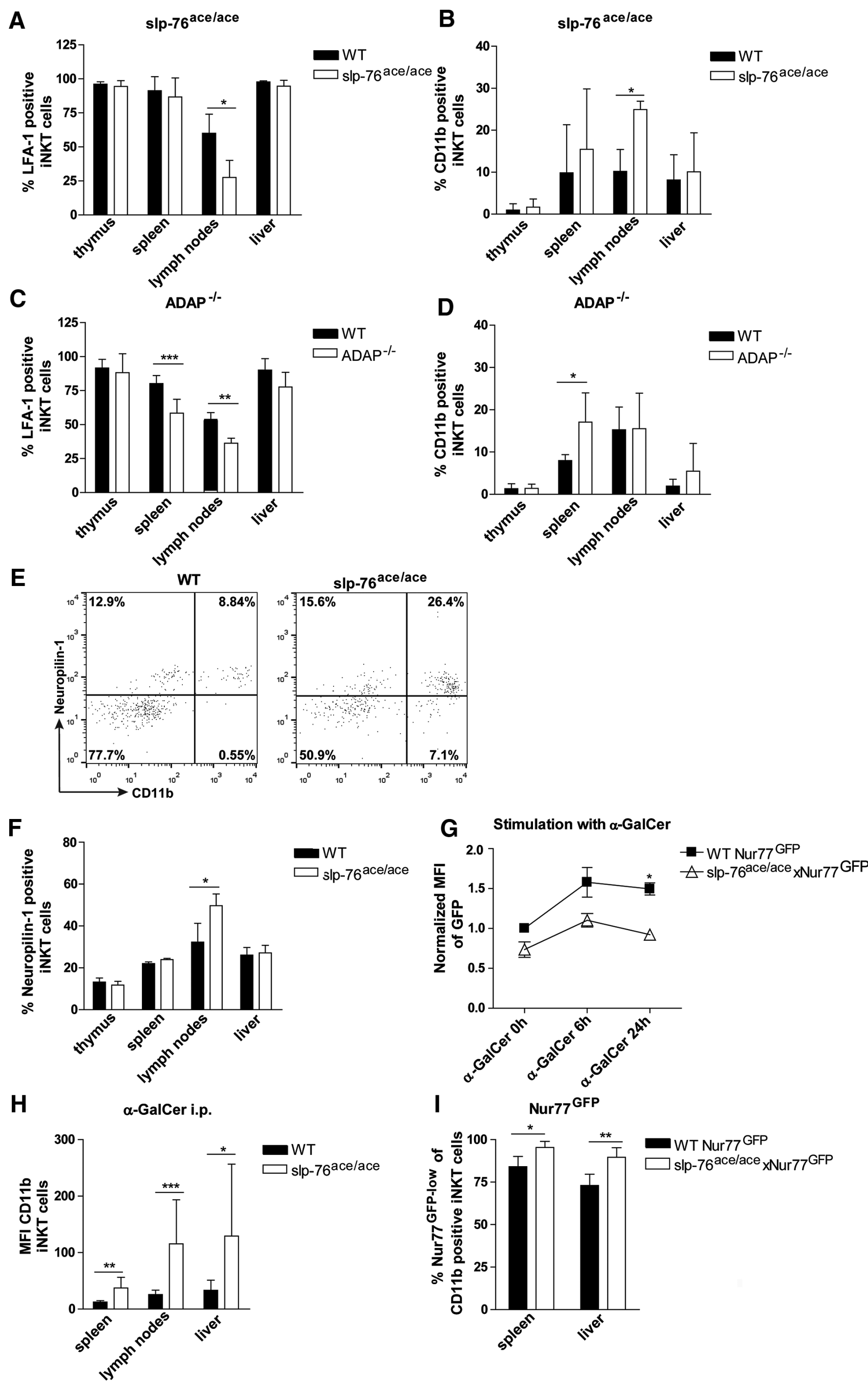

Figure 5. Slp-76-ADAP interactions and the TCR signal strength regulate the expression of CD11b. (A–F) iNKT cells were isolated from the indicated organs of (A, B, F) —three to six individual B6 and *slp-76*$^{ace/ace}$ mice and (C, D) —four to six individual ADAP$^{-/-}$ and control mice. The expression of (A, C) LFA-1, (B, D) CD11b, and (F) NP-1 was measured by flow cytometry and shown as mean ±SD and are representative of three independent experiments. (E) The co-expression of CD11b and NP-1 on iNKT cells in the lymph nodes from B6 and *slp-76*$^{ace/ace}$ mice is displayed in representative FACS dot plots. (G) Splenocyte suspensions were stimulated for 3 and 6 h with 100 ng/mL α-GalCer. The mean fluorescence intensity (MFI) of Nur77^{GFP} within iNKT cells of six individual B6 *Nur77*^{GFP} and *slp-76*$^{ace/ace}$x*Nur77*^{GFP} mice was normalized to the average MFI of unstimulated B6 Nur77^{GFP} iNKT cells. Data shown as mean ±SD and are representative of three independent experiments performed. (H) CD11b-expression on iNKT cells within the indicated organs of eight individual B6 Nur77^{GFP} and *slp-76*$^{ace/ace}$xNur77^{GFP}was measured by flow cytometry 4 days after the intraperitoneal application of 1 μg α-GalCer. The average mean fluorescence intensity (MFI) of CD11b on CD11b^{+} iNKT cells at the indicated organ sites is displayed as mean ±SD and data are representative of four independent experiments. (I) CD11b expression of on Nur77-GFP^{low} iNKT cells was analyzed in the indicated organs of five individual B6xNur77^{GFP} and *slp-76*$^{ace/ace}$xNur77^{GFP} mice injected 6 h before with 1 μg α-GalCer. Data are shown as mean ±SD and are representative of two independent experiments. Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*), <0.01 (**) or <0.001 (***) was considered significant.

(Fig. 5H; data not shown). While NP-1 and LFA-1 were comparably distributed on Nur77-GFP^{low} and -GFP^{high} cells in B6 and *slp-76*$^{ace/ace}$ mice (data not shown), CD11b was mainly detected on Nur77- GFP^{low} iNKT cells on the *slp-76*$^{ace/ace}$ background (Fig. 5I; Supporting Information Fig. 3C). Thus, the attenuated TCR signal contributes to an enhanced expression of CD11b on *slp-76*$^{ace/ace}$ iNKT cells.

