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Functional Education of Invariant NKT Cells by Dendritic Cell Tuning of SHP-1

Anna Napolitano,* Paola Pittoni,*-† Lucie Beaudoin,‡ Agnes Lehuen,‡ David Voehringer,‡ H. Robson MacDonald,§ Paolo Dellabona,* and Giulia Casorati*

Invariant NKT (iNKT) cells play key roles in host defense by recognizing lipid Ags presented by CD1d. iNKT cells are activated by bacterial-derived lipids and are also strongly autoreactive toward self-lipids. iNKT cell responsiveness must be regulated to maintain effective host defense while preventing uncontrolled stimulation and potential autoimmunity. CD1d-expressing thymocytes support iNKT cell development, but thymocyte-restricted expression of CD1d gives rise to Ag hyperresponsive iNKT cells. We hypothesized that iNKT cells require functional education by CD1d+ cells other than thymocytes to set their correct responsiveness. In mice that expressed CD1d only on thymocytes, hyperresponsive iNKT cells in the periphery expressed significantly reduced levels of tyrosine phosphatase SHP-1, a negative regulator of TCR signaling. Accordingly, heterozygous SHP-1 mutant mice displaying reduced SHP-1 expression developed a comparable population of Ag hyperresponsive iNKT cells. Restoring nonthymocyte CD1d expression in transgenic mice normalized SHP-1 expression and iNKT cell reactivity. Radiation chimera revealed that CD1d+ dendritic cells supported iNKT cell upregulation of SHP-1 and decreased responsiveness after thymic emigration. Hence, dendritic cells functionally educate iNKT cells by tuning SHP-1 expression to limit reactivity. The Journal of Immunology, 2013, 190: 3299–3308.

In this study, we sought to gain mechanistic insight into the functional education of iNKT cells and identify the CD1d-mediated cellular interactions that regulate their responsiveness.

Materials and Methods

Mice

C57BL/6N mice were from Charles River. pLck-hCD1d, mCD1d+/−, pLck-mCD1d, μT, and ΔDC mice were previously described (8, 10–12). TCRα+/− mice (13) were obtained from Centre de Distribution, Typage, Archivage Animal–Centre National de la Recherche Scientifique (Orleans, France). Nonviable motheaten (me+/+) C57BL/J-Hcphme mice were purchased from The Jackson Laboratories. Mice were kept in a specific pathogen–free environment. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee, San Raffaele Scientific Institute (protocol N.509).

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42835) under accession number GSE42835.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; DP, double-positive; iNKT, invariant NKT; iTCR, invariant TCR; LN, lymph node; RFI, relative fluorescence intensity; tg, transgenic; WT, wild-type.

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Flow cytometry

iNKT cells were stained with αGalCer-loaded mouse or human CD1d-IgG1 dimers (BD Pharmingen) (8) or with PBS-treated mCD1d-PE tetramers (NIH Tetramer Core Facility), and with mAbs specific for TCRβ, CD44, NK1.1, hCD1d, CD3e, anti-CD45.1, CD24/HA, CD19, and H-2-Kb (BioLegend). Nonspecific binding was blocked by pre-incubation with anti-CD32/CD16 mAb. Intracellular SHP-1 or cytokines were detected by staining fixed/permeabilized cells with anti–SHP-1 mAb (Abcam) and secondary Ab (Southern Biotech), or with anti–IFN-γ or anti–IL-4 mAbs (BioLegend). The specificity of intracellular SHP-1 expression detection is shown in Supplemental Fig. 2. Samples were acquired on Canto-II flow cytometer (BD Biosciences), excluding aggregates and dead cells, and analyzed using FlowJo (Tree Star). CD69 and SHP-1 expression level was calculated as relative fluorescence intensity (RFI) (sample mean fluorescence intensity/isotype control mean fluorescence intensity).

