ego, CA) followed by a horseradish peroxidase–conjugated secondary antibody (Amersham, Arlington Heights, IL) and visualized with the use of an Amersham ECL kit (Amersham). Diethylaminoethyl (DEAE) agarose (Bio-Rad, Hercules, CA) column chromatography was performed as in (*21*). Approximately 0.5 μg of antibody to human Myc (Oncogene Science) was incubated with 100 to 200 μg of N2A S100 lysate from cells expressing either the Myc-tagged, full-length TP1 (TP1), or with no plasmid (N2A), for 1 hour, followed by incubation with 5 to 10 μl of protein G–Sepharose beads at 4°C for 1 to 3 hours. For the Myc peptide competition, 10 μg of Myc peptide (Oncogene Science) was added before incubation with anti-Myc. The supernatant was removed, and the beads were washed twice with 1 ml of 2.3× hypobuffer (*7*) containing 10% (v/v) glycerol and 0.1 M NaCl. The lysates (lysate) and immunoprecipitates (IP) were assayed by the telomerase repeat amplification protocol (TRAP) (*30*). For the anti-peptide immunoprecipitations, sera was adsorbed to 10 μl of protein A–Sepharose beads (Pierce, Rockford, IL), washed with hypobuffer, and incubated with DEAE-purified S100 lysates of NIH 3T3 cells. For peptide competition, 60 μg of each peptide was incubated with the washed protein A beads before the addition of lysate.

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Immunoglobulin E Production in the Absence of Interleukin-4–Secreting CD1-Dependent Cells

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A lymphocyte population that expresses surface markers found on T cells and natural killer (NK) cells secretes large amounts of interleukin-4 (IL-4) immediately after T cell receptor ligation. These NK-like T cells are thus thought to be important for the initiation of type 2 T helper cell (T_H2) responses. CD1-deficient mice were found to lack this lymphocyte subset, but they could nevertheless mount a protypical T_H2 response; after immunization with antibody to immunoglobulin D (IgD), CD1-deficient mice produced IgE. Thus, although dependent on CD1 for their development, IL-4–secreting NK-like T cells are not required for T_H2 responses.

Interleukin-4 is critical for the differentiation of naïve T cells into T_H2 cells. Perhaps the most compelling evidence for this are studies showing that knockout mice harboring gene disruptions for either IL-4 (*1*) or STAT6, the signaling molecule activated by IL-4 (*2*, *3*), fail to generate T_H2 cells and the IgE responses they promote. Despite the importance of this cytokine, the identity of the cell type that produces the IL-4 needed for the development of T_H2 cells remains controversial. In addition to T_H2 cells themselves, mast cells (*4*), $CD8^+$ T cells (*5*), $\gamma\delta^+$ T cells (*6*), and a subset of $\alpha\beta^+$ T cells expressing NK cell markers (*7*) are all capable of producing IL-4.

NK-like T cells are notable in that they promptly secrete large amounts of IL-4 after in vivo administration of a monoclonal antibody (mAb) to CD3 (*8*). Certain mouse strains, such as SJL and β_2-microglobulin (β_2M)–deficient mice, lack NK-like T cells and do not produce high titers of IL-4 after primary stimulation of their T cell receptors (TCRs) in vivo (*9*, *10*). These animals are also impaired in their ability to produce IgE after immunization with the polyclonal stimulus antibody to IgD (anti-IgD) (*9*, *10*). The correlation between this inability to produce IgE in response to anti-IgD and an absence of IL-4–producing NK-like T cells has led to the suggestion that IL-4 production by NK-like T cells is important for the initiation of T_H2 responses.

Several lines of evidence suggest that NK-like T cells are restricted by the nonpolymorphic major histocompatibility complex (MHC) class I–like molecule CD1. First, $NK1.1^+$ T cells, including those that are $CD4^+$, are easily identified in MHC class II–deficient mice (*11*–*13*), whereas disruption of the gene encoding β_2M, which impairs the expression of CD1, results in the loss of these cells (*13*, *14*). Second, $NK1.1^+$ T cells express a limited repertoire of TCRs comprising an invariant α chain, $V_\alpha14J_\alpha281$, usually paired with a $V_\beta8$, $V_\beta7$, or $V_\beta2$ chain (*15*, *16*), which suggests that they are restricted by a monomorphic ligand. Finally, $NK1.1^+$ T cells can be activated by CD1-expressing cells (*12*, *17*).

