Supporting information

Facciotti et al. 10.1073/pnas.1108809108

SI Materials and Methods

Immunofluorescence and Biochemical Analyses. Dendritic cells (DCs) from Eα-CD1e transgenic (Tg) mice and non-Tg littermates were fixed, permeabilized, stained with sheep anti-TGN38 Abs (Serotec), revealed with A488-conjugated goat anti-sheep Abs (Jackson ImmunoResearch), and then labeled with the anti-CD1e 20.6 mAb and Cy3-conjugated polyclonal goat anti-mouse Ig (Jackson ImmunoResearch) as previously described (1). Cells were also stained with the 20.6 mAb, counterstained with Cy3-conjugated polyclonal goat anti-mouse Abs, followed by a blocking step with 5% nonimmune mouse serum. Then the cells were labeled with biotinylated anti-MHCII mAb ER-TR3 (Abcam) for class II-associated compartments followed by Cy5-conjugated streptavidin (Invitrogen). The biochemical maturation of CD1e was analyzed by pulse–chase labeling experiments followed by immunoprecipitation, as previously described (2).

Tg Mice. Human CD1E cDNA was cloned into the EcoRI site of the pDOI5 vector under the control of the H-2 Eα promoter (3). The construct was microinjected into B6 × (B6D2F1) fertilized oocytes. Two Tg founders transmitting the transgene to the progeny were obtained; from them, two B6 congenic Tg mouse lines were derived by backcrossing at least seven times to C57BL/6J. The analysis of both Tg lines showed a similar pattern of expression; therefore, all of the experiments described here were performed with animals derived from one such line, B6.Cg–Tg (H2-Eα–CD1E)1Dfim, termed Eα-CD1e Tg. Mice were bred and kept at the animal facility of the University Hospital Basel. This study was approved by and performed according to the regulatory standards of the Kantonales Veterinäramt Basel-Stadt (Cantonal Veterinary Office of Basel City), Switzerland. Mice were genotyped by PCR using the following primer pairs: 5′-CD1e, 5′-CGAGGGTCTCTGCTGTCCTGG-3′ and 3′-CD1e, 5′ AGACTGCTGTGTTTCACCCGAC-3′.

Analysis of Mouse Cells. Mouse thymocytes were prepared by grinding thymi through tea strainers. For further analysis of autoreactive type 1 natural killer T (iNKT) cells, thymocytes were depleted of $CD24^+$ cells by treatment with B2A2 (rat IgM anti-mouse CD24) and rabbit complement. Viable recovered cells were purified over a Lympholyte M gradient (Cedarlane Laboratories). Mouse liver mononuclear cells were prepared as described previously (4). Mouse splenocytes, peritoneal macrophages, and bone marrow cells were obtained by using standard methods. Mouse DCs were prepared by culturing bone marrow progenitors with mouse GM-CSF for 8 d.

Flow Cytometry. iNKT cells were identified by mouse CD1d–α-galactosylceramide (α-GalCer) dimers (4). Cells were stained with the following mAbs: anti–T-cell receptor β (TCRβ) (H57-597), anti-CD8α (53-6.7), anti-CD4 (RM4-5), anti-CD1d (1B1), anti-TCRγδ (GL3), anti-Vβ8.2 (F23-2), anti-Vβ7 (TR310), anti-CD69 (H1.2F3), anti-CD11b (M1/70), and anti-CD11c (HL3) (BD Biosciences or Biolegend). L363 (5) was used to detect CD1d–α-GalCer complexes on DCs. To detect early appearance of CD1d–α-GalCer complexes, mouse bone marrow-derived DCs (BMDCs) were incubated for 1 h with 500 nM α-GalCer, then washed and chased at 37 °C for 1–4 h before staining. To detect disappearance of CD1d–α-GalCer complexes, mouse BMDCs were incubated for 1 h with different doses of α-GalCer, then chased for 24 h before staining. Biotinylated mAbs were revealed with streptavidin–allophycocyanin or streptavidin–Alexa Fluor 750.

Lipid Loading to and Unloading from CD1d by Isoelectrofocusing (IEF). Stock solutions of liposomes were prepared by mixing appropriate amounts of lipids (Avanti Polar Lipids) in chloroform/methanol, followed by drying and rehydration in 10 mM Bis-Tris/10 mM NaCl (pH 6.5) for 1 h and 10 freeze–thaw cycles. Aliquots of CD1d and CD1d–GD3 (10 μM final concentration), previously purified by chromatofocusing, were shaken (700 rpm, 37 °C) in glass inserts in the presence or absence of CD1e (10 μM for lipid loading and 15 μM for CD1d–GD3 unloading experiments) and tested with lipid/phosphatidylcholine/phosphatidylethanolamine/sphingomyelin/cholesterol liposomes (200/500/200/200/250 μM final concentrations) in 50 mM Na acetate/50 mM NaCl/1 mM DTT/1 mM EDTA (pH 5.0). After 1 h, the samples were cooled on ice and analyzed on IEF3-9 gels (GE Healthcare) as reported (6). Recombinant CD1e was generated as reported (1).

