

T_H2 dominance and defective development of a CD8⁺ dendritic cell subset in Id2-deficient mice

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Background: Although the T_H1/T_H2 balance is important in many clinical situations, the regulatory mechanisms in vivo have not been well elucidated.

Objective: We sought to characterize the immunologic status of mice lacking Id2, an inhibitor of basic helix-loop-helix transcription factors.

Methods: We analyzed serum immunoglobulin levels, gene-expression profiles in the spleen, T_H1/T_H2 balance, and dendritic cell (DC) populations of Id2^{-/-} mice.

Results: Serum levels of T_H2-mediated IgG1 and IgE were increased more than 10-fold in Id2^{-/-} mice without antigenic stimulation. Gene-expression analysis in Id2^{-/-} splenocytes revealed enhanced expression of T_H2-related genes, such as IL-4, and reduced expression of T_H1-related genes, including IFN- γ and IL-12. Intracellular cytokine staining also confirmed that Id2^{-/-} splenic CD4⁺ T cells are substantially skewed to T_H2 cells. However, Id2^{-/-} naive CD4⁺ T cells differentiated into T_H1 cells comparably with wild-type T cells under the appropriate culture conditions. Id2^{-/-} mice displayed a selective and remarkable reduction of the CD8 α ⁺ DC subset, which is known to induce preferential T_H1 differentiation.

Conclusion: Id2 is an indispensable regulator of the T_H1/T_H2 balance, possibly through the proper development of CD8 α ⁺ DCs, and could be a novel target to treat allergic diseases. (J Allergy Clin Immunol 2003;111:136-42.)

Key words: Id2, basic helix-loop-helix factor, T_H1, T_H2, dendritic cell

During immune responses, naive CD4⁺ T cells differentiate into at least 2 types of effector cells, which are

Abbreviations used

bHLH: Basic helix-loop-helix
DC: Dendritic cell
NK: Natural killer
PE: Phycoerythrin

characterized by their distinct patterns of cytokine expression: T_H1 cells secrete IFN- γ , which promotes predominantly cell-mediated immunity, whereas T_H2 cells produce IL-4, IL-5, IL-10, and IL-13 and promote humoral immunity characterized by IgE production.^{1,2} This dichotomy of T_H cell differentiation determines the fundamental pathologic features of many disease conditions, such as infection, autoimmune diseases, and allergy.^{1,2} It has been well established that the T_H1/T_H2 balance is controlled by cytokines. In particular, IL-12 and IFN- γ facilitate T_H1 development, whereas IL-4 promotes the generation of T_H2 cells.^{1,2} Furthermore, T_H1 and T_H2 cells interfere with each other in the polarization of the T-cell response.^{1,2} However, the regulation of the T_H1/T_H2 balance in vivo is more complicated because of cross-talk among various cell populations in the immune system.

Dendritic cells (DCs) have been well documented to be the most potent antigen-presenting cells for activating naive T cells.³⁻⁶ Their potency is ascribed to their high expression of costimulatory molecules, as well as MHC class I and class II molecules.³⁻⁶ DCs affect the quality of the T-cell response. In mice CD11c⁺ DCs have been classified into at least 2 populations, CD8 α ⁺ and CD8 α ⁻, and the CD8 α ⁻ subset is further divided into CD4⁺ and CD4⁻ cells.⁷ The CD8 α ⁺ DCs effectively prime naive CD4⁺ T cells and preferentially induce T_H1 differentiation in vivo, whereas the CD8 α ⁻ DCs induce T_H2 differentiation.⁵⁻⁸ CD8 α ⁺ DCs can produce higher amounts of IL-12, a key cytokine for T_H1 development, than CD8 α ⁻ DCs, and CD8 α ⁺ DCs isolated from mice deficient in IL-12 show remarkably reduced T_H1-inducing activity.⁸ Another DC subset that displays characteristics of human plasmacytoid DCs has just recently been identified in the mouse.⁹⁻¹¹ Although CD8 α ⁺ and CD8 α ⁻ DCs are thought to be of lymphoid and myeloid origin, respectively,⁴⁻⁶ the origin and differentiation pathway of DC subsets have still not been completely clarified. Recent studies favor the idea that the development and function of DCs are modulated by exogenous factors.¹²⁻¹⁶

