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Selective Defects in the Development of the Fetal and Adult Lymphoid System in Mice with an Ikaros Null Mutation

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Summary

Mice homozygous for an Ikaros null mutation display distinct defects in the development of fetal and adult lymphocytes. Fetal T lymphocytes, and fetal and adult B lymphocytes and their earliest progenitors are absent. Postnatally, hematopoietic stem cells give rise to thymocyte precursors that undergo aberrant differentiation into the CD4 lineage and clonal expansion. The lack of NK cells and some γδ T cell subsets and a large reduction in thymic dendritic APCs suggest that Ikaros is essential for establishing early branch points in the postnatal T cell pathway. The lymphoid defects detected in Ikaros null mice reveal critical molecular differences between fetal and postnatal hematopoietic progenitors that dictate their ability to give rise to T cells. These studies also establish *Ikaros* as a tumor suppressor gene acting during thymocyte differentiation. Phenotypic comparison of this null mutation with a severe dominant-negative Ikaros mutation identifies molecular redundancy in the postnatal hemolymphoid system.

Introduction

The molecular mechanisms that control lineage commitment and differentiation in the hemo-lymphopoietic system are being elucidated. In part, these mechanisms involve transcription factors that mediate selective changes in gene expression required for the developmental restriction of a pluripotent hematopoietic stem cell (HSC) to one or more of its lineages (Clevers and Grosschedl, 1996). Ikaros, a member of the Kruppel family of zinc finger DNA-binding proteins, was described as a master regulator for the specification of all lymphoid lineages. A mutation that deletes the N-terminal zinc finger DNA-binding domain from the Ikaros proteins blocks lymphocyte development at its earliest recognizable stage (Georgopoulos et al., 1994). Mice homozygous for this mutation (DN−/−) lack mature T and B lymphocytes and natural killer (NK) cells as well as their earliest described precursors during both fetal and adult life. In sharp contrast, mice heterozygous for this Ikaros mutation are born with apparently normal lymphocyte populations, but their thymocytes display augmented proliferative responses. These mice rapidly develop lymphoproliferative disorders and ultimately die of T cell leukemias and lymphomas manifested in 100% of the heterozygous population (Winandy et al., 1995).

The *Ikaros* gene encodes by means of alternate splicing a family of zinc finger transcription factors expressed only in the fetal and adult hemo-lymphoid system and in the developing corpus striatum (Georgopoulos et al., 1992; Hahm et al., 1994; Molnar and Georgopoulos, 1994). The Ikaros proteins all share a common C-terminal domain with two zinc fingers attached to a domain with different combinations of one to four N-terminal zinc fingers. At least three N-terminal zinc fingers are required for the sequence-specific and high affinity DNA interactions of the Ikaros isoforms (Molnar and Georgopoulos, 1994). In addition, homodimerization and heterodimerization of Ikaros proteins through their C-terminal zinc fingers is essential for high affinity DNA interactions (Sun et al., 1996). Mutations that disrupt the structure of these C-terminal zinc fingers interfere with the ability of Ikaros proteins to bind DNA and prevent them from activating transcription from their cognate sequences (Sun et al., 1996). In addition, heterodimers consisting of an Ikaros isoform that has an intact DNA-binding domain and an Ikaros isoform that lacks a DNA-binding domain do not bind DNA and are transcriptionally inactive. Therefore, Ikaros proteins with fewer than three N-terminal zinc fingers can play a dominant-negative role in transcription by interfering with the activity of isoforms that can bind DNA (Sun et al., 1996).