Bacteria. *Sphingomonas paucimobilis* P1 was provided by J. Mattner (University of Erlangen-Nürnberg, Erlangen, Germany) (7). Bacterial cultures were grown to logarithmic phase in Mueller–Hinton broth at 37 °C. For stimulation experiments, heat-inactivated *S. paucimobilis* P1 (1 h, 70 °C) was used to stimulate 10^5 BMDCs. In some experiments, 10^5 DCs were pulsed for 2 h with heat-inactivated *S. paucimobilis* at a ratio 200:1 bacteria:APC before fixation and addition of 10^5 mouse iNKT FF13 hybridoma cells. For infection experiments, 10^5 DCs were infected for 1 h with *S. paucimobilis* P1 at a multiplicity of infection (MOI) of 1:1 before addition of 15 μg/mL gentamicin. Freshly isolated iNKT cells from spleen were added, and up-regulation of CD69 was measured at different time points by flow cytometry.

1. de la Salle H, et al. (2005) Assistance of microbial glycolipid antigen processing by CD1e. *Science* 310:1321–1324.
2. Maître B, et al. (2009) The assembly of CD1e is controlled by an N-terminal propeptide which is processed in endosomal compartments. *Biochem J* 419:661–668.
3. Kouskoff V, Fehling HJ, Lemeur M, Benoist C, Mathis D (1993) A vector driving the expression of foreign cDNAs in the MHC class II-positive cells of transgenic mice. *J Immunol Methods* 166:287–291.
4. Schümann J, et al. (2007) Differential alteration of lipid antigen presentation to NKT cells due to imbalances in lipid metabolism. *Eur J Immunol* 37:1431–1441.
5. Yu KO, et al. (2007) Production and characterization of monoclonal antibodies against complexes of the NKT cell ligand α-galactosylceramide bound to mouse CD1d. *J Immunol Methods* 323:11–23.
6. Garcia-Alles LF, et al. (2006) Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *EMBO J* 25:3684–3692.
7. Mattner J, et al. (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434:525–529.

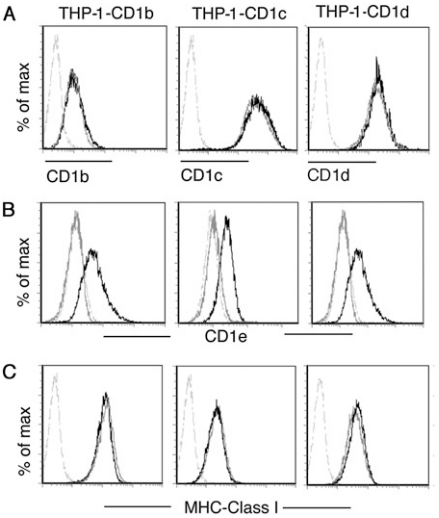

Fig. S1. THP-1 single and double transfectants express the same levels of CD1 molecules. Gray lines illustrate expression levels of the various molecules in single CD1 transfectants, and black lines indicate levels in double transfectants. Hatched lines represent background staining with irrelevant antibodies. (*A*) CD1b (*Left*), CD1c (*Center*), and CD1d (*Right*) are shown. (*B*) Intracellular CD1e. (*C*) MHCI. Similarly, C1R single and double transfectants were selected by sorting, analyzed by flow cytometry, and used in some experiments.