To confirm that NK-like T cells require CD1 for their development and to assess the role of CD1-dependent cells in the generation of T_H2 responses, we used gene targeting in embryonic stem (ES) cells to produce CD1-deficient mice. The murine CD1 locus is composed of two closely linked genes, *CD1.1* and *CD1.2*, arranged in opposite transcriptional orientations and spaced approximately 9 kb apart (*18*). We used a gene-targeting construct that deleted almost all of the coding regions of both *CD1.1* and *CD1.2* while maintaining the intergenic sequence (Fig. 1A). Correctly targeted ES cells of strain 129 mice were identified at a frequency of 1 in 75 drug-resistant clones and injected into BALB/c embryos to generate germline chimeras. Southern (DNA) blot analysis confirmed transmission of the targeted allele and deletion of coding sequences from both CD1 genes (Fig. 1B). Homozygous mice were generated at the expected Mendelian frequency and were grossly normal in appearance. To determine whether homozygous mice lacked CD1 expression, thymocytes were analyzed by flow cytometry with the CD1-specific mAb 1B1 (Fig. 1C). CD1 expression was detected on thymocytes from wild-type littermates but was reduced on cells from heterozygous mice and was absent on cells from homozygous mice. Moreover, the CD1-restricted hybridoma DN32.D3 (*16*) could not be activated by thymocytes from homozygous mice (Fig. 1D). Thus, homozygous mice were phenotypically and functionally deficient for CD1 expression.

Several studies have suggested that NK-like T cells may be dependent on CD1 for their maturation (*13*, *14*). In the thymus, NK-like T cells are phenotypically characterized as heat-stable antigen $(HSA)^{-/low}$, $NK1.1^+$, TCR^{int}, $CD44^{high}$, and $CD122^+$ (*19*). In addition, their TCR expression is greatly biased toward $V_\alpha14$ and $V_\beta8$ (*15*, *16*). To determine whether NK-like T cells require CD1 for their development, thymocytes from control and CD1-deficient mice were analyzed by flow cytometry (Fig. 2A). Approximately 10% of the $HSA^{-/low}$ thymocytes from C57BL/6 mice were $V_\beta8^{int}$ $NK1.1^+$, and these cells could also be defined as $V_\beta8^{int}$ $CD44^{high}$ or $V_\beta8^{int}$ $CD122^+$ (*20*) (Fig. 2A). As was consistent with previous observations (*13*, *14*), this population was greatly diminished in β_2M-deficient C57BL/6 mice. When CD1-deficient mice were analyzed for the presence of $HSA^{-/low}$ $V_\beta8^{int}$ $CD44^{high}$ thymocytes (the NK1.1 marker is not expressed in the 129 and BALB/c genetic backgrounds of the CD1-deficient mice), this population was similarly decreased in number (Fig. 2A). Thus, CD1 is the β_2M-dependent molecule required for the development of this thymocyte subset.

Unlike mature peripheral T cells, $HSA^{-/low}$ thymocytes secrete large amounts of both IL-4 and interferon-γ (IFN-γ) after stimulation through their TCRs (*7*, *21*). In

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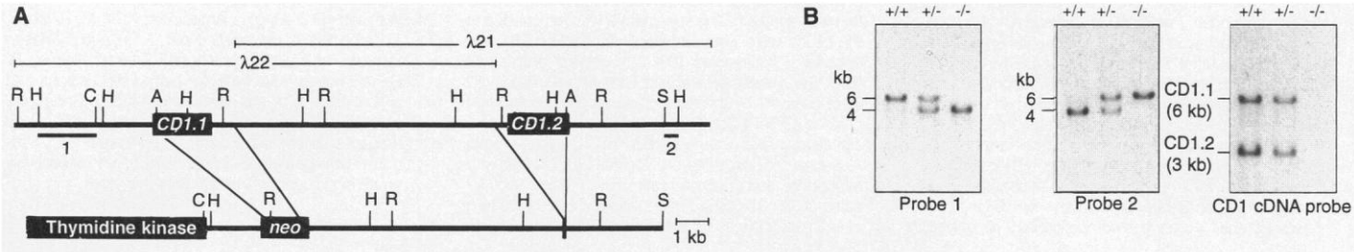