CD11b inhibits the accumulation of iNKT cells in the liver and the release of cytokines

To evaluate the function of CD11b on iNKT cells, we analyzed CD11b$^{-/-}$ and control mice. We recovered two times more iNKT cells from the livers of CD11b$^{-/-}$ compared to B6 mice (Fig. 6A). The expression of LFA-1 and of NP-1 was comparable on B6 and

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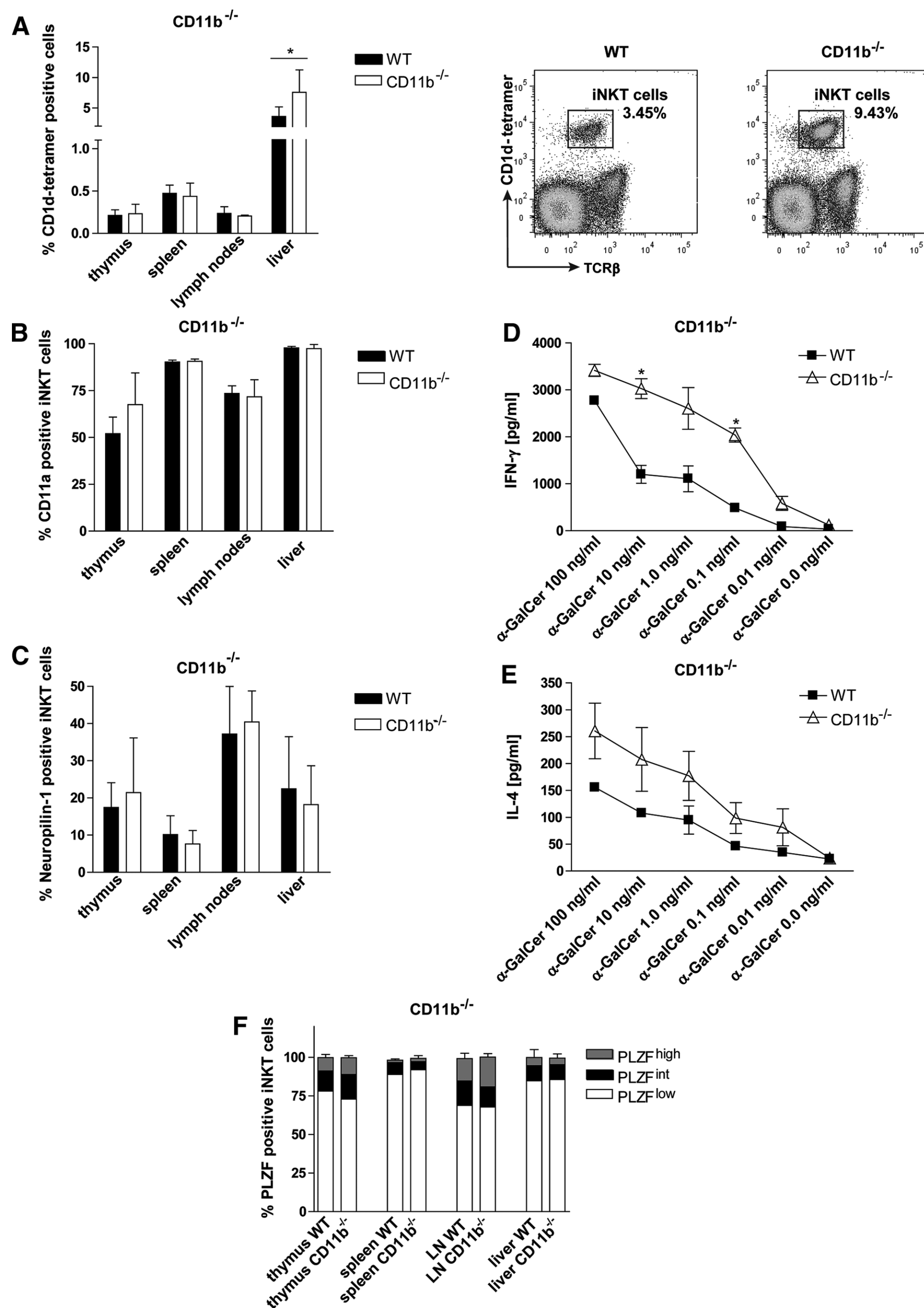

Figure 6. CD11b inhibits iNKT-cell functions. (A-C) iNKT cells isolated from the indicated organ sites of six individual B6 WT and CD11b-deficient (CD11b$^{-/-}$) mice and (A) the relative distribution of iNKT cells as well as expression of (B) LFA-1 and (C) NP-1-expression was measured by flow cytometry. Data are shown as mean ±SD and are representative of three independent experiments. A representative FACS dot plot for the distribution of iNKT cells in the livers of B6 and CD11b$^{-/-}$ mice is displayed (A, middle and right). (D, E) Splenocytes from four individual B6 and CD11b$^{-/-}$ mice were stimulated with increasing concentrations of α-GalCer and the amounts of (D) IFN-γ and (E) IL-4 released into the cell culture supernatants were determined 72 h later by ELISA. Data are shown as mean ±SD and are representative of three independent experiments. (F) iNKT cells in the thymi, spleens, lymph nodes (LN) and livers of six individual B6 and CD11b$^{-/-}$ mice were divided into PLZF^{high}-, PLZF^{int}-, and PLZF^{low}-expressing subsets and the relative distribution of the three subsets was summarized for the indicated organs. Data are shown as mean ±SD and are representative of three independent experiments. Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*) was considered significant.

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CD11b$^{-/-}$ iNKT cells (Fig. 6B, C), indicating that CD11b does not directly interfere with the expression of the other two integrins regulated by the slp-76 mutation.

As the reduced induction of the TCR immediate early gene *Nur77* and the decreased cytokine production of iNKT cells from *slp-76$^{ace/ace}$* mice upon TCR ligation was paralleled by an enhanced CD11b-expression, we explored the release of cytokines in splenocyte cultures from CD11b$^{-/-}$ and B6 mice, in which comparable numbers of iNKT cells were detected (Fig. 6A). The application of α-GalCer induced a significantly enhanced cytokine release in CD11b$^{-/-}$ mice (Fig. 6D, E) without affecting the polarization of iNKT cells or their PLZF-expression (Fig. 6F). In addition, significantly more IFN-γ^{+} iNKT cells were detected in CD11b$^{-/-}$ compared to B6 mice (13.43% ± 3.71 versus 9.91% ± 2.38; $p<0.05$; data are mean ±SD of two experiments with a total of 6 mice upon PMA/ionomycin stimulation of splenocyte cultures).

CD11b-deficiency exacerbates hepatic inflammation

iNKT cells play a pivotal role in the induction of liver damage [2]. Thus, we evaluated the impact of CD11b on ConA-induced hepatitis. CD11b$^{-/-}$ mice exhibited a significantly enhanced liver damage compared to littermate controls (Fig. 7A, B), similar as observed in a poly IC-induced liver damage model [76]. To elucidate the role of CD11b on iNKT cells, we reconstituted irradiated Ly5.1 mice with a 1:4 mixture of CD11b$^{-/-}$ and Jα18$^{-/-}$ bone marrow. Another set of irradiated Ly5.1 mice received a 1:4 mixture of B6and Jα18$^{-/-}$ bone marrow as control. Similar to CD11b$^{-/-}$ mice, CD11b$^{-/-}$/Jα18$^{-/-}$ bone chimeras developed a more severe hepatitis than B6/Jα18$^{-/-}$ control chimeras (Fig. 7C) suggesting an intrinsic, inhibitory effect of CD11b on iNKT cells. In accordance with the enhanced CD11b-expression on *slp-76$^{ace/ace}$* compared to B6 iNKT cells (Fig. 5B), ConA-induced hepatitis was ameliorated in *slp-76$^{ace/ace}$* mice compared to B6 controls (Fig. 7D).

Thus, the loss of function mutation within slp-76 is associated with an enhanced expression of CD11b on iNKT cells, which inhibits the induction of immediate early genes, the release of cytokines, iNKT cell accumulation and liver inflammation.