Cell preparations

Thymocytes, splenic lymphocytes, and hepatic lymphocytes were obtained as described previously (8). Thymic iNKT cells (TCRβ<sup>+</sup>CD1d-dimers<sup>+</sup>) and peripheral iNKT cells (CD19<sup>-</sup>MHC-II<sup>-</sup> TCRβ<sup>+</sup>CD1d<sup>-</sup>dimer<sup>+</sup> or TCRβ<sup>+</sup>CD1d-tetramer<sup>+</sup>) were sorted (95% purity) using a MoFlo cell sorter (Beckman Coulter). Dendritic cells (DCs) were obtained from bone marrow (BM) cells cultured 5 d with GM-CSF (25 ng/ml) and IL-4 (5 ng/ml; R&D Systems) (14).

iNKT cell activation

For iTCR cross-linking, sorted iNKT cells were placed in 96-well plates precoated with 10 μg/well goat anti-rat IgG and 2 μg/ml anti-CD28 mAb (BD Pharmingen). Goat anti-rat IgGs cross-linking the iTCR by binding rat anti-mouse IgG1-PE mAbs used to detect the CD1d-dimer<sup>+</sup> iNKT cells. For activation by DCs, sorted iNKT cells and DCs were cocultured with increasing αGalCer concentrations; PMA/iodoacetamide stimulation was performed with 25 ng/ml PMA and 1 μg/ml iodonium (Sigma) for 1 h at 37°C plus brefeldin A (10 μg/ml) for 1 h. iNKT cells were activated in vivo by injecting 1.25 μg anti-CD3 mAb 3C11 i.v. into mice.

Whole-gene expression analysis by DNA microarray

Total RNA from hepatic iNKT cells was amplified twice, biotin-labeled, and purified using the RNeasy Mini Kit (Qiagen). The cRNA (10 μg) was hybridized to M430 2.0 chips (Affymetrix). All samples were generated in triplicate. Raw data were normalized using the RMA algorithm of GenePattern (15). Nonvariable low expression values were excluded using “Preprocess Dataset” of GenePattern. Data were visualized using the “Multiplot Process”,” “Multiplot,” and “Hierarchical Cluster” of GenePattern. Gene ontology analysis and visualization were performed using Gorilla software (16). Gene expression data are deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE42835 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42835).

Real-time PCR

RNA was amplified using MessageAmp Premier aRNA Amplification (Ambion) and reverse transcribed. TaqMan was performed in triplicate with 100 ng cDNA/reaction using specific primers and Gadph or Hprt1 endogenous controls (Applied Biosystems) on ABI Prism 7900 and analyzed with SDS 2.2.1 software. Relative quantitation of gene expression was determined using the comparative cycle threshold method (17). For absolute quantitation, Pip96 exons 8–12 cDNA was cloned into ZeroBlunt TOPO Cloning plasmid (Invitrogen). Plasmid DNA was diluted from 10<sup>9</sup> to 0 copy number into irrelevant DNA. Gadph and Hprt1 genes were amplified concurrently to normalize the cDNA of samples. Standard curves for both housekeeping genes were prepared using serial dilution of a cDNA pool (from 150 to 12.5 ng) derived from wild-type (WT) iNKT cells. Pip96 mRNA copy number present in each sample after normalization was calculated using the standard curves.

BM chimeras

BM chimeras were generated as described previously (11) and analyzed 6–8 wk after transplantation.

Statistical analysis

The two-tailed Student t test was used for unpaired samples, applying correction for unequal variances where required. The p values < 0.05 were considered significant.