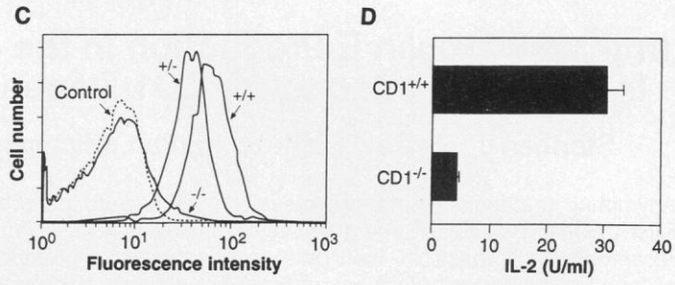

Fig. 1. Generation and analysis of CD1-deficient mice. (**A**) The CD1 gene-targeting construct. Two phage clones isolated from a genomic library of strain 129 mice are depicted above the deduced restriction endonuclease map of the *CD1* locus. The coding regions of the *CD1.1* and *CD1.2* genes are indicated, and the targeting construct derived from the phage clones is shown below. Probes 1 and 2 used for Southern analyses are also indicated. R, Eco RI; H, Hind III; C, Cla I; A, Asp 718; S, Spe I. (**B**) Southern blot analysis of tail DNA from (BALB/c × 129Sv) F_2 CD1-deficient mice. DNA was digested with Eco RI (left and right panels) or Hind III (middle panel) and hybridized with the indicated probes. The cDNA probe was derived from nucleotides 110 through 387 of the CD1 cDNA (*24*) by polymerase chain reaction. (**C**) Flow cytometric analysis of CD1 expression. Total thymoctes from wild-type (+/+), heterozygous (+/−), and CD1-deficient (−/−) mice were incubated with the CD1-specific rat mAb 1B1 (Pharmingen) or an isotype-matched control mAb. Cells were subsequently treated with fluorescein-conjugated mouse mAb to rat IgG (Pharmingen) and analyzed by flow cytometry. (**D**) Stimulation of the CD1-restricted T cell hybridoma DN32.D3. Hybridoma cells were stimulated with thymocytes from the indicated mice (*25*).

contrast, activated $HSA^{-/low}$ thymocytes from β_2M-deficient mice (of either the C57BL/6 or BALB/c genetic backgrounds) produced little IL-4 and IFN-γ (Fig. 2B), which suggests that CD1-dependent cells may be the source of these cytokines. $HSA^{-/low}$ thymocytes from CD1-deficient mice were impaired in their capacity to secrete IL-4 after TCR cross-linking (Fig. 2B). However, in contrast to $HSA^{-/low}$ thymocytes from β_2M-deficient mice, CD1-deficient $HSA^{-/low}$ thymocytes produced IFN-γ in amounts comparable to those produced by thymocytes from control littermates. Thus, IL-4–secreting $HSA^{-/low}$ thymocytes are CD1-dependent, but IFN-γ–producing $HSA^{-/low}$ thymocytes comprise a distinct subset of cells that is β_2M-dependent and CD1-independent.

Intravenous injection of antibody to CD3 (anti-CD3) leads to secretion of large amounts of IL-4 and IFN-γ by splenic cells from wild-type but not β_2M-deficient mice (*8*, *10*). To determine whether CD1-dependent cells are required for this response, control and CD1-deficient mice were injected with anti-CD3. Spleens were removed 90 min later, and RNA was prepared for Northern (RNA) analysis (Fig. 3A). In vivo administration of anti-CD3 induced IL-4 mRNA in splenic cells from control but not β_2M-deficient mice, independently of their genetic background. CD1-deficient mice also did not produce IL-4 mRNA after injection of anti-CD3. However, in contrast to β_2M-