ICAM-1 binds preferably to LFA-1^{+} iNKT cells

LFA-1 binds better to ICAM-1 than to CD11b on neutrophils [77]. To evaluate whether similar mechanisms apply for iNKT cells, we exposed liver MNCs enriched for iNKT cells from B6 and *slp-76$^{ace/ace}$* mice to plate bound ICAM-1. Significantly more B6 than *slp-76$^{ace/ace}$* iNKT cells adhered to ICAM-1 (Fig. 8A). To evaluate the contribution of LFA-1 and CD11b on the adhesion capacity of iNKT cells, we blocked both integrins by antibodies. Similar to neutrophils, only the blockade of LFA-1 significantly reduced the number of iNKT cells bound to ICAM-1 (Fig. 8B). Furthermore, in line with previous results [21] the number of liver iNKT cells in B6 WT mice dropped up to three-fold after the combined application of anti-LFA-1 and anti-ICAM-1 antibodies. In *slp-76$^{ace/ace}$* mice, however, the respective reduction of iNKT cells was not higher than 2-fold (data not shown). The injection of anti-CD11b antibodies slightly increased the number of iNKT cells in the liver, particularly in *slp-76$^{ace/ace}$* mice (26.7% ± 7.36 versus 33.1% ± 9.19 for naïve versus anti-CD11b treated; $p<0.05$). Thus, due to the insufficient capacity of CD11b^{+} iNKT cells to bind ICAM-1, these data suggest that the shift towards an enhanced CD11b- and decreased LFA-1-expression inhibits the accumulation of *slp-76$^{ace/ace}$* iNKT cells to the liver.

NP-1 promotes the accumulation of iNKT cells in peripheral lymph nodes

As the greater pool of iNKT cells in the peripheral lymph nodes of *slp-76$^{ace/ace}$* mice correlated with an enhanced NP-1-expression compared to B6 controls, we characterized the role of NP-1 in the biology of iNKT cells. Thus, we injected blocking anti-NP-1 and anti-VEGF antibodies into B6 and *slp-76$^{ace/ace}$* mice every other day for 8 days and compared the tissue distribution and cytokine production of iNKT cells to untreated control mice. Whereas the application of anti-NP-1 antibodies inhibited the accumulation of iNKT cells in the peripheral lymph nodes of *slp-76$^{ace/ace}$* mice (Fig. 8C and D), the blockade of VEGF had no effect (data not shown). Furthermore, none of the two antibodies affected the production of cytokines upon stimulation with α-GalCer in either mouse strain (Fig. 8E and F; data not shown). Thus, these data identify NP-1 as critical molecule for the accumulation of iNKT cells within peripheral lymph nodes.

Discussion

In this study we report a role of slp-76 for the regulation of PLZF-expression and the tissue distribution of iNKT cells. Despite the accumulation of PLZF^{low} iNKT cells in the thymi of *slp-76*$^{ace/ace}$ mice and an enhanced expression of different NK-cell receptors, iNKT cells were not Th1-polarized and even strikingly reduced in spleens and livers, organ sites in which PLZF^{low}, Th1-polarized [8] iNKT cells are usually abundantly detected [15]. Instead, iNKT cells accumulated in the peripheral lymph nodes and the thymi of *slp-76*$^{ace/ace}$ mice and were hyporeactive following TCR ligation, resulting in an overall reduced cytokine release and inability to induce tissue inflammation.

Slp-76$^{ace/ace}$ iNKT cells showed a significantly decreased expression of ADAP, the primary interaction partner for the SH2 domain of slp-76 [78]. The strikingly reduced accumulation of iNKT cells in livers and spleens was paralleled by an extenuated LFA-1- and enhanced CD11b-expression in both ADAP$^{-/-}$ and *slp-76$^{ace/ace}$* mice. These findings support the concept that ADAP is pivotal for the transmission of slp-76-mediated signals. As iNKT cells with a *slp-76$^{ace/ace}$* mutation or a ADAP-deletion exhibited no differences in the expression of apoptotic or proliferation markers compared to control mice (data not shown) and as the expression of CD1d or ICAM-1 on myeloid cells was not affected in both mouse strains

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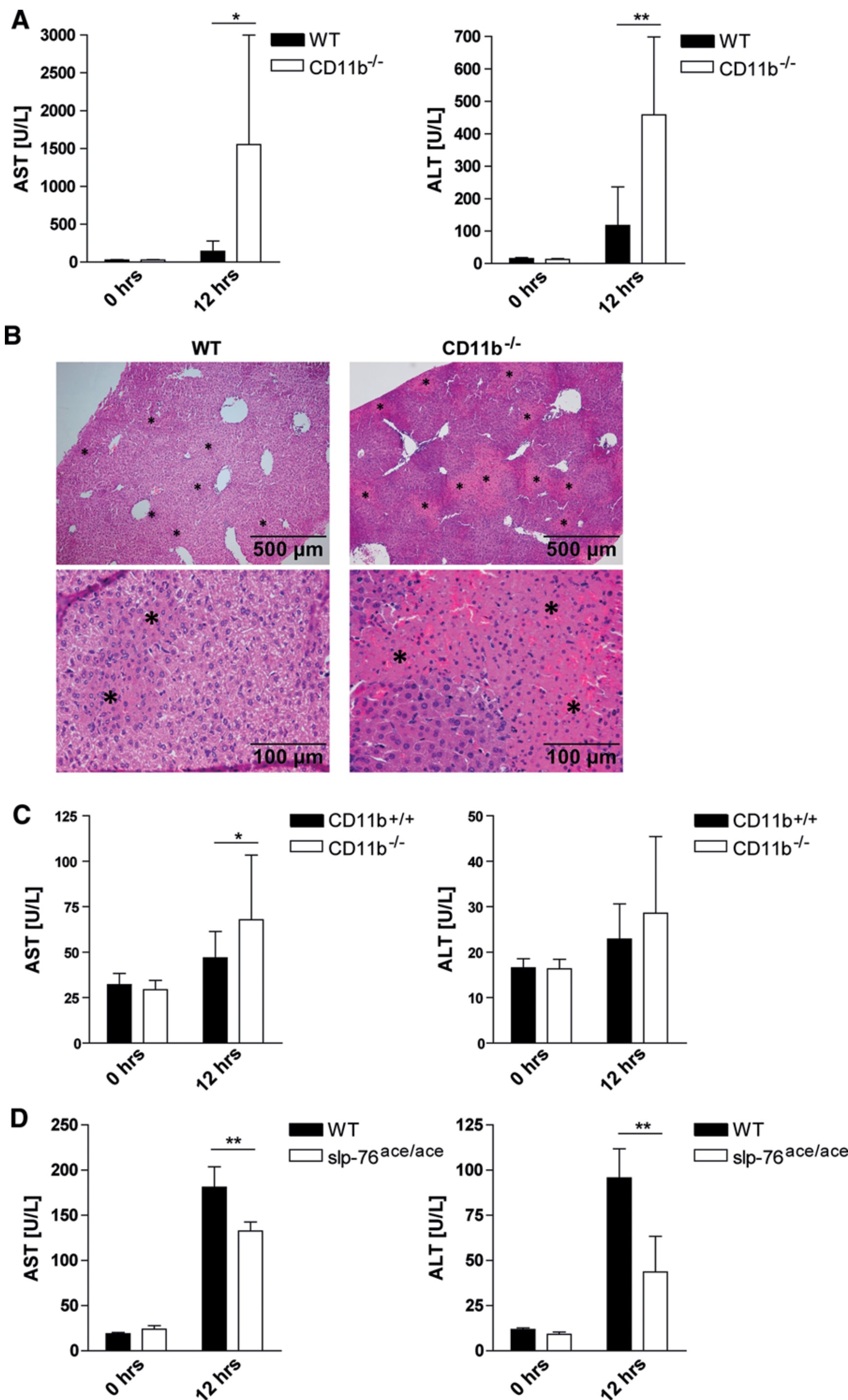