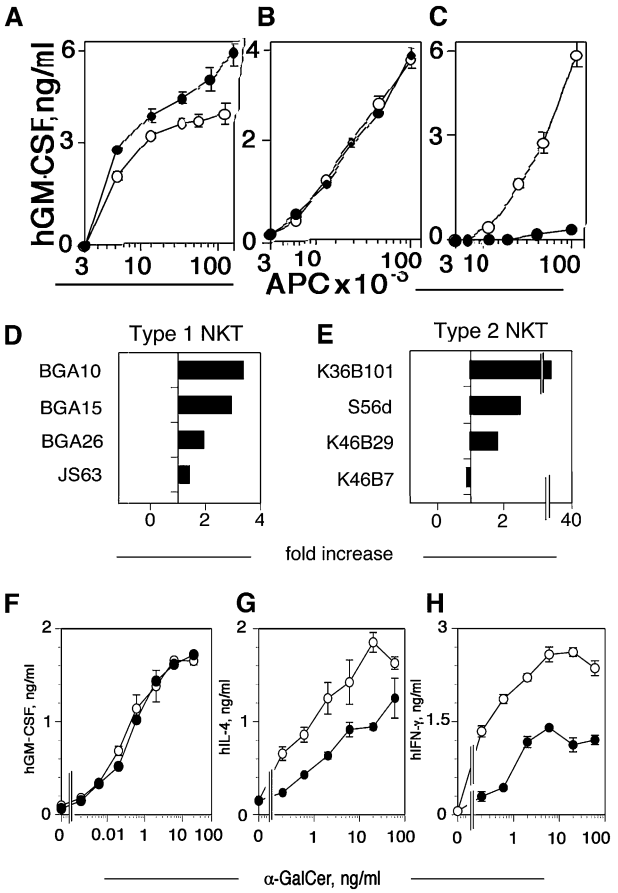

Fig. S2. CD1d-restricted clones are influenced by CD1e. (*A–C*) A human type 1 NKT cell clone (*A*) or type 2 NKT cell clones (*B* and *C*) (5×10^4 cells per well) were incubated with increasing numbers of THP-1 cells transfected with either the *CD1D* gene (○) or with the *CD1D* and *CD1E* genes (●). (*D* and *E*) Autoreactive type 1 (*D*) and type 2 (*E*) NKT cell clones were incubated with single-transfected (*CD1D* only) and double-transfected (*CD1D* and *CD1E*) C1R cells. The difference in activation is shown as fold increase [ratio of human GM-CSF (hGM-CSF) release in response to double- and single-transfected C1R cells]. In each experiment, 5×10^4 T cells were stimulated with 10^5 C1R cells. (*F–H*) C1R cells transfected with the *CD1D* gene (○) or with the *CD1D* and *CD1E* genes (●) were incubated for 2 h with different concentrations of α-GalCer before addition of the human nonself-reactive iNKT clone VM-D5. Released cytokines are expressed as mean ng/mL ± SD (n = 3). Data shown are representative of at least three experiments.