Results

iNKT cells from pLck-hCD1d tg mice are hyperresponsive

Functional maturation of iNKT cells requires interaction with CD1d<sup>+</sup> APCs, so we investigated reactivity of iNKT cells that developed in pLck-hCD1d<sup>+</sup> mCD1d<sup>-</sup> tg mice (pLck-hCD1d tg) in the absence of “educational” CD1d<sup>+</sup> APCs (8). iNKT cells were sorted from the liver of WT and pLck-hCD1d tg mice using αGalCer-loaded human CD1d-IgG dimers (hCD1d-dim) and were activated by either iTCR cross-linking or αGalCer presentation by DCs. hCD1d-dim bind selectively to mouse V<sub>β</sub>8.2<sup>+</sup> iNKT cells (18), and the hCD1d transgene selects essentially the same population (8), thus enabling robust identification of the same iNKT cell repertoire in both hCD1d tg and WT mice. pLck-hCD1d tg-derived hepatic iNKT cells produced significantly more IFN-γ and IL-4 than WT iNKT cells after either iTCR cross-linking (Fig. 1A) or αGalCer-loaded DCs (Fig. 1B). The cells from both mice expressed similar TCR levels (data not shown). To assess whether iNKT cell hyperreactivity in tg mice depended on enhanced early TCR signaling or instead reflected increased capacity for cytokine expression, we activated hepatic lymphocytes from tg and WT mice with PMA/iodonium, which induce cytokine production bypassing the TCR–proximal complex. Similar fractions of iNKT cells from tg and WT mice expressed CD69 (Fig. 1C, left panels) and produced IFN-γ (Fig. 1C, right panels), suggesting that the hyperreactivity of tg iNKT cells depended on enhanced early TCR signaling.

We next assessed the responsiveness of tg iNKT cells to rapid activation with anti-CD3 mAb in vivo, because the absence of CD1d<sup>+</sup> APCs in these animals precludes the use of αGalCer. Within 90 min of mAb injection, both splenic and hepatic iNKT cells in tg mice upregulated CD69 and produced significantly more IFN-γ than iNKT cells from WT mice (Fig. 1D). Similarly, IL-4 production was upregulated by tg iNKT cells in liver. In these same assays, T cells began to upregulate CD69 similarly in both animals, whereas they did not produce cytokines (data not shown).

Consistent with the intracellular staining data, tg mice administered with anti-CD3 mAb exhibited substantially increased IFN-γ, and slightly higher IL-4, levels in serum compared with WT mice, upon normalization for the total iNKT cell number present in the spleens of each type of mice (Fig. 1E).

Mature T cells of pLck-hCD1d tg mice express low hCD1d levels (8). However, this leaky hCD1d expression on peripheral T cell played a role in the acquisition of NK1.1<sup>+</sup>, but not in the altered responsiveness, of iNKT cells from pLck-hCD1d tg mice, as shown by the phenotype and TCR hyperreactivity exhibited by peripheral iNKT cells developing in mixed BM chimeras in which hCD1d expression was confined to DP thymocytes (Supplemental Fig. 1). Furthermore, the iNKT cell hyperresponsiveness was not due to xenogenic CD1d expression, because iNKT cells isolated from an alternative model in which the mouse CD1d cDNA is expressed under the proximal Lck promoter (10) (pLck-mCD1d tg) exhibited comparable Ag hyperreactivity (Fig. 1F). Together, these data suggested that iNKT cells that develop in pLck-hCD1d tg mice display increased responsiveness to TCR-dependent stimulation.

Distinct gene expression profile in iNKT cells from pLck-hCD1d tg mice

To define the molecular basis of iNKT cell hyperreactivity in pLck-hCD1d tg mice, we compared the global gene expression profile of WT and tg iNKT cells using DNA microarrays. Hepatic iNKT cells were sorted from tg and WT mice using hCD1d-dimers before RNA was extracted for hybridization to genome-wide microarrays (M430v2). iNKT cells from WT and tg mice exhibited very similar
transcription profiles, with only 38 known genes upregulated and 14 downregulated 2-fold in tg cells compared with WT iNKT cells (Fig. 2A, 2B, Supplemental Table I). Gene ontology analysis of the biological processes associated with these differentially expressed genes did not reveal any specific function or pathway (data not shown). The differential expression of a set of
these genes with potential roles in the functional regulation of iNKT cells was validated by RT-quantitative PCR (Fig. 2C). Among the candidate genes downregulated ~2-fold in tg iNKT cells, 

Ptpn6 encodes the SHP-1 tyrosine phosphatase that is involved in negative regulation of TCR signaling (19, 20). Among the confirmed upregulated genes, Cceam1 encodes Carcinoembryonic-Ag-related adhesion molecule 1, which is expressed by activated T cells and inhibits TCR signaling through interaction with SHP-1 (21). Also upregulated were genes Tyrobp, encoding for the NK adaptor protein DAP12, which can exerts both activating and inhibitory effects (22), and gene FcgRIIb, encoding the low-affinity IgG receptor expressed by B cells and activated T cells that mediates inhibitory functions by recruiting SHP-1, SHP-2, and SH2-containing complex inositol 5-phosphatase SHIP1 (23). Finally, upregulated genes Kira5 and Kira7 encode the inhibitory NK receptors Ly49E and Ly49G2 that recruit SHP-1 via their cytoplasmic ITIM regions (24).