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Id proteins are inhibitors of basic helix-loop-helix (bHLH) transcription factors that are crucial for various cell-differentiation processes, such as hematopoiesis and myogenesis, of multicellular organisms.^{17,18} In general, Id proteins heterodimerize with and sequester ubiquitously expressed bHLH factors, so-called E proteins consisting of HEB, E2-2, and E2A gene products, and this interaction inhibits tissue-specific bHLH factors from forming functional heterodimerized transcription factors.¹⁷⁻¹⁹ Id2 is a member of the family of Id proteins, and we have reported that Id2^{-/-} mice lack lymph nodes and Peyer patches and exhibit a greatly reduced population of natural killer (NK) cells, although their splenic architecture is normal.²⁰

We show that Id2^{-/-} mice are in a spontaneous T_H2-dominant state and exhibit a remarkable and specific reduction of splenic CD8⁺ DCs and hepatic CD11b^{low} DCs in Id2^{-/-} mice but not of the other DC subsets. Our results suggest that Id2 is required for the development of the CD8⁺ DC subset, which plays an important role in regulating the T_H1/T_H2 balance.

METHODS

Mice

Id2 mutant mice²⁰ were of the 129/Sv genetic background and were reared under specific pathogen-free conditions. Id2^{+/-} mice displayed characteristics similar to those of wild-type litter mates in all experiments conducted unless otherwise specified. All animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Kyoto University Graduate School of Medicine and Graduate School of Biostudies and Fukui Medical University.

Determination of serum levels of IgE and IgG subclasses

Total IgE and IgG subclasses in sera were determined by means of ELISA with capture antibodies for IgE (MCA419; Serotec Ltd, Oxford, United Kingdom), IgG1 (A83-3), IgG2a (R11-89), or IgG2b (R9-91) and biotinylated detection antibodies for IgE (MCA420B, Serotec), IgG1 (A85-1), IgG2a (R19-15), or IgG2b (R12-3), with standard immunoglobulins of IgE (M-IgE; Yamasa Shoyu, Tokyo Japan), IgG1 (A112-2), IgG2a (G155-178), or IgG2b (49.2). After incubation with poly-horseradish peroxidase-labeled streptavidin 20 (Research Diagnostics, Flanders, NJ), the signal was detected by using 3,3',5,5'-tetramethyl benzidine (Wako Pure Chemicals, Osaka, Japan). All the capture antibodies, standard antibodies, and detecting antibodies for IgG subclasses were purchased from BD-PharMingen (San Diego, Calif) unless otherwise noted.

RT-PCR

Total RNA was extracted from whole splenocytes or purified DC subsets by using Trizol (Gibco/BRL, Grand Island, NY) and was reverse transcribed with oligo d(T)₁₂₋₁₈. The RT products were then amplified by means of PCR for 30 cycles. Each cycle consisted of incubation at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. Primers used for PCR were designed according to the sequence deposited in the GenBank. The primer sequences are available on request. Reduced glyceraldehyde-phosphate dehydrogenase or β -actin was used as an internal loading control. For semi-quantitative RT-PCR analysis, RT product mixture derived from 250 ng of total RNA was serially diluted by a factor of 5 and amplified

in a reaction mixture of 30 μ L with the program indicated above. Half of each RT-PCR product was electrophoresed on a gel. For reduced glyceraldehyde-phosphate dehydrogenase, 5 ng of total RNA was used as the starting material. CD4 instead of CD8 α was used to examine the purity of the DC subsets because the gene expression of CD8 α is detected even in CD8 α ⁻ DCs by means of RT-PCR in spite of its lack of detectable expression at the protein level (data not shown).

Purification of naive CD4⁺ T cells

Splenocytes were treated with a mixture of culture supernatants of anti-human serum albumin (J11D), Fc γ R2/III (2.4G2), and CD8 (H0-2.2) hybridomas and purified monoclonal antibodies (mAbs) against B220 (RAS-6B2; Caltag Laboratories, Burlingame, Calif), Mac-1 (M1.70; Immunotech, Marseilles, France), and pan-NK (DX5, BD-PharMingen). They were incubated in anti-rat IgG (Caltag Laboratories)-coated culture dishes for 1 hour at 37°C to collect unbound cells as CD4⁺ T cells. Then L-selectin (CD62L^{high}) naive T cells were enriched with MACS by using anti-L-selectin antibody (Caltag Laboratories) and anti-rat IgG magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of naive CD4⁺ T cells was reproducibly greater than 95% (data not shown).