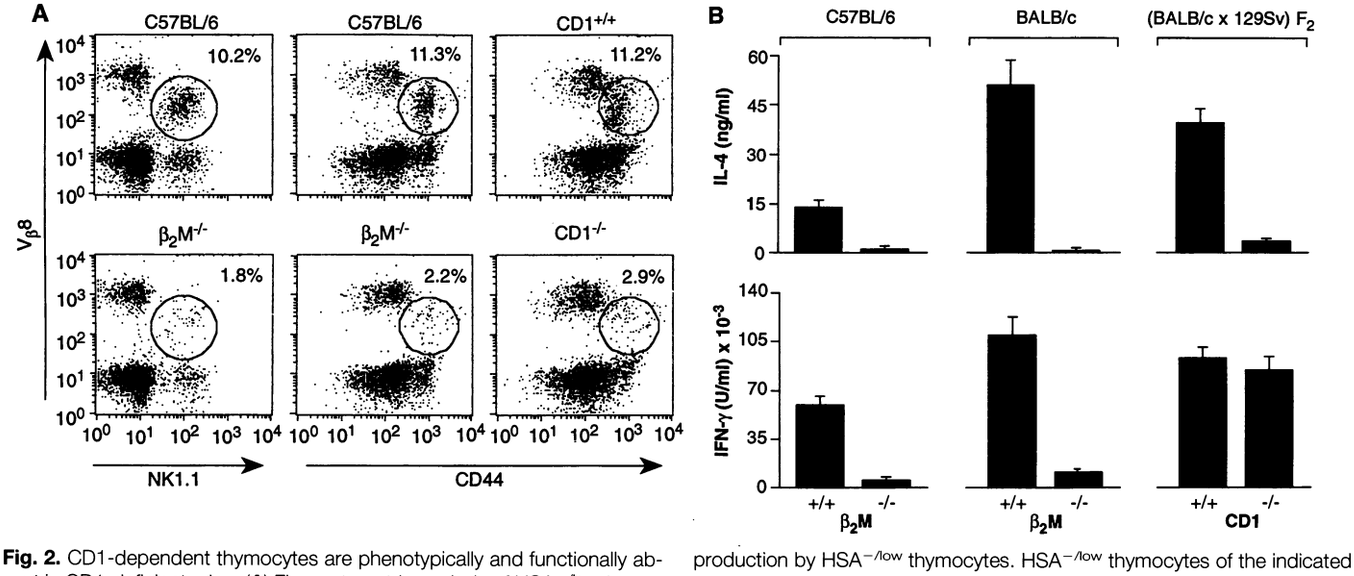

Fig. 2. CD1-dependent thymocytes are phenotypically and functionally absent in CD1-deficient mice. (**A**) Flow cytometric analysis of $HSA^{-/low}$ thymocytes from CD1-deficient mice. $HSA^{-/low}$ thymocytes were purified from control or β_2M-deficient C57BL/6 mice and from control or CD1-deficient (BALB/c × 129Sv) F_2 mice and analyzed by flow cytometry (*26*). (**B**) Cytokine production by $HSA^{-/low}$ thymocytes. $HSA^{-/low}$ thymocytes of the indicated genotypes were stimulated with anti-CD3 and assayed for cytokine production (*27*). Results depict the average and standard deviation of duplicate determinations of cytokine production per 1×10^6 cells. Similar results were obtained in three independent experiments.

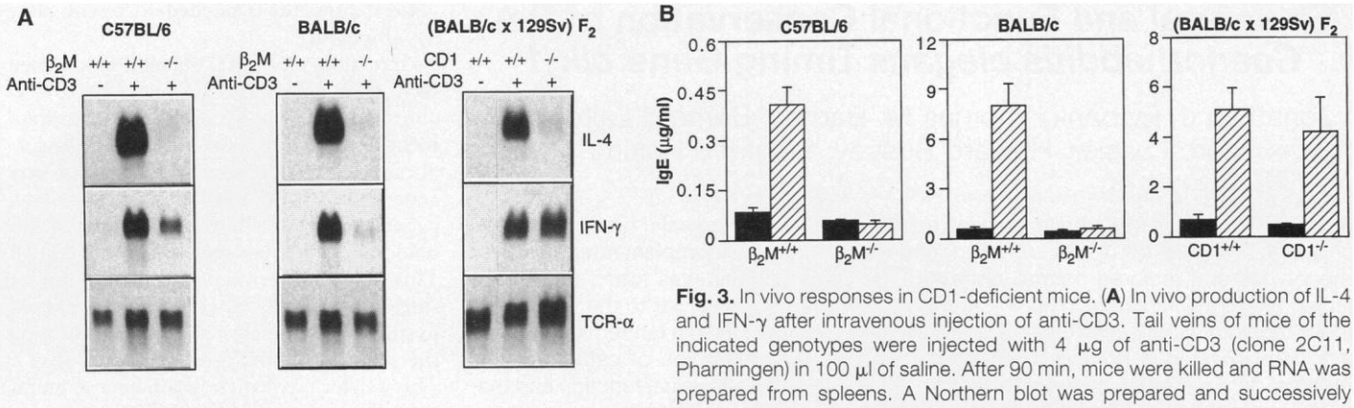

Fig. 3. In vivo responses in CD1-deficient mice. (**A**) In vivo production of IL-4 and IFN-γ after intravenous injection of anti-CD3. Tail veins of mice of the indicated genotypes were injected with 4 μg of anti-CD3 (clone 2C11, Pharmingen) in 100 μl of saline. After 90 min, mice were killed and RNA was prepared from spleens. A Northern blot was prepared and successively hybridized with probes specific for IL-4, IFN-γ, and the TCR-α chain. Similar results were obtained in two independent experiments. (**B**) In vivo IgE production in response to immunization with anti-IgD. Mice of the indicated genotypes were immunized; 9 days later, animals were killed and sera were analyzed for IgE (*2*). Basal IgE concentrations were obtained from uninjected control mice. Results depict the average and standard deviation from three mice of each genotype. Solid bars indicate uninjected mice; striped bars indicate injected mice.