Figure 7. CD11b deficiency exacerbates hepatitis. (A–D) The following groups of mice were each injected intravenously with ConA: (A, B) 6 B6 or CD11b$^{-/-}$ mice, (C) 8 CD11b^{-}/Jα18^{-} or B6/Jα18^{-} bone marrow chimeras or (D) 5 B6 and *slp-76*$^{ace/ace}$ mice. 12 h later (A, C, D) sera and (B) liver tissue were evaluated for the accumulation of liver enzymes and hepatic inflammation and necrosis by biochemical and H&E analysis. (B) Necrotic regions within ConA injured livers are indicated by asterisks. Bar – 500 μm, 40× magnification (top) and – 100 μm, 200× magnification (bottom). Data are shown as mean ±SD and are representative of two independent experiments. Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*) or <0.01 (**) was considered significant.

either, we concluded that the inverse regulation of CD11b- with LFA-1-expression by slp-76-ADAP interactions inhibited the accumulation of iNKT cells in the liver due to an altered competition of CD11b with LFA-1 for their common ligand ICAM-1 [79]. Thus, iNKT cells, which failed to adhere to ICAM-1^{+} cells in the liver, frequently expressed CD11b and were redistributed to the peripheral lymph nodes due to an enhanced simultaneous NP-1-expression. Although slp-76-ADAP interactions controlled the accumulation of iNKT cells in the spleen, the participating chemokine(s) and/or adhesion factor(s) still need to be identified.

In contrast to CD11b and LFA-1, slp-76 ADAP-independently regulated the expression of NP-1, a transmembrane glycoprotein, which binds to VEGF receptors and exhibits various functions, many of them yet to be defined [80–83]. Here, we identified NP-1 as critical molecule for the accumulation of iNKT cells in peripheral lymph nodes. As only the application of anti-NP-1-antibodies,

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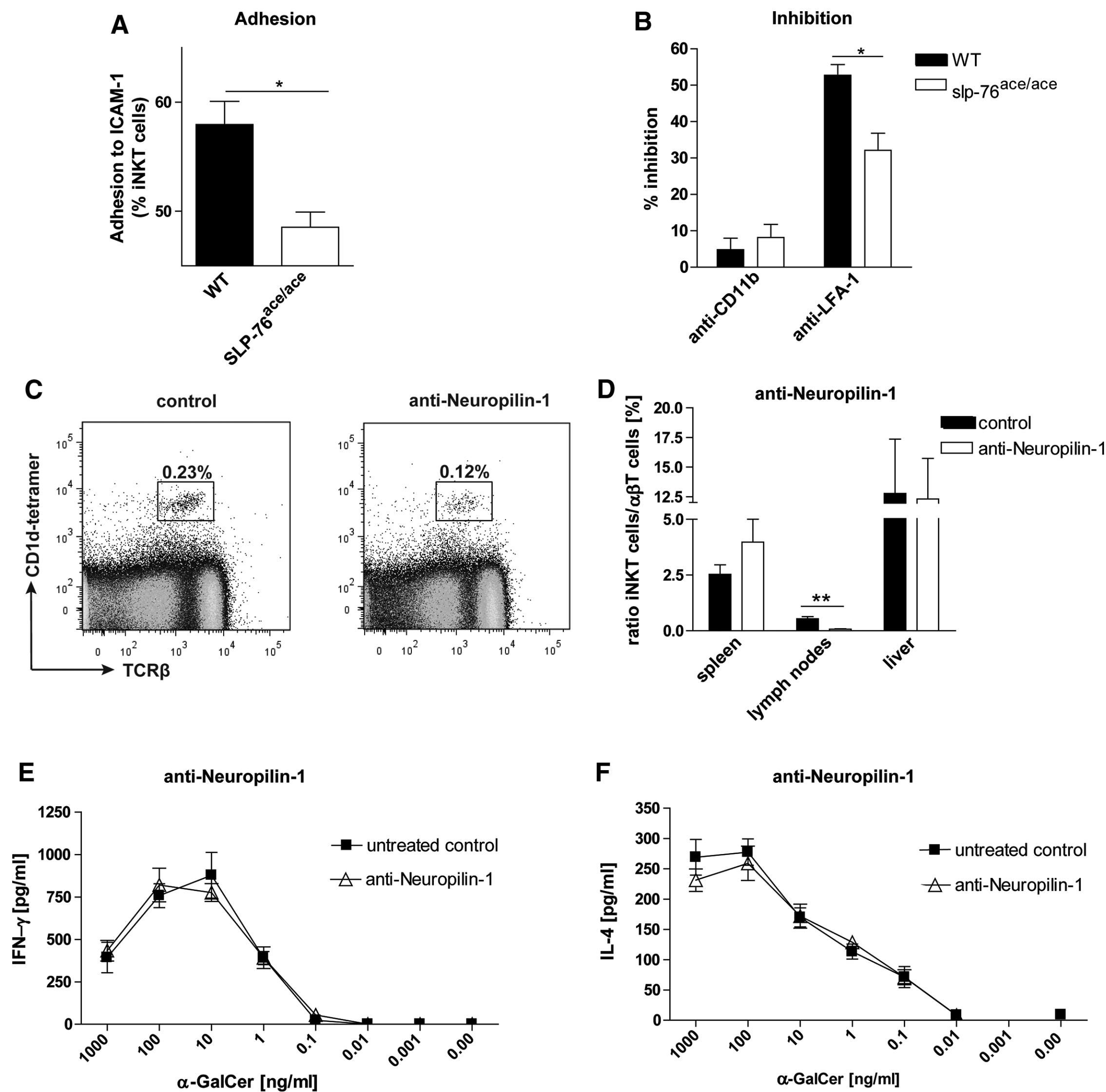

Figure 8. CD11b and NP-1 regulate the accumulation of iNKT cells. (A, B) 500,000 liver MNCs from five individual B6 and *slp-76^ace/ace^* mice enriched for iNKT cells were exposed to plate-bound ICAM-1 with or without anti-LFA-1 or (B) anti-CD11b blocking antibodies. The numbers of adherent and nonadherent iNKT cells were determined by flow cytometry. The mean percent adhesion (A) or the mean percent inhibition of adhesion by blocking antibodies adhesion relative to isotype-matched control antibodies (B) are shown as mean ±SD and are representative of two independent experiments. (C, D) The distribution of iNKT cells within the spleens, peripheral lymph nodes and livers of 4 individual *slp-76^ace/ace^* mice treated every other day with anti-NP-1 for 8 days was compared to four untreated control mice 24 h after the last injection of antibody. Representative FACS dot plots for peripheral lymph nodes (C) and the respective summary (D) are displayed as mean ±SD and are representative of two independent experiments. (E, F) Splenocytes from four individual *slp-76^ace/ace^* mice were stimulated in the presence or absence of blocking anti-NP-1-antibody with the indicated concentrations of α-GalCer. The amount of (E) IFN-γ and (F) IL-4 released into the cell culture supernatants was determined 72 h later by ELISA and shown as mean ±SD and are representative of four independent experiments. Statistical significant differences were determined using a Student's *t*-test. A *p*-value <0.5 (*), <0.01 (**) or <0.001 (***) was considered significant.

but not of antibodies against VEGF (data not shown), inhibited the accumulation of iNKT cells at this tissue site, homotypic interactions between NP-1^+ iNKT cells and/or other NP-1^+ cell populations are likely to be more critical for the retention of iNKT in peripheral lymph nodes than interactions of iNKT cells with VEGF. Intrathymic iNKT cell numbers as well as PLZF- and Id3-expression were comparable between ADAP$^{-/-}$ and control mice. As the expression of PLZF and of Id3 [63], however, was reduced in iNKT cells from *slp-76*$^{ace/ace}$ compared to B6 mice, our data suggest that slp-76 regulates Id3 and PLZF ADAP-independently. The reduced expression of Id3, which has been described as suppressor of iNKT cell expansion in the thymus [17], probably contributes to the thymic accumulation of iNKT cells in *slp-76*$^{ace/ace}$ mice. In contrast, the contribution of chemokines and/or integrins might be only subsidiary. Accordingly we did not detect any variations in the expression of CXCR3 (data not shown), which has been described as critical for the retention of iNKT cells in the thymus [84].