Intracellular staining of cytokines

Intracellular cytokine staining was performed as described previously²¹ by using a Cytotfix/Cytoperm Plus Kit (BD-PharMingen). Freshly prepared splenocytes or splenocytes cultured for 4 days in the presence of anti-CD3 mAb were subjected to intracellular cytokine staining. T_H1 or T_H2 cells from naive CD4⁺ T cells were selectively induced for 6 days with 5 ng/mL IL-12 (Genzyme, Cambridge, Mass) plus 10 μ g/mL anti-IL-4 mAb (Endogen, Woburn, Mass) or 50 ng/mL IL-4 (Genzyme) plus 10 μ g/mL anti-IFN- γ mAb (Endogen), respectively, in the presence of 5 ng/mL IL-2 (Genzyme) in RPMI 1640 medium supplemented with 10% FCS and 50 μ mol/L 2-mercaptoethanol in tissue-culture plates precoated with 5 μ g/mL anti-CD3 mAb. These cells were subjected to intracellular cytokine staining.

Preparation and analysis of DCs

For subset analysis, spleen and hepatic leukocytes were prepared by means of collagenase (Boehringer-Ingerheim, Indianapolis, Ind) digestion. Low-density cells were separated with BSA gradient centrifugation (Sigma-Aldrich, St Louis, Mo) for splenocytes and with Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient for hepatic leukocytes, which were subjected to flow cytometric analysis, as previously described.²² The cells were stained with FITC-CD11c in combination with phycoerythrin (PE)-CD8 α for spleen or PE-CD11b for liver, followed by either biotinylated B220 or CD4 plus avidin-conjugated Cy-Chrome (BD-PharMingen). The DCs were acquired as CD11c⁺ by using a FACSCalibur flow cytometer and analyzed with CellQuest software (Becton Dickinson Immunocytometry & Cellular Imaging, San Jose, Calif). The numbers of individual DC subsets in the spleen and liver were calculated on the basis of the cell yield and recovery of cell preparations and proportional analysis by means of flow cytometry.

For RT-PCR analyses, spleen DC subsets were purified from low-density cells. In brief, the cells were depleted of cells expressing B220⁺ Thy1⁺ and CD3⁺ by using MACS columns (Miltenyi Biotec). Subsequently, CD11c⁺ cells were positively selected by using FITC-CD11c (BD-PharMingen) and anti-FITC microbeads (Miltenyi Biotec). Then the cells were stained with PE-CD8 (BD-PharMingen), and CD11c⁺CD8⁺ and CD11c⁺CD8⁻ DCs were purified by means of sorting with an EPICS ALTRA flow cytometer (Coulter Corp, Hialeah, Fla).