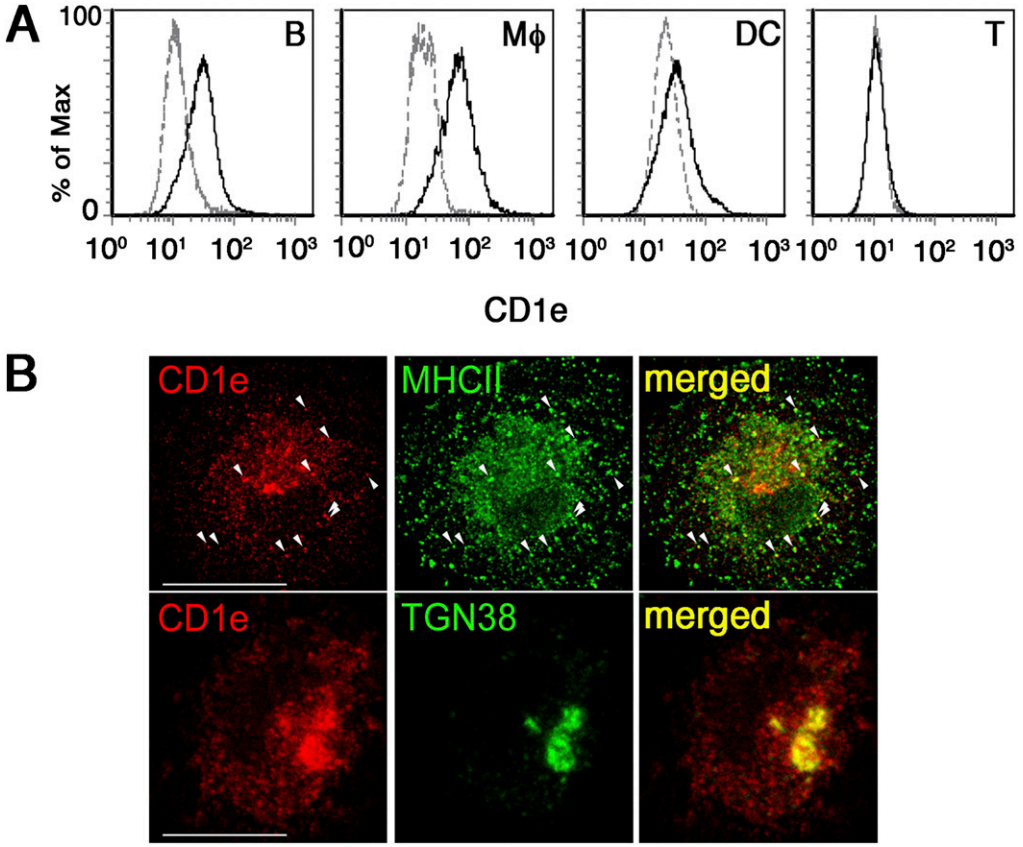

Fig. S3. Generation and characterization of Eα-CD1e Tg mice. (*A*) CD1e is expressed by B cells, macrophages, and BMDCs of Eα-CD1e Tg mice, but not by T cells. Intracellular staining with anti-CD1e mAbs in cells from Eα-CD1e Tg (black lines) and non-Tg littermates (gray lines). Each plot illustrates staining of gated cell populations as indicated. (*B*) CD1e protein in Eα-CD1e Tg mice is present in MHC class II compartment and the trans-Golgi network (TGN). Cells were intracellularly stained with anti-CD1e and anti-MHCII mAbs (*Upper*) or with anti-TGN38 antiserum and CD1e mAb (*Lower*) and analyzed by confocal microscopy. White arrowheads indicate colocalization of CD1e and MHCII molecules. (Bars: 10 μm.)

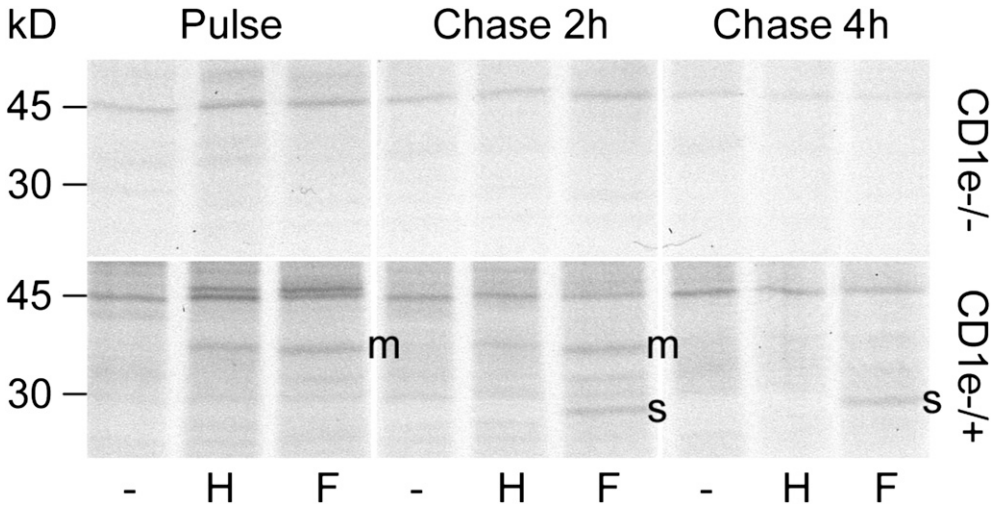

Fig. S4. CD1e in DCs from Tg mice is cleaved in endosomal compartments. BMDCs were metabolically labeled with [^{35}S]methionine and cysteine for 30 min. Cells were lysed either directly or after chase at 37 °C for 2 or 4 h in normal culture medium. CD1e was immunoprecipitated with the 20.6 mAb, deglycosylated with EndoH (H) or EndoF (F) or left untreated (–), separated on denaturing electrophoresis gels, and analyzed by autofluorography. Letters indicate the position of cleaved CD1e. m, membrane CD1e; s, soluble CD1e. Molecular mass markers are indicated in kDa on the left side.