These data indicated that downregulation of SHP-1 may underpin the increased responsiveness of iNKT cells that develops in the absence of peripheral CD1d. The upregulation of receptor molecules able to interact with SHP-1 in tg iNKT cells suggested some level of functional compensation, as described in NK cells expressing inactive SHP-1 (25).

**SHP-1 downregulation functionally linked to iNKT cell hyperresponsiveness**

To assess a functional link between SHP-1 and iNKT cell reactivity, we first determined that the similarly hyperreactive iNKT cells from pLck-hCD1d and pLck-mCD1d tg mice exhibited also a comparable downregulation of SHP-1 transcript (Fig. 2D). SHP-1 transcript was also similarly downregulated in the hyperresponsive iNKT cells from pLck-hCD1d mice and from BM chimera expressing hCD1d only on DP thymocytes, described earlier (Supplemental Fig. 1). Next, we investigated iNKT cell responses in motheaten mice (me−/−), which bear a defective SHP-1 protein because of a single nucleotide deletion in the Ptpn6 gene (26). Mice heterozygous for the motheaten mutation (me+/−) exhibit a 50% reduction in SHP-1 transcript and protein in hematopoietic cells, resulting in T cell hyperreactivity upon TCR stimulation (26, 27). Unlike the homozygous me−/−, the heterozygous me+/− mice are healthy and do not exhibit systemic inflammation (28). Frequency and NK1.1 expression of thymic and hepatic iNKT cells in me+/− and WT mice were comparable (data not shown). We confirmed that both SHP-1 transcript and protein levels were similarly reduced in hepatic iNKT cells from both me+/− and pLck-hCD1d tg mice compared with WT animals (Fig. 3A, 3B, Supplemental Fig. 2). As expected, a lower SHP-1 protein level was observed in T cells from me+/− mice, but not in T cells from pLckhCD1d tg mice (Fig. 3B), thereby confirming the selective downregulation of SHP-1 in the tg iNKT cells. Hepatic iNKT cells sorted from me+/− mice produced more cytokines than cells from controls (me−/−), upon activation either by αGalCer-pulsed DCs (Fig. 3C) or by iTCR cross-linking (Fig. 3D), supporting a key role for SHP-1 in regulating iNKT cell responsiveness.

**Restoring contacts with nonthymocyte mCD1d normalizes iNKT cell responsiveness**

We next determined whether reintroducing physiological contact with endogenous mCD1d molecules normalized iNKT cell responsiveness in pLck-hCD1d tg mice. We hence crossed tg and C57BL6 mice to generate pLck-hCD1d tg mCD1d+/+ mice and investigated the reactivity of the peripheral iNKT cells. Hepatic iNKT cells from pLck-hCD1d tg, pLck-hCD1d tg mCD1d+/+, and WT mice were sorted using hCD1d-dimers and activated in vitro either by αGalCer-pulsed DCs (Fig. 4A, upper panels) or by iTCR cross-linking (Fig. 4A, lower panels). Both pLck-hCD1d tg mCD1d+/+ and WT iNKT cells produced levels of cytokines significantly lower than those observed in iNKT cells from pLck-hCD1d tg mice (Fig. 4A). Accordingly, SHP-1 expression was significantly increased in both pLck-hCD1d tg mCD1d+/+ and WT iNKT cells compared with pLck-hCD1d tg iNKT cells (Fig. 4B). The hyperreactivity of iNKT cells that develop in pLck-hCD1d tg...
mice was thus normalized by changes in SHP-1 expression induced by contact with CD1d<sup>+</sup> cells other than thymocytes.