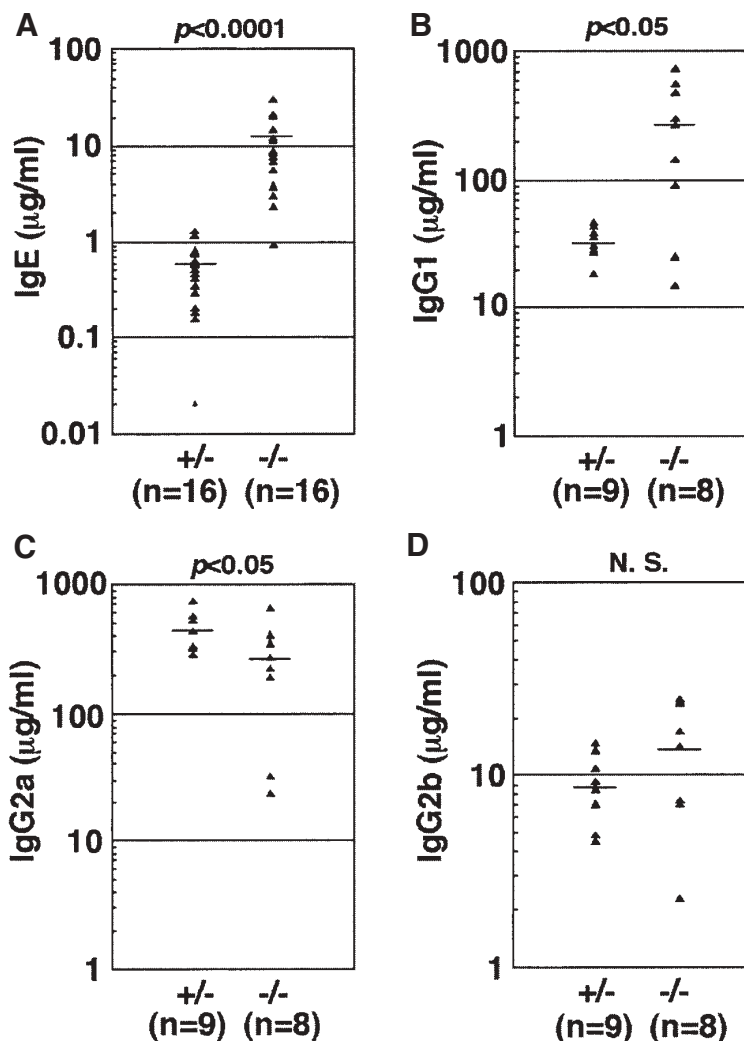


FIG 1. Serum levels of IgE and IgG subclasses in $Id2^{-/-}$ and control mice. Sera were obtained from $Id2^{-/-}$ and control mice aged 10 to 20 weeks, and the levels of IgE (**A**) and the IgG subclasses IgG1 (**B**), IgG2a (**C**), and IgG2b (**D**) were measured by means of ELISA. The numbers of samples are indicated at the bottom of each panel. N.S., Not significant.

Statistical analysis

Differences between groups were analyzed by using the Mann-Whitney *U* test. *P* values of less than .05 were considered statistically significant.

RESULTS

Increased serum levels of T_H2 -mediated immunoglobulins in $Id2^{-/-}$ mice

To evaluate the immunologic status of $Id2^{-/-}$ mice, we analyzed serum levels of IgE and IgG subclasses in $Id2^{-/-}$ mice by means of ELISA. The levels of IgE and IgG1 were substantially increased (by approximately 20-fold and 10-fold, respectively) in $Id2^{-/-}$ mice compared with those in control mice (Fig 1, **A** and **B**). In contrast, the level of IgG2a in the sera of $Id2^{-/-}$ mice was lower than that in control mice (Fig 1, **C**; $P < .05$), and the level of IgG2b did not differ significantly between these 2 groups

(Fig 1, **D**). These data indicated that levels of the T_H2 -mediated (IL-4-dependent) immunoglobulins IgE and IgG1 are increased in $Id2^{-/-}$ mice, whereas levels of the T_H1 -mediated (IFN- γ -dependent) immunoglobulin IgG2a is decreased. Thus $Id2^{-/-}$ mice seemed to be in a T_H2 -dominant state.

Dominant expression of T_H2 -related genes in the spleens of $Id2^{-/-}$ mice

We next examined the expression of various T_H1 or T_H2 response-related genes in spleens by means of RT-PCR after immunization with sheep red blood cells (Fig 2). IL-4 was expressed more abundantly in $Id2^{-/-}$ spleen cells than in $Id2^{+/+}$ spleen cells. The expression of IL-6 and IL-10, other T_H2 -related cytokines, was also enhanced in $Id2^{-/-}$ splenocytes. In contrast, the expression of IFN- γ , a signature cytokine of the T_H1 response, was hardly detected in $Id2^{-/-}$ splenocytes. Consistent

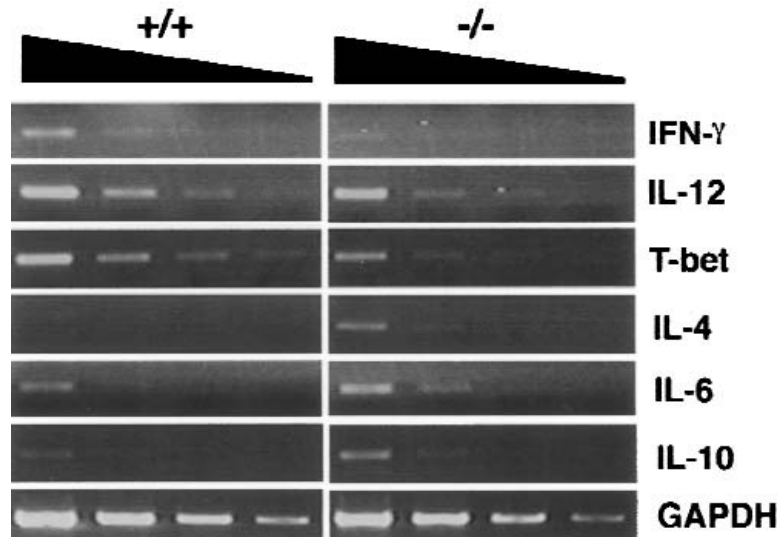


FIG 2. Expression of T_H1 - or T_H2 -related genes in the spleen. RNA was purified from the spleen 4 days after intraperitoneal injection of 1×10^8 sheep red blood cells and was reverse transcribed. RT products were serially diluted by a factor of 5, as indicated, and subjected to PCR. Reduced glyceraldehyde-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Representative results of at least 3 mice in each genotype are shown.