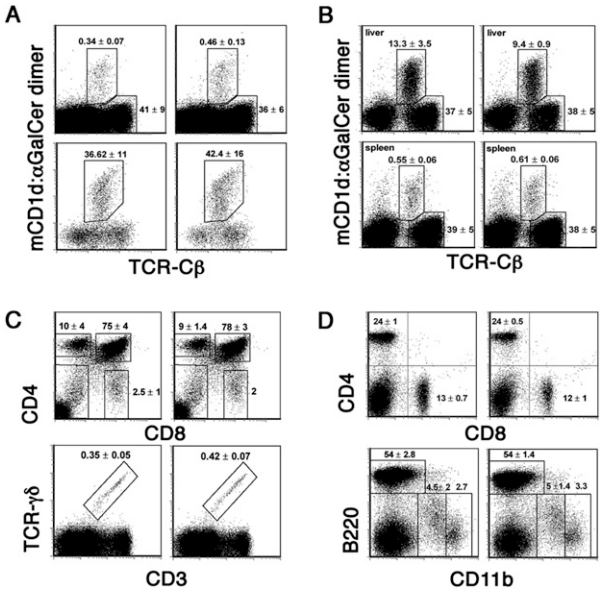

Fig. S5. Eα-CD1e Tg mice have normal numbers and phenotype of iNKT, other T- and B-cell, and monocyte populations. (*A*) Total (*Upper*) or $CD24^+/CD8^+$-depleted (*Lower*) thymocytes from Eα-CD1e Tg (*Right*) and WT (*Left*) mice were costained with mouse CD1d–α-GalCer dimers and anti–TCR-Cβ mAbs and analyzed by flow cytometry. The gates defining iNKT (TCR-$C\beta^+$ and CD1d–α-GalCer $dimer^+$) and conventional TCRαβ T cells (TCR-$C\beta^+$ and CD1d–α-GalCer $dimer^-$) are shown. (*B*) Liver mononuclear cells (*Upper*) or splenocytes (*Lower*) from Eα-CD1e Tg (*Right*) or WT (*Left*) mice stained with mouse CD1d–α-GalCer dimers and anti–TCR-Cβ mAbs. (*C*) Expression of CD4 and CD8α (*Upper*) and TCRγδ (*Lower*) cells on thymocytes from Eα-CD1e Tg (*Right*) and WT (*Left*) mice. (*D*) Splenocytes from Eα-CD1e (*Right*) or WT (*Left*) mice were analyzed for expression of CD4 and CD8α (*Upper*) and B220 and CD11b (*Lower*). Numbers represent the mean percentage of positive cells (±SD) in the indicated gates (n = 3).

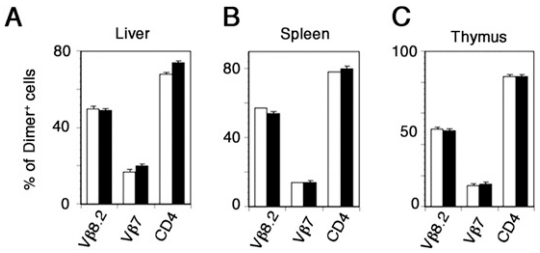

Fig. S6. iNKT cells in Eα-CD1e Tg mice express normal levels of CD4 and TCR-Vβ repertoire. iNKT cells (gated as CD1d–α-GalCer $dimer^+$ and TCR-$C\beta^+$) from liver (*A*), spleen (*B*), and thymus (*C*) from Eα-CD1e Tg mice (black bars) or negative littermates (white bars) were stained with anti-CD4, anti–TCR-Vβ8.2, and anti–TCR-Vβ7 mAbs. Numbers represent the mean percentage of positive cells (±SD) (n = 3).

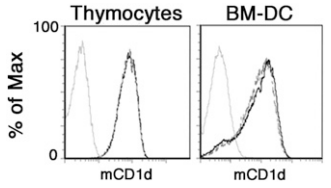

Fig. S7. Eα-CD1e Tg mice express normal levels of CD1d molecules. (*Left*) Expression levels of mouse CD1d on thymocytes from Eα-CD1e Tg mice (gray line) and negative littermates (black line). (*Right*) Expression levels of mouse CD1d in BMDCs from Eα-CD1e Tg mice (gray line) and negative littermates (black line). Light gray lines in both plots represent background staining with irrelevant antibodies.

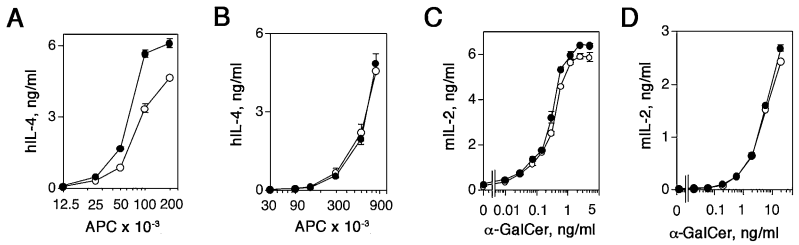

Fig. S8. CD1e expressed in Tg DCs influences presentation of self-lipids. (*A* and *B*) DCs (*A*) or thymocytes (*B*) from Eα-CD1e Tg mice (●) or non-Tg littermates (○) were incubated with the human self-reactive iNKT clone JS63. Human IL-4 (hIL-4) (ng/mL ± SD) release was determined by ELISA. (*C* and *D*) DCs (*C*) or thymocytes (*D*) from Eα-CD1e mice (●) or non-Tg littermates (○) were incubated for 2 h with α-GalCer before addition of FF13 iNKT mouse hybridoma cells.