**iNKT cell responsiveness and SHP-1 expression are not imprinted in thymus**

To determine whether the iNKT cell hyperreactivity and SHP-1 downregulation in pLck-hCD1d<sup>tg</sup> mice is imprinted in thymus, iNKT cells from the thymus, spleen, and liver of tg and WT mice were sorted with hCD1d-dimers and then activated by TCR cross-linking to directly compare their effector functions. Thymic iNKT cells from pLck-hCD1d<sup>tg</sup> and WT mice secreted comparable amounts of IFN-γ and IL-4 (Fig. 5A), and displayed equivalent SHP-1 protein expression (Fig. 5B, Supplemental Fig. 3). In WT animals, peripheral iNKT cells secreted significantly less IFN-γ and IL-4 than thymic iNKT cells. These results suggest that the hyperreactivity of iNKT cells in pLck-hCD1d<sup>tg</sup> mice is not the result of a thymic imprinting event.

**FIGURE 3.** SHP-1 downregulation and hyperresponsiveness in iNKT cells from motheaten me<sup>+/+</sup> mice. (A) RNA was extracted from iNKT cells sorted from the liver of WT, pLck-hCD1d<sup>tg</sup>, and me<sup>+/+</sup> mice. Relative quantification of Ptpn6 transcript by RT-qPCR (left panel). The amount of Ptpn6 transcript was normalized to Gapdh, and results are shown as fold change compared with iNKT cells from WT mice. Absolute quantification of Ptpn6 transcript by RT-qPCR (right panel). The number of copies of Ptpn6 mRNA in iNKT cells from WT, pLck-hCD1d<sup>tg</sup>, and me<sup>+/+</sup> mice was determined using a Ptpn6 standard curve. Samples were normalized to Gapdh RNA. (B) Upper histograms show intracellular SHP-1 expression in hepatic T and iNKT cells determined by FACS in one representative mouse per group. Lower panels show SHP-1 mean ± SD RFI in three mice per group. See also Supplemental Fig. 1. (C) Hepatic iNKT cells were sorted from the liver of me<sup>+/+</sup> and littermate WT mice, and activated either by coculture at 5:1 ratio with BM-derived αGalCer-loaded DCs, or (D) by iTCR cross-linking (1 × 10<sup>4</sup> cell/well). Cytokine production was measured by ELISA after 48 h. Shown is mean ± SD cytokine production from three mice in one representative experiment of three performed. *t test, *p < 0.05, **p < 0.01, ***p < 0.005.
cytokine than thymic iNKT cells. In contrast, cytokine secretion by peripheral iNKT cells from pLck-hCD1d tg was sustained at levels comparable with those detected in thymic iNKT cells, and cytokine production was significantly higher than that displayed by WT peripheral iNKT cells. The expression of SHP-1 protein increased markedly from thymus to periphery in iNKT cells from WT mice, but not in iNKT cells from pLck-hCD1d iNKT cells (asterisk); values significantly different between pLck-hCD1d mCD1d+/+ and pLck-hCD1d iNKT cells (boxed asterisk). Shown are mean ± SD of cytokine produced by three mice per group from one representative experiment of three. (B) SHP-1 intracellular (i.c.) staining of T and iNKT cells from the liver of the indicated mice. Upper panels show SHP-1 i.c. expression from one representative mouse per group. Lower panels show SHP-1 mean ± SD RFI from all three mice per group. One representative experiment of three is shown. *p < 0.05, **p < 0.01.

FIGURE 4. Coexpression of mCD1d and hCD1d normalizes iNKT cell responsiveness and SHP-1 expression. (A) Hepatic iNKT cells were sorted from pLck-hCD1d tg, pLck-hCD1d mCD1d+/+, and WT mice. iNKT cells (2 × 10⁴/well) were activated by αGalCer-loaded WT DCs (4 × 10³/well; upper panels) or by iTCR cross-linking (10⁴ cells/well; lower panels). Cytokines were measured by ELISA after 48 h. Values significantly different between WT and pLck-hCD1d iNKT cells (asterisk); values significantly different between pLck-hCD1d mCD1d+/+ and pLck-hCD1d iNKT cells (boxed asterisk). Shown are mean ± SD of cytokine produced by three mice per group from one representative experiment of three. (B) SHP-1 intracellular (i.c.) staining of T and iNKT cells from the liver of the indicated mice. Upper panels show SHP-1 i.c. expression from one representative mouse per group. Lower panels show SHP-1 mean ± SD RFI from all three mice per group. One representative experiment of three is shown. *p < 0.05, **p < 0.01.