with this, $Id2^{-/-}$ splenocytes also showed lower expression of IL-12, an important cytokine for the T_H1 response, and T-bet, a critical transcription factor for IFN- γ expression, than did $Id2^{+/+}$ splenocytes.²³ No significant difference was detected in the expression of signal transducer and activator of transcription 6, IL-5, IL-18, and GATA-3 (data not shown). These findings demonstrated that the T_H2 response dominates in the $Id2^{-/-}$ mouse spleen.

Skewed differentiation of $Id2^{-/-}$ naive $CD4^+$ T cells to T_H2 is not intrinsic to $CD4^+$ T cells

Next we analyzed the balance between T_H1 and T_H2 cells in the spleen by means of intracellular cytokine staining of $CD4^+$ T cells without prior immunization. A significantly lower percentage of IFN- γ^+ T_H1 cells was observed in $CD4^+$ T cells from $Id2^{-/-}$ mice than in those from control mice, whereas the percentage of IL-4 $^+$ T_H2 cells was increased in $Id2^{-/-}$ splenocytes (Fig 3, A). We then stimulated splenocytes with anti-CD3 antibody for 4 days in vitro and examined their cytokine-producing profiles (Fig 3, B). Again, fewer IFN- γ^+ and more IL-4 $^+$ $CD4^+$ T cells were detected in $Id2^{-/-}$ splenocytes than in $Id2^{+/+}$ splenocytes. These data indicate that splenic $CD4^+$ T cells in $Id2^{-/-}$ mice are skewed to differentiated T_H2 . However, when CD62L^{high} naive $CD4^+$ T cells were purified from $Id2^{-/-}$ splenocytes and stimulated with anti-CD3 antibody in the presence of IL-2 under either T_H1 - or T_H2 -inducing conditions, they differentiated into both T_H1 and T_H2 cells, depending on the culture conditions, similarly to those from control mice (Fig 3, C). These data showed that the apparently polarized T_H2 differentiation in $Id2^{-/-}$ mice in vivo is not ascribable to a defect intrinsic to $CD4^+$ T cells and suggested that the

physiologic milieu surrounding $CD4^+$ T cells in situ is responsible for the polarization of T_H development in $Id2^{-/-}$ mice.

Remarkable decrease in $CD8^+/CD11b^{low}$ DCs in spleen and liver

Macrophages are known to be one of the major sources of IL-12,²⁴ and they might be the cause of T_H2 dominance in $Id2^{-/-}$ mice. However, there was no significant difference of IL-12 production of thioglycollate-induced peritoneal macrophages between $Id2^{-/-}$ and wild-type macrophages in response to LPS and IFN- γ (data not shown). One possibility that remained to be examined was whether there are functional defects or alteration of proportions of DC subsets in $Id2^{-/-}$ mice because DCs act as the major antigen-presenting cells in the induction of immune responses, and phenotypically distinguishable DC subsets have been demonstrated to play a role in skewing the immune response.^{3-6,8,25,26} Therefore we examined the phenotype of $CD11c^+$ DCs in the spleen. There were no significant differences in the levels of expression of MHC class II or class I molecules, CD40, CD54, CD80, or CD86 on $CD11c^+$ cells among $Id2^{-/-}$, $Id2^{+/-}$, and $Id2^{+/+}$ mice (data not shown). However, a remarkable decrease in $CD8^+$ DCs was observed in $Id2^{-/-}$ mice (Fig 4, A). Therefore we further examined the proportion and cell number of each DC subset in individual mice. Only $CD8^+$ DCs were decreased in proportion and number; the other DC subsets, such as $CD8-CD4^+$, $CD8-CD4^+$ DCs, or $CD11c^{int}$ B220 $^+$ plasmacytoid DC precursor cells, were not (Fig 4). Similar results were obtained in the liver, where $CD11b^{low}$ DCs, a subset corresponding to splenic $CD8^+$ DCs, were dramatically reduced in number, but $CD11b^{high}$ DCs were not (Fig 4).