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Thus, the slp-76 mutation causes ADAP-dependent and ADAP-independent effects on iNKT cells in *slp-76*$^{ace/ace}$ mice. On the one hand, the decreased expression and/or conformational changes of slp-76 protein in *slp-76*$^{ace/ace}$ mice below the level of slp-76$^{+/-}$ mice might account for the ADAP-independent effects observed in *slp-76*$^{ace/ace}$ mice. On the other hand, the reduced association of ADAP with slp-76 protein in *slp-76*$^{ace/ace}$ mice accounts for the ADAP-dependent effects of the slp-76 mutation, but is not sufficient to resemble the phenotype of ADAP$^{-/-}$ mice.

In summary, we show that a loss of function mutation within the SH2 domain of slp-76 promotes the accumulation of PLZF^{low} iNKT cells in the thymus and a striking redistribution of iNKT cells from the livers and spleens to the peripheral lymph nodes. While ADAP was not required for the accumulation of iNKT cells in the thymus and the peripheral lymph nodes or the regulation of NP-1-, PLZF-, and Id3-expression, it was pivotal for the inverse expression of LFA-1 and CD11b by slp-76 and the inhibition of iNKT cell accumulation in the livers. Despite an enhanced expression of different NK cell receptors, suggesting an increased Th1-polarization according to published models of iNKT cell differentiation [1, 7, 8], we discovered a simultaneous reduction of both IL-4- and IFN-γ-expression by CD11b^{+} iNKT cells and an overall reduced TCR-reactivity in iNKT cells from mice carrying the *slp-76*$^{ace/ace}$-mutation. Thus, variations in the tissue distribution rather than the cytokine polarization need to be considered in patients with allelic mutations in TCR signaling molecules before pursuing vaccination strategies involving α-GalCer as an adjuvant.

Materials and methods

Mice

All mice were bred and kept at the University of Erlangen (Erlangen, Germany) and at Cincinnati Children´s Hospital (Cincinnati, OH, USA). *Slp-76*$^{ace/ace}$ mice with a threonine to isoleucine amino acid change at position 428 were recently described [51, 52]. *Nur77*^{GFP} mice were obtained from Dr. Kristin Hogquist (University of Minnesota, Minneapolis, MN, USA) [74] and crossed with *slp-76*$^{ace/ace}$ mice. ADAP$^{-/-}$ mice and littermate controls were obtained from Dr. Annegret Reinhold (Otto von Guericke University, Magdeburg, Germany) [49]. CD11b$^{-/-}$ and slp-76$^{-/-}$ mice were from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were raised in a specific pathogen-free environment and used at the age of 6–8 weeks. The animal studies were conducted according to the Institutional and Governmental Animal Care and Use Guidelines.

ConA hepatitis

ConA (Sigma) was dissolved in sterile PBS and injected intravenously in a final volume of 100 μL at a dose of 25 mg/kg. CD11b$^{-/-}$ and B6 mice were euthanized for the collection of blood and livers 12 h later. Biochemical as well as histopathological analyses from the plasma (ALT, AST) and tissues (H&E staining) were performed.

Blocking antibody experiments

Blocking antibodies against mouse CD11a (LFA-1; M17/4), CD54 (ICAM-1; YNI.7.4), CD11b (Mac-1; M1/70) and rat IgG2a Control (2A3) or NP-1 (761704) were obtained from BioXCell or R&D Systems. Two hundred micrograms isotype control, 100 μg anti-LFA-1, 200 μg anti-ICAM-1 or 200 μg anti-CD11b antibodies were injected intravenously 3 h before the harvest of the respective tissues. Anti-NP-1 was injected intraperitoneally every other day (10 mg/kg body weight) for 8 days.

Bone marrow chimeras

For the preparation of bone marrow radiation chimeras, a 1:4 mixture of 2 × 10^{6} CD11b$^{-/-}$ or B6 and 8 × 10^{6} Jα18$^{-/-}$ bone marrow cells was injected intravenously into 7–12 weeks-old Ly5.1 mice which had been irradiated twice within a 4-h interval (first 500 Rad, then 400 Rad; Gamma Cell 2000, Molsgaard Dental A/S, Copenhagen, Denmark) one day prior to the transfer. The reconstitution of mixed bone marrow chimeras was analyzed by flow cytometry 6–8 weeks after the bone marrow injection.

Flow cytometry

Single cell suspensions were prepared by gently passing the respective organs through 70 μm pore size cell strainers (Corning Incorporated, Corning, NY, USA). Liver homogenates were further purified by density gradient centrifugation using a Bicoll solution with a density of 1077 g/mL (Biochrom, Berlin, Germany). Single cell suspensions were stained with antibodies purchased from eBioscience (San Diego, CA, USA) or R&D Systems (Minneapolis, MN, USA): anti-mouse TCRβ-APCeFluor780, CD45.1-PE-Cyanine7, CD45.1-FITC, CD45.2-FITC, CD45.2-PerCP-Cyanine5.5, CD11b-PE-Cyanine7, CD11b-FITC, CD11b-PerCP-Cyanine5.5, CD11a-PerCP-eFluor710, RORγt-PerCP-eFluor710, PLZF-PE, IFN-γ-PE, T-bet-PerCP-Cyanine5.5, NK1.1-PE, CD122-PE, IL-17RB-APC, NKG2D-PE-Cyanine7, CD1d-PE, Ly49G2-FITC, Neuropilin-1(CD304)-FITC, gamma-delta TCR-PerCP-Cyanine5.5, PD-1(CD279)-PerCP-Cyanine5.5, CD54(ICAM-1)-PE, B220(CD45R)-PE-Cyanine7, FoxP3-PE and CD11c-APCeFluor780.

α-GalCer-loaded CD1d-tetramers were obtained from the NIH Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA). Fixation and permeabilization of cells for intracellular staining of PLZF, FoxP3, T-bet and IFN-γ were performed using the FoxP3 staining Kit from eBioscience (San Diego, CA, USA). Cells were measured on a FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (Ashland, OR, USA).

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Purification of iNKT cells

The single cell suspensions were pre-sorted for CD8- and CD19-negativity with MACS Microbead Technology (Miltenyi Biotec, Bergisch Gladbach, Germany), subsequently stained with anti-TCRβ mAb and CD1d tetramer and sorted on a FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA).