Functional education of iNKT cells is mediated by CD1d+ DCs

To identify the CD1d+ cells that might regulate iNKT cell responsiveness in peripheral compartments, we considered B cells and DCs as primary candidates, because both cell types express CD1d and interact with iNKT lymphocytes (29). Furthermore, hyperreactivity of peripheral iNKT cells from pLck-mCD1d tg mice has been reported to normalize upon crossing these animals with tg mice that express mCD1d on MHC II+ APCs (7).

We first investigated the reactivity of iNKT cells in μT mice that lack B lymphocytes (30). The percentage of iNKT cells in these animals was normal in thymus and in periphery, but the numbers were decreased in periphery (data not shown), consistent with the observed T and NK cell reduction in these animals (31, 32). Splenic and hepatic iNKT cells of μT mice that were sorted and activated in vitro by TCR cross-linking were comparably responsive, although IL-4 secretion by splenic iNKT cells was somewhat reduced in μT mice (Fig. 6A). The normal responsiveness of peripheral iNKT cells from μT mice was associated with normal levels of SHP-1 protein expression (Fig. 6B).

The role of DCs in the regulation of iNKT cell responsiveness was investigated using mice in which DCs are constitutively deleted by the expression of an inducible tg diphtheria toxin (DCt mice) (12). DCt mice display a 90% reduction in myeloid DCs, lymphoid DCs, plasmacytoid DCs, and Langerhans cells in thymus, spleen, lymph nodes (LNs), and skin, leading to lethal systemic autoimmunity by 7–8 wk of age (12). We therefore sorted liver iNKT cells from 5-wk-old DCt mice and WT mice using mCD1d dimers, and activated these cells by iTCR cross-linking in vitro. iNKT cells from DCt mice were hyperresponsive (Fig. 6C), suggesting that DCs are key regulators of the iNKT cell activation threshold. To confirm these data in mice not affected...
by autoimmune pathology, we reconstituted lethally irradiated CD1d<sup>−/−</sup> mice with 20% BM cells from either WT mice or CD1d<sup>−/−</sup> mice in combination with 80% BM cells from D<sub>DC</sub> mice (Fig. 6D). The frequencies of iNKT cells were similar in the two types of BM chimera (Fig. 6E), but the proportion of mature NK1.1<sup>+</sup> iNKT cells in the liver and spleen was reduced in animals that lacked CD1d<sup>+</sup> DCs (Fig. 6E). iNKT cells from mice that lacked CD1d<sup>+</sup> DCs were also hyperresponsive to activation in vitro (Fig. 6F). Consistent with this hyperreactivity, iNKT cells from chimera deficient in CD1d<sup>+</sup> DCs expressed a lower SHP-1 mRNA copy number compared with cells from control chimera (data not shown).

Collectively, these data indicated that stimulation by CD1d expressed on peripheral DCs functionally educates iNKT cells by tuning SHP-1 expression.

**Discussion**

Our study reveals a novel mechanism of iNKT cell functional education that occurs in the periphery and is exerted by tuning the TCR signaling threshold. This process is mediated by the upregulation of SHP-1 in iNKT cells, induced upon engaging CD1d<sup>+</sup> DCs. In the absence of this functional checkpoint, expression of SHP-1 remains low, resulting in altered reactivity of iNKT cells. Tyrosine phosphatase SHP-1 exerts a global negative regulatory function on TCR-mediated activation of T lymphocytes by dephosphorylating several proximal signal transduction mediators, thereby setting the threshold for thymocyte selection and activation of mature T cells (20). SHP-1 levels are physiologically modulated during T cell development or upon activation, increasing the avidity of immature thymocytes and recently activated T cells for Ag signals (20). Our finding that iNKT cells upregulate SHP-1 and reduce TCR responsiveness in the periphery is consistent with recent data from tg mice expressing a GFP reporter that directly responds to TCR signaling (33). Thymic iNKT cells from these animals are brightly fluorescent, whereas peripheral iNKT cells are not, suggesting that these cells may become desensitized to CD1d stimulation in the periphery.