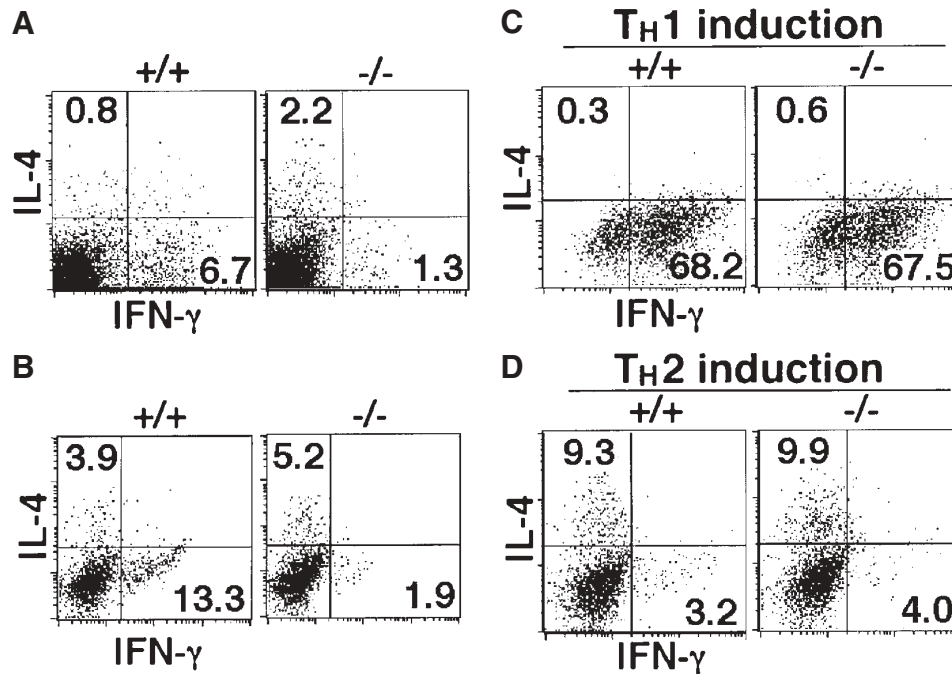


FIG 3. CD4⁺ T-cell subsets and differentiation ability of naive CD4⁺ T cells. **A**, Cytokine profile of splenic CD4⁺ T cells after a 4-hour stimulation of freshly prepared whole splenocytes with phorbol 12-myristate 13-acetate and ionomycin. **B**, Cytokine profile of CD4⁺ T cells after culturing whole splenocytes with anti-CD3 mAb for 4 days. **C**, Differentiation of naive CD4⁺ T cells in vitro. Intracellular cytokine staining was performed after culturing naive CD4⁺ T cells under T_H1- or T_H2-favoring conditions for 6 days. Representative results of at least 4 independent experiments are shown in each panel.

Gene-expression analysis of Id proteins determined by means of RT-PCR

To explore the gene-expression profiles specific to the CD8α⁺ DC subset with respect to Id proteins, we performed RT-PCR analysis by using RNA purified from CD8α⁺ and CD8α⁻ DC subsets of the wild-type spleen (Fig 5). Id2 was readily detectable in both the CD8α⁺ and CD8α⁻ DC subsets, and Id3 was found to be expressed weakly only in the CD8α⁺ DC subset. Id1 and Id4 were not detected in either DC subset under the conditions used in this study. These results indicate that Id2 is abundantly expressed in both splenic CD8α⁺ and CD8α⁻ DC subsets, although we expected its specific expression in the CD8α⁺ DC subset.

DISCUSSION

In this article we have shown that Id2^{-/-} mice are in a T_H2-dominant state and have a selective defect in the CD8⁺ DC subset. The T_H2 dominance in the mutant mice was shown by examining serum immunoglobulin levels and verified with gene-expression analysis in the spleen and intracellular staining of cytokines in splenic CD4⁺ T cells. We initially speculated that Id2 regulates T_H1/T_H2 differentiation at the T_H0 level as an intrinsic factor. However, purified naive CD4⁺ T cells of Id2^{-/-} mice differentiated normally to both T_H1 and T_H2 cells under appropriate culture conditions. We then analyzed antigen-presenting cells to determine whether they play a role in the T_H2 dominance in Id2^{-/-} mice. Although we failed to detect any differences

in macrophage counts, flow cytometric analysis revealed a remarkable reduction in the number of CD8α⁺ DC subset cells of Id2^{-/-} mice. Other DC subsets were present in numbers and percentages similar to those of control mice. Expression of Id2 in CD8α⁺ DCs was confirmed by means of RT-PCR. Our current study has thus demonstrated that Id2 is indispensable for proper differentiation control of T_H cells and for CD8α⁺ DC development in vivo.