In vitro stimulation of iNKT cells

Spleen cell suspensions were incubated in round-bottom plates in RPMI1640 medium supplemented with glutamine, antibiotics, and 10% FCS (Sigma Aldrich, St. Louis, MO, USA) with 50 ng/mL plate-bound anti-CD3 antibody (eBioscience, San Diego, CA, USA), PMA/Ionomycin (Cell Stimulation Cocktail, eBioscience, San Diego, CA, USA), 0.1–100 ng/mL α-GalCer [85] or an equivalent amount of DMSO (Carl Roth, Karlsruhe, Germany). CD11b- and Nur77-GFP-expression on as well as IFN-γ-and T-bet-expression in iNKT cells were analyzed using FACS. Cytokines in the cell culture supernatants were measured using the respective DuoSet ELISAs from R&D Systems (Minneapolis, MN, USA) following the manufacturer's instructions.

Cell adhesion assay

The 96-well round bottom plates were pre-coated over night with 10 μg/mL ICAM-1 (Sino Biol. Inc., China) in a 10% BSA solution. 5×10^5 liver mononuclear cells (MNCs) enriched for iNKT cells by CD8 and CD19 depletion were added to the pre-coated wells. To some cultures blocking antibodies to CD11b (clone M1/70; eBioscience) and LFA-1 (clone M 17/4; eBioscience) were added at a concentration of 20 μg/mL. After incubation at 37°C in 5% CO_2 for 3 h, non-adherent cells were removed by washing three times with PBS. Adherent cells were scraped off and collected as well. Both, adherent and non-adherent cells were stained separately with anti-TCRβ mAb and CD1d tetramer. The percentages and total numbers of adherent and non-adherent iNKT cells were estimated by flow cytometry and compared to the original number of iNKT cells seeded in the respective chambers.

Western blotting

In vitro stimulated splenocytes, purified CD4-positive T cells or iNKT cells were lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) according to manufacturer's instructions. Cell lysates were subjected to SDS-PAGE and transferred to PVDF-membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked with 5% milk powder in TBS with 0.05% Tween (Sigma Aldrich, St. Louis, MO, USA) and subsequently incubated with primary and secondary antibodies. The primary antibodies against mouse slp-76, ADAP, HPK-1, GAPDH, Hsp90, phospho-PLCγ1, PLCγ1, phospho-PLCγ2, PLCγ2, phospho-ERK1/2, ERK1/2, Itk, phospho-Vav-1, Vav-1, Id2 and Id3, and the HRP-linked secondary antibody goat anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse actin was from Sigma Aldrich (St. Louis, MO, USA). Signals were developed with Immobilon Western, Chemiluminescent HRP substrate (Merck Millipore, Darmstadt, Germany) and the ChemiLux Imager (Intas, Göttingen, Germany).

Statistical analysis

Samples were analyzed for normal distribution by a Kolmogorov–Smirnov test. Statistical significance between groups was calculated using unpaired Student's *t*-test with GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). Data represent the mean and standard deviation. A *p*-value <0.05 was considered significant.

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Conflicts of interest: The authors declare no commercial or financial conflict of interest.

References

1 **Bendelac, A., Savage, P. B. and Teyton, L.**, The biology of NKT cells. *Annu. Rev. Immunol.* 2007. **25**: 297–336.

2 **Mattner, J.**, Natural killer T (NKT) cells in autoimmune hepatitis. *Curr. Opin. Immunol.* 2013. **25**: 697–703.

3 **Godfrey, D. I., Stankovic, S. and Baxter, A. G.**, Raising the NKT cell family. *Nat. Immunol.* 2010. **11**: 197–206.

4 **Griewank, K., Borowski, C., Rietdijk, S., Wang, N., Julien, A., Wei, D. G., Mamchak, A. A. et al.**, Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 2007. **27**: 751–762.

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5 **Savage, A. K., Constantinides, M. G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O. et al.**, The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 2008. **29**: 391–403.

6 **Seiler, M. P., Mathew, R., Liszewski, M. K., Spooner, C. J., Barr, K., Meng, F., Singh, H. et al.**, Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat. Immunol.* 2012. **13**: 264–271.

7 **Benlagha, K., Kyin, T., Beavis, A., Teyton, L. and Bendelac, A.**, A thymic precursor to the NK T cell lineage. *Science* 2002. **296**: 553–555.

8 **Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C. and Hogquist, K. A.**, Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat. Immunol.* 2013. **14**: 1146–1154.

9 **Matsuda, J. L., Gapin, L., Baron, J. L., Sidobre, S., Stetson, D. B., Mohrs, M., Locksley, R. M. et al.**, Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 2003. **100**: 8395–8400.

10 **Michel, M. L., Keller, A. C., Paget, C., Fujio, M., Trottein, F., Savage, P. B., Wong, C. H. et al.**, Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 2007. **204**: 995–1001.

11 **Coquet, J. M., Chakravarti, S., Kyparissoudis, K., McNab, F. W., Pitt, L. A., McKenzie, B. S., Berzins, S. P. et al.**, Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4- NK1.1- NKT cell population. *Proc. Natl. Acad. Sci. U. S. A.* 2008. **105**: 11287–11292.

12 **Doisne, J. M., Becourt, C., Amniai, L., Duarte, N., Le Luduec, J. B., Eberl, G. and Benlagha, K.**, Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J. Immunol.* 2009. **183**: 2142–2149.

13 **Enders, A., Stankovic, S., Teh, C., Uldrich, A. P., Yabas, M., Juelich, T., Altin, J. A. et al.**, ZBTB7B (Th-POK) regulates the development of IL-17-producing CD1d-restricted mouse NKT cells. *J. Immunol.* 2012. **189**: 5240–5249.

14 **Engel, I., Zhao, M., Kappes, D., Taniuchi, I. and Kronenberg, M.**, The transcription factor Th-POK negatively regulates Th17 differentiation in Valpha14i NKT cells. *Blood* 2012. **120**: 4524–4532.

15 **Constantinides, M. G. and Bendelac, A.**, Transcriptional regulation of the NKT cell lineage. *Curr. Opin. Immunol.* 2013. **25**: 161–167.

16 **Schaeffer, E. M., Yap, G. S., Lewis, C. M., Czar, M. J., McVicar, D. W., Cheever, A. W., Sher, A. et al.**, Mutation of Tec family kinases alters T helper cell differentiation. *Nat. Immunol.* 2001. **2**: 1183–1188.

17 **D'Cruz, L. M., Stradner, M. H., Yang, C. Y. and Goldrath, A. W.**, E and Id proteins influence invariant NKT cell sublineage differentiation and proliferation. *J. Immunol.* 2014. **192**: 2227–2236.

18 **Watarai, H., Sekine-Kondo, E., Shigeura, T., Motomura, Y., Yasuda, T., Satoh, R., Yoshida, H. et al.**, Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* 2012. **10**: e1001255.

19 **Milpied, P., Massot, B., Renand, A., Diem, S., Herbelin, A., Leite-de-Moraes, M., Rubio, M. T. et al.**, IL-17-producing invariant NKT cells in lymphoid organs are recent thymic emigrants identified by neuropilin-1 expression. *Blood* 2011. **118**: 2993–3002.

20 **Geissmann, F., Cameron, T. O., Sidobre, S., Manlongat, N., Kronenberg, M., Briskin, M. J., Dustin, M. L. et al.**, Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol.* 2005. **3**: e113.

21 **Thomas, S. Y., Scanlon, S. T., Griewank, K. G., Constantinides, M. G., Savage, A. K., Barr, K. A., Meng, F. et al.**, PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *J. Exp. Med.* 2011. **208**: 1179–1188.

22 **Marlin, S. D. and Springer, T. A.**, Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 1987. **51**: 813–819.