DCs play a key role in the activation of iNKT cells (29) and mediate their thymic negative selection (8, 34). B cells are abundant CD1d<sup>+</sup> APCs in secondary lymphoid organs and are also able to interact with iNKT cells (35–37). Yet, our data suggest that DCs...
are key in tuning the TCR responsiveness of iNKT cells in peripheral tissues in the steady-state. DCs, unlike B cells, might provide costimulatory signals that, in addition to the CD1d-dependent one, may cooperate to upregulate SHP-1 expression by peripheral iNKT cells. iNKT cells might also preferentially contact DCs in unchallenged situations, as they colocalize with DCs in the spleen marginal zone and periarteriolar lymphoid sheaths, LNs subcapsular area, and liver sinusoids (38–40). We cannot exclude that also CD1d+ macrophages, which locate proximally to iNKT cells in the splenic marginal zone and in LNs (38, 40), and may be depleted in DDC mice, may contribute to tuning iNKT cell responsiveness. Conditional deletion of CD1d expression by DCs was recently reported to exert little effect on iNKT responsiveness (41). This model is different from ours, and a differential persistence of residual CD1d+ DCs in these mice and in the DDC mice used in this study might account for the variable responsiveness observed.

We show that B lymphocytes do not play a major role in regulating SHP-1 expression in iNKT cells. However, upon TCR cross-linking in vitro, we find a reduced IL-4 secretion by splenic iNKT cells from μT mice. Upon αGalCer injection in vivo, a normal iNKT cell response was found either in μT mice (42) or in mice that exhibit only CD1d-deficient B cells (41), whereas other studies suggested a possible CD1d-dependent suppressive role for B cells in regulating iNKT cell function (29). It is difficult to compare the different iNKT cell activation regimens used in these studies; nevertheless, these data imply that B cells might also regulate iNKT cell effector response, possibly in specific anatomical sites and likely by mechanisms distinct from DCs.

Peripheral contacts with CD1d are required for the final phenotypic maturation of iNKT cells (7, 43, 44). We find that contacts with peripheral CD1d+ DCs results in the upregulation of both NK1.1 and SHP-1 expression by iNKT cells. iNKT cells may complete their maturation upon engaging self-lipid Ags (45), as well as exogenous lipid(s) derived from the intestinal microbiota of mice (46), following uptake and presentation by CD1d-expressing DCs. We also show that hCD1d expression by peripheral T cells in the pLck-hCD1d tg mice can upregulate NK1.1, but not SHP-1, in iNKT cells. Human CD1d may deliver a stronger “basic” stimulation to peripheral iNKT cells than mouse CD1d (8), sufficient
for NK1.1 upregulation. SHP-1 upregulation in peripheral iNKT cells may require additional costimulatory signals, selectively provided by DCs and not by B or T lymphocytes.

Unlike their peripheral counterparts, thymic iNKT cells from pLck-hCD1d tg mice exhibited normal reactivity and SHP-1 levels, in line with published data showing that thymic and peripheral NKT cells represent different functional subsets (7, 47), controlled by distinct molecular and/or environmental cues (7, 48).

The mechanisms by which CD1d+ DC-dependent signaling up-regulates SHP-1 expression in peripheral iNKT cells remain to be defined. In a different context, it was recently shown that weak stimulation of human iNKT cells by CD1d-expressing APCs in vitro opens the IFNG locus and makes it accessible to transcription triggered by innate signaling (49). The TCR triggering of mouse iNKT cells by CD1d+ DCs may poise the PTTP6 locus to transcription, induced by concomitant costimulatory signals directly delivered by DCs or present in the microenvironment.

In conclusion, our study defines a functional education step of iNKT cells occurring in the periphery, which may be critical to control the innate effector state of iNKT cells by tuning their reactivity toward stronger stimulatory signals, generated in stress-related conditions and bacterial infections.

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References