Several lines of recent evidence lead us to consider it probable that the T_H2 dominance observed in Id2^{-/-} mice is caused, at least in part, by the defect in CD8α⁺ DCs. It has been demonstrated that distinct DC subsets participate in T_H1 and T_H2 cell development.^{4,5,8,25,26} In particular, CD8α⁺ DCs produce a large amount of IL-12, a crucial cytokine for T_H1 development, and prime naive CD4⁺ T cells, leading to T_H1 differentiation.^{8,25,26} T_H1 development can be induced in vivo by administration of antigen-loaded wild-type CD8α⁺ DCs into syngeneic mouse footpads, whereas those from IL-12^{-/-} mice fail to do so, showing that the T_H1-inducing activity of CD8α⁺ DCs is IL-12 dependent.²⁶ Therefore it is conceivable that the defect in CD8α⁺ DCs is responsible for the T_H2 dominance in Id2^{-/-} mice. It would be very interesting to see whether the transfer of CD8α⁺ DCs into Id2^{-/-} mice would correct their T_H1/T_H2 imbalance.

Do Id2^{-/-} mice contain any other factors that might cause the shift to T_H2 dominance? Id2^{-/-} mice exhibit a greatly reduced number of NK cells.²⁰ NK cells produce a large amount of IFN-γ in an IL-12-dependent manner, and

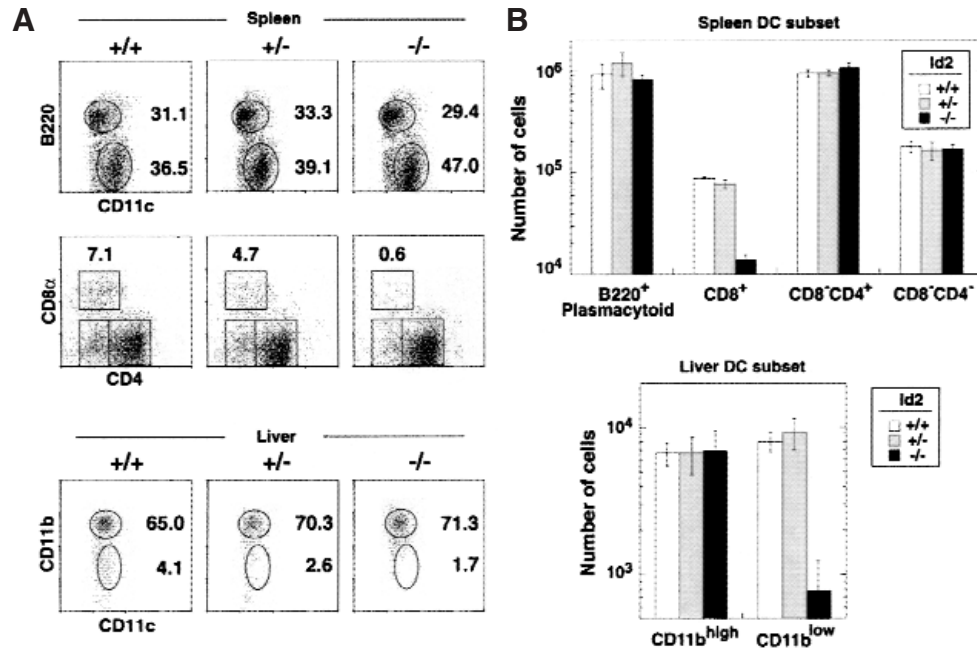


FIG 4. Remarkable decrease in CD8 α ⁺ DCs in the spleens and corresponding CD11b^{low} DCs in the livers of Id2^{-/-} mice. **A**, Spleen DC subsets (top 2 rows) and liver DC subsets (bottom row). The percentage of each subset is indicated. The results shown here are representative of those from 4 mice in each group. **B**, The numbers of individual DC subsets in the spleen and liver. Results are shown as means $\pm$ SD of 4 individual mice for the spleen and 3 experimental analyses of 2 independent experiments with pooled nonhepatic leukocytes of 2 mice each for the liver.