23 **Smith, C. W., Rothlein, R., Hughes, B. J., Mariscalco, M. M., Rudloff, H. E., Schmalstieg, F. C. and Anderson, D. C.**, Recognition of an endothelial determinant for CD 18-dependent human neutrophil adherence and transendothelial migration. *J. Clin. Invest.* 1988. **82**: 1746–1756.

24 **Smith, C. W., Marlin, S. D., Rothlein, R., Toman, C. and Anderson, D. C.**, Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils In vitro. *J. Clin. Invest.* 1989. **83**: 2008–2017.

25 **Han, Y., Jiang, Z., Chen, Z., Gu, Y., Liu, Y., Zhang, X., Cao, X.**, Pathogen-expanded CD11b invariant NKT cells feedback inhibit T cell proliferation via membrane-bound TGF-beta1. *J. Autoimmun.* 2015. **58**: 21–35.

26 **Subleski, J. J., Hall, V. L., Wolfe, T. B., Scarzello, A. J., Weiss, J. M., Chan, T., Hodge, D. L. et al.**, TCR-dependent and -independent activation underlie liver-specific regulation of NKT cells. *J. Immunol.* 2011. **186**: 838–847.

27 **Jackman, J. K., Motto, D. G., Sun, Q., Tanemoto, M., Turck, C. W., Peltz, G. A., Koretzky, G. A. et al.**, Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J. Biol. Chem.* 1995. **270**: 7029–7032.

28 **Clements, J. L., Ross-Barta, S. E., Tygrett, L. T., Waldschmidt, T. J. and Koretzky, G. A.**, SLP-76 expression is restricted to hemopoietic cells of monocyte, granulocyte, and T lymphocyte lineage and is regulated during T cell maturation and activation. *J. Immunol.* 1998. **161**: 3880–3889.

29 **Clements, J. L., Yang, B., Ross-Barta, S. E., Eliason, S. L., Hrstka, R. F., Williamson, R. A. and Koretzky, G. A.**, Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science* 1998. **281**: 416–419.

30 **Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W. and Geha, R. S.**, Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. *Cell* 1998. **94**: 229-238.

31 **Jordan, M. S., Smith, J. E., Burns, J. C., Austin, J. E., Nichols, K. E., Aschenbrenner, A. C. and Koretzky, G. A.**, Complementation in trans of altered thymocyte development in mice expressing mutant forms of the adaptor molecule SLP76. *Immunity* 2008. **28**: 359–369.

32 **Motto, D. G., Ross, S. E., Wu, J., Hendricks-Taylor, L. R. and Koretzky, G. A.**, Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. *J. Exp. Med.* 1996. **183**: 1937–1943.

33 **Bubeck Wardenburg, J., Fu, C., Jackman, J. K., Flotow, H., Wilkinson, S. E., Williams, D. H., Johnson, R. et al.**, Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *J. Biol. Chem.* 1996. **271**: 19641–19644.

34 **Raab, M., da Silva, A. J., Findell, P. R. and Rudd, C. E.**, Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. *Immunity* 1997. **6**: 155–164.

35 **Tuosto, L., Michel, F. and Acuto, O.**, p95vav associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. *J. Exp. Med.* 1996. **184**: 1161–1166.

36 **Wu, J., Motto, D. G., Koretzky, G. A. and Weiss, A.**, Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. *Immunity* 1996. **4**: 593–602.

37 **Bubeck Wardenburg, J., Pappu, R., Bu, J. Y., Mayer, B., Chernoff, J., Straus, D. and Chan, A. C.**, Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity* 1998. **9**: 607–616.

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38 **Wunderlich, L., Farago, A., Downward, J. and Buday, L.**, Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. *Eur. J. Immunol.* 1999. **29**: 1068–1075.

39 **Su, Y. W., Zhang, Y., Schweikert, J., Koretzky, G. A., Reth, M. and Wienands, J.**, Interaction of SLP adaptors with the SH2 domain of Tec family kinases. *Eur. J. Immunol.* 1999. **29**: 3702–3711.

40 **Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C. and Berg, L. J.**, Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. *J. Biol. Chem.* 2000. **275**: 2219–2230.

41 **Yablonski, D., Kadlecek, T. and Weiss, A.**, Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. *Mol. Cell Biol.* 2001. **21**: 4208–4218.

42 **Liu, S. K., Fang, N., Koretzky, G. A. and McGlade, C. J.**, The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Curr. Biol.* 1999. **9**: 67–75.

43 **Sauer, K., Liou, J., Singh, S. B., Yablonski, D., Weiss, A. and Perlmutter, R. M.**, Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP-76 in lymphocytes. *J. Biol. Chem.* 2001. **276**: 45207–45216.

44 **da Silva, A. J., Li, Z., de Vera, C., Canto, E., Findell, P. and Rudd, C. E.**, Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. *Proc. Natl. Acad. Sci. U. S. A.* 1997. **94**: 7493–7498.

45 **Musci, M. A., Hendricks-Taylor, L. R., Motto, D. G., Paskind, M., Kamens, J., Turck, C. W. and Koretzky, G. A.**, Molecular cloning of SLAP-130, an SLP-76-associated substrate of the T cell antigen receptor-stimulated protein tyrosine kinases. *J. Biol. Chem.* 1997. **272**: 11674–11677.

46 **Shui, J. W., Boomer, J. S., Han, J., Xu, J., Dement, G. A., Zhou, G. and Tan, T. H.**, Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* 2007. **8**: 84–91.

47 **Wu, J. N., Gheith, S., Bezman, N. A., Liu, Q. H., Fostel, L. V., Swanson, A. M., Freedman, B. D. et al.**, Adhesion- and degranulation-promoting adapter protein is required for efficient thymocyte development and selection. *J. Immunol.* 2006. **176**: 6681–6689.

48 **Griffiths, E. K., Krawczyk, C., Kong, Y. Y., Raab, M., Hyduk, S. J., Bouchard, D., Chan, V. S. et al.**, Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science* 2001. **293**: 2260–2263.

49 **Peterson, E. J., Woods, M. L., Dmowski, S. A., Derimanov, G., Jordan, M. S., Wu, J. N., Myung, P. S. et al.**, Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science* 2001. **293**: 2263–2265.

50 **Griffiths, E. K. and Penninger, J. M.**, Communication between the TCR and integrins: role of the molecular adapter ADAP/Fyb/Slap. *Curr. Opin. Immunol.* 2002. **14**: 317–322.

51 **Cashman, S., Lampe, K., Sheridan, R. and Hoebe, K.**, An ENU mutagenesis approach to dissect “self”-induced immune responses: Unraveling the genetic footprint of immunosurveillance. *Oncoimmunology* 2012. **1**: 856–862.

52 **Lampe, K., Endale, M., Cashman, S., Fang, H., Mattner, J., Hildeman, D. and Hoebe, K.**, Slp-76 is a critical determinant of NK-cell mediated recognition of missing-self targets. *Eur. J. Immunol.* 2015. **45**: 2072–2083.

53 **Kumar, L., Pivniouk, V., de la Fuente, M. A., Laouini, D. and Geha, R. S.**, Differential role of SLP-76 domains in T cell development and function. *Proc. Natl. Acad. Sci. U. S. A.* 2002. **99**: 884–889.

54 **Kettner, A., Pivniouk, V., Kumar, L., Falet, H., Lee, J. S., Mulligan, R. and Geha, R. S.**, Structural requirements of SLP-76 in signaling via the high-affinity immunoglobulin E receptor (Fc epsilon RI) in mast cells. *Mol. Cell Biol.* 2003. **23**: 2395–2406.