deficient mice, the induction of IFN-γ mRNA was normal in CD1-deficient mice. Thus, in both the thymus and periphery, distinct subsets of β_2M-dependent cells are probably responsible for the prompt production of IL-4 and IFN-γ after primary stimulation through their TCRs. IL-4–producing cells are CD1-dependent, whereas IFN-γ–producing cells are CD1-independent.

Certain strains of mice, such as SJL and β_2M-deficient mice, lack NK-like T cells and do not produce IgE after immunization with anti-IgD (*9*, *10*). This observation has led to the suggestion that NK-like T cells are important for the initiation of this T_H2 response. To evaluate whether CD1-dependent cells are required for this response, control and CD1-deficient mice were injected with anti-IgD. Nine days after immunization, mice were bled and sera were analyzed for total IgE (Fig. 3B). Administration of anti-IgD to C57BL/6 or BALB/c mice resulted in an approximately 10-fold increase in serum IgE titers, but this response was absent in genetically matched β_2M-deficient mice. In contrast, CD1-deficient mice produced IgE in amounts comparable to those produced by their control littermates after immunization with anti-IgD. Thus, CD1-dependent cells are not required for IgE production in vivo.

Our analysis of CD1-deficient mice establishes that the development of the subset of cells expressing surface markers of both the T cell and NK cell lineages is CD1-dependent. In addition, we have shown that CD1-dependent cells are responsible for the prompt production of IL-4 after primary TCR stimulation, but that a distinct subset of β_2M-dependent CD1-independent cells is responsible for IFN-γ production under this condition. Finally, we showed that CD1-dependent cells, and the IL-4 that they produce, are not required for the generation of an IgE response after immunization with anti-IgD.

Although our results indicate that β_2M-dependent CD1-independent cells can promote the generation of an IgE response to anti-IgD, recent evidence suggests that β_2M-independent cells can also initiate IgE responses to a variety of protein antigens and pathogens (*22*). Thus, multiple cell types have the capacity to initiate T_H2 responses in vivo. CD1-deficient mice complement a growing list of strains lacking distinct populations of IL-4–producing cells, including SJL and β_2M-, $\gamma\delta^+$ T cell– and mast cell–deficient mice. Crosses between these strains may help to further define the relative contributions of individual cell types in initiating T_H2 responses. As CD1 is strongly expressed on gastrointestinal epithelium (*23*), an extensive analysis of mucosal immune responses in CD1-deficient mice should be particularly useful in revealing the role of CD1-dependent cells.

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25. Hybridoma cells (5×10^4) were cultured with 5×10^5 thymocytes from the indicated mice in 96-well microcultures. After 24 hours, supernatants were harvested, and IL-2 production was quantitated by HT-2 cell proliferation. Proliferation was converted to IL-2 units with the use of a standard curve generated with recombinant IL-2 (Genzyme).
26. $HSA^{-/low}$ thymocytes were obtained by treatment of total thymocytes with antibody to HSA (clone J11d2) plus rabbit complement (Cederlane, Hornby, Ontario, Canada) for 45 min at 37°C, followed by isolation of viable cells on lympholyte M gradients (Cederlane). After Fc receptors were blocked by treatment with mAb 24G2, cells were stained with phycoerythrin-conjugated mAb specific for V_β8.1/8.2 and fluorescein-conjugated mAb specific for NK1.1 or CD44, and then analyzed by flow cytometry. All mAbs were purchased from Pharmingen.
27. Anti-CD3–coated plates (10 μg/ml, clone 2C11; Pharmingen) were prepared and used to stimulate 5×10^5 $HSA^{-/low}$ thymocytes in 1 ml of media. After 48 hours, supernatants were harvested and assayed for cytokine production (*2*). Recombinant IL-4 and IFN-γ (Genzyme) were used to generate standard curves.

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