IFN- γ is a key factor directing the differentiation of naive CD4⁺ T cells into T_H1 cells.²⁷ Thus the decreased NK cell population is one of the potential causes of the skewed T_H2 development in Id2^{-/-} mice. Recent reports, however, have suggested that NK cells contribute to T_H1 development to a relatively small extent.²⁸⁻³⁰ Enhanced class switching favoring T_H2-mediated immunoglobulins in Id2^{-/-} B cells is another possible mechanism underlying the increased serum levels of IgE and IgG1. However, skewing is obvious at the level of CD4⁺ T cells, and Id2^{-/-} mice show normal distributions of B-cell populations in the bone marrow and spleen (Sugai et al, unpublished observations).

The molecular mechanisms underlying the developmental defect of the CD8 α ⁺ DC subset in Id2^{-/-} mice remain to be determined at present because Id2 expression was observed in both CD8 α ⁻ and CD8 α ⁺ DCs. Although the developmental pathways of the respective DC subsets have not yet been conclusively elucidated, CD8 α expression seems to be a maturation or differentiation stage marker rather than a lineage marker distinguishing lymphoid-related DCs from myeloid-related DCs.^{12-16,31,32} As in the situation of NK cell development,³³ Id2 might inhibit a bHLH factor and thereby promote the transition from the CD8 α ⁻ stage to the CD8 α ⁺ stage of DC maturation or differentiation. On the other hand, the functional relationship between Id2 and factors required for DC development,³⁴⁻³⁹ such as RelB, is unclear.

In human subjects retrovirus-mediated gene transduction of Id2 or Id3 into hematopoietic stem cells strongly inhibits the development of plasmacytoid pre-DC-2 with-

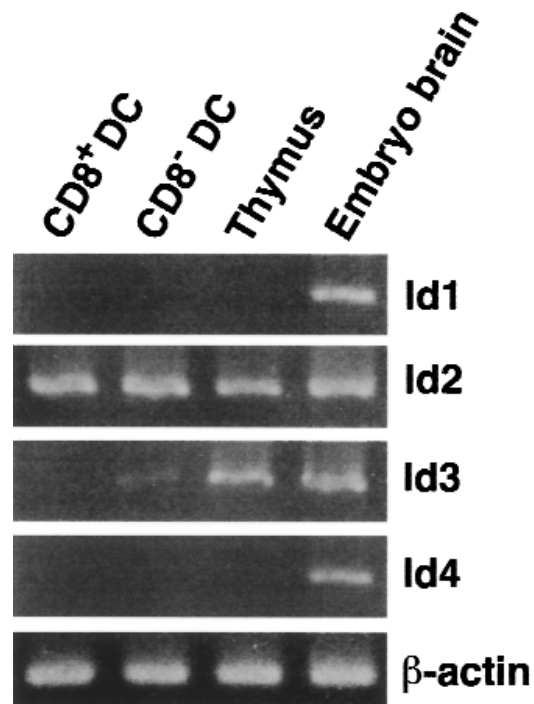


FIG 5. RT-PCR analysis of gene expression of Id proteins in DC subsets. RNA sources are indicated. RNA of embryonic brain at embryonic day 14 and adult thymi served as positive controls. β -Actin was used as a loading control. Representative results of 2 independent RNA sources are shown.

out affecting that of CD11c⁺ pre-DC-1, suggesting a role for bHLH factors in the regulation of DC subset development.⁴⁰ However, Id2^{-/-} mice show normal development of B220⁺ DCs, which correspond to human plasmacytoid pre-DC-2. Thus at least in mice, Id2 is dispensable for the development of this particular DC subset.

From the clinical point of view, the spontaneous Th2-biased and hyper-IgE phenotype of Id2^{-/-} mice shown here has a significant similarity to the situation seen in atopic human subjects. The mouse thus might be a useful model to elucidate the mechanisms underlying the atopic state, especially focusing on the role of DCs. Both genetic and environmental factors contribute to the development of allergic diseases.⁴¹ Thus far, the mutant mice have been reared under specific pathogen-free conditions and showed no detectable allergic symptoms. It will be interesting to see whether any such symptoms occur on specific antigenic stimulation, environmental stimulation, or both.

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