55 **Felices, M., Yin, C. C., Kosaka, Y., Kang, J. and Berg, L. J.**, Tec kinase Itk in gammadeltaT cells is pivotal for controlling IgE production in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 2009. **106**: 8308–8313.

56 **Verykokakis, M., Boos, M. D., Bendelac, A., Adams, E. J., Pereira, P. and Kee, B. L.**, Inhibitor of DNA binding 3 limits development of murine slam-associated adaptor protein-dependent “innate” gammadelta T cells. *PLoS One* 2010. **5**: e9303.

57 **Qi, Q., Xia, M., Hu, J., Hicks, E., Iyer, A., Xiong, N. and August, A.**, Enhanced development of CD4+ gammadelta T cells in the absence of Itk results in elevated IgE production. *Blood* 2009. **114**: 564–571.

58 **Ueda-Hayakawa, I., Mahlios, J. and Zhuang, Y.**, Id3 restricts the developmental potential of gamma delta lineage during thymopoiesis. *J. Immunol.* 2009. **182**: 5306–5316.

59 **Lauritsen, J. P., Wong, G. W., Lee, S. Y., Lefebvre, J. M., Ciofani, M., Rhodes, M., Kappes, D. J. et al.**, Marked induction of the helix-loop-helix protein Id3 promotes the gammadelta T cell fate and renders their functional maturation Notch independent. *Immunity* 2009. **31**: 565–575.

60 **Alonzo, E. S., Gottschalk, R. A., Das, J., Egawa, T., Hobbs, R. M., Pandolfi, P. P., Pereira, P. et al.**, Development of promyelocytic zinc finger and ThPOK-expressing innate gamma delta T cells is controlled by strength of TCR signaling and Id3. *J. Immunol.* 2010. **184**: 1268–1279.

61 **Gordon, S. M., Carty, S. A., Kim, J. S., Zou, T., Smith-Garvin, J., Alonzo, E. S., Haimm, E. et al.**, Requirements for eomesodermin and promyelocytic leukemia zinc finger in the development of innate-like CD8+ T cells. *J. Immunol.* 2011. **186**: 4573–4578.

62 **Brennan, P. J., Brigl, M. and Brenner, M. B.**, Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat. Rev. Immunol.* 2013. **13**: 101–117.

63 **Murre, C.**, Helix-loop-helix proteins and lymphocyte development. *Nat. Immunol.* 2005. **6**: 1079–1086.

64 **Emoto, M., Mittrucker, H. W., Schmits, R., Mak, T. W. and Kaufmann, S. H.**, Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. *J. Immunol.* 1999. **162**: 5094–5098.

65 **Springer, T. A.**, Adhesion receptors of the immune system. *Nature* 1990. **346**: 425–434.

66 **Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguilar, J., Hibbs, M. L. and Springer, T. A.**, ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J. Cell Biol.* 1990. **111**: 3129–3139.

67 **Diamond, M. S., Staunton, D. E., Marlin, S. D. and Springer, T. A.**, Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell* 1991. **65**: 961–971.

68 **Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L. and Springer, T. A.**, The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J. Cell Biol.* 1993. **120**: 1031–1043.

69 **Wu, H., Rodgers, J. R., Perrard, X. Y., Perrard, J. L., Prince, J. E., Abe, Y., Davis, B. K. et al.**, Deficiency of CD11b or CD11d results in reduced staphylococcal enterotoxin-induced T cell response and T cell phenotypic changes. *J. Immunol.* 2004. **173**: 297–306.

70 **Donovan, J. A., Wange, R. L., Langdon, W. Y. and Samelson, L. E.**, The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor. *J. Biol. Chem.* 1994. **269**: 22921–22924.

71 **Buday, L., Egan, S. E., Rodriguez Viciana, P., Cantrell, D. A. and Downward, J.**, A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells. *J. Biol. Chem.* 1994. **269**: 9019–9023.

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72 **Sieh, M., Batzer, A., Schlessinger, J. and Weiss, A.**, GRB2 and phospholipase C-gamma 1 associate with a 36- to 38-kilodalton phosphotyrosine protein after T-cell receptor stimulation. *Mol. Cell Biol.* 1994. **14**: 4435–4442.

73 **Maltzman, J. S., Kovoor, L., Clements, J. L. and Koretzky, G. A.**, Conditional deletion reveals a cell-autonomous requirement of SLP-76 for thymocyte selection. *J. Exp. Med.* 2005. **202**: 893–900.

74 **Moran, A. E., Holzapfel, K. L., Xing, Y., Cunningham, N. R., Maltzman, J. S., Punt, J. and Hogquist, K. A.**, T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* 2011. **208**: 1279–1289.

75 **Chang, W. S., Kim, J. Y., Kim, Y. J., Kim, Y. S., Lee, J. M., Azuma, M., Yagita, H. Y. et al.**, Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *J. Immunol.* 2008. **181**: 6707–6710.

76 **Zhang, M., Han, Y., Han, C., Xu, S., Bao, Y., Chen, Z., Gu, Y. et al.**, The beta2 integrin CD11b attenuates polyinosinic:polycytidylic acid-induced hepatitis by negatively regulating natural killer cell functions. *Hepatology.* 2009. **50**: 1606–1616.

77 **Ding, Z. M., Babensee, J. E., Simon, S. I., Lu, H., Perrard, J. L., Bullard, D. C., Dai, X. Y. et al.**, Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J. Immunol.* 1999. **163**: 5029–5038.

78 **Koretzky, G. A., Abtahian, F. and Silverman, M. A.**, SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* 2006. **6**: 67–78.

79 **Springer, T. A.**, Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* 1995. **57**: 827–872.

80 **Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z.**, Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 1999. **13**: 9–22.

81 **Raper, J. A.**, Semaphorins and their receptors in vertebrates and invertebrates. *Curr. Opin. Neurobiol.* 2000. **10**: 88–94.

82 **Bagri, A. and Tessier-Lavigne, M.**, Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance. *Adv. Exp. Med. Biol.* 2002. **515**: 13–31.

83 **Staton, C. A., Kumar, I., Reed, M. W. and Brown, N. J.**, Neuropilins in physiological and pathological angiogenesis. *J. Pathol.* 2007. **212**: 237–248.

84 **Drennan, M. B., Franki, A. S., Dewint, P., Van Beneden, K., Seeuws, S., van de Pavert, S. A., Reilly, E. C. et al.**, Cutting edge: the chemokine receptor CXCR3 retains invariant NK T cells in the thymus. *J. Immunol.* 2009. **183**: 2213–2216.

85 **Liu, Y., Goff, R. D., Zhou, D., Mattner, J., Sullivan, B. A., Khurana, A., Cantu, C. et al.**, A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells. *J. Immunol. Methods* 2006. **312**: 34–39.

Abbreviations: **α-GalCer**: alpha-Galactosylceramide · **APC**: antigen presenting cell · **ADAP**: adhesion and degranulation-promoting adaptor protein · **B6**: C57BL/6J · **Fig.**: figure · **iNKT cell**: invariant natural killer T cell · **int**: intermediate · **LN**: lymph nodes · **mAb**: monoclonal antibody · **MFI**: mean fluorescence intensity · **MNCs**: mononuclear cells · **NK cell**: natural killer cell · **NP-1**: Neuropilin-1 · **PLZF**: promyelocytic leukaemia zinc finger · **SH2 domain**: src homology 2 domain · **slp-76**: SH2 domain-containing leukocyte protein of 76 kDa · **TCR**: T cell receptor

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