Proteins generated by the *Ikaros* locus from which the DNA-binding domain has been deleted lack essential zinc finger motifs required for DNA interactions, but have an intact C-terminal zinc finger dimerization domain. In heterozygous lymphocytes, these Ikaros mutant proteins can sequester the DNA-binding isoforms made by the wild-type locus in transcriptionally inactive heterodimers and drastically reduce Ikaros activity (Sun et al., 1996; Winandy et al., 1995). These mutant Ikaros proteins may also interfere with the activity of other factors (e.g., the *Aiolos* gene) that share similar dimerization properties and work in concert with Ikaros during lymphocyte development (Morgan et al., 1996). In mice homozygous for the dominant-negative N-terminal Ikaros mutation, expression of Ikaros mutant proteins in multipotent progenitors may interfere with activity of such factors that work in concert with Ikaros to determine the subsequent fate of progenitors in lymphoid differentiation (Morgan et al., 1996).

To investigate the role of Ikaros protein interactions in the development of the hemo-lymphoid system, we targeted a different deletion at the C-terminus of the *Ikaros* gene. This deletion removes the last translated exon that includes domains involved in activation, dimerization, and other protein interactions (Sun et al., 1996; J. Koipally, unpublished data). The functionally

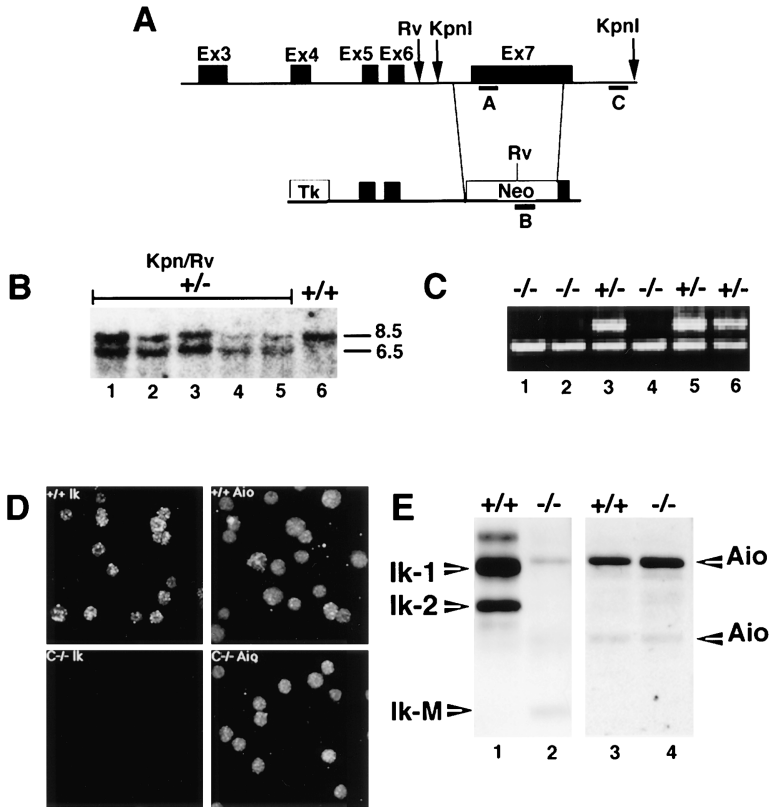


Figure 1. Functional Inactivation of the *Ikaros* Gene by Targeting Its Last Translated Exon

(A) Recombination strategy for targeting a deletion of a 1.35 kb genomic fragment encompassing the 5′ coding region of exon 7.

(B) Analysis of genomic DNA from six selected ES cell clones. The respective 8.5 kb and 6.5 kb KpnI and KpnI–EcoRV genomic fragments derived from the wild-type and targeted *Ikaros* alleles hybridized to probe A derived from a region outside the recombination locus. Single integration events of the recombination vector were determined using a neomycin-derived probe B (data not shown).

(C) PCR analysis of tail DNAs from a 2-week-old F3 ($-/- \times +/-$) litter revealed the occurrence of homozygous offspring at the expected Mendelian frequency.

(D) Confocal immunofluorescence analysis of Ikaros C$-/-$ and $+/+$ thymocytes for expression of Ikaros and Aiolos proteins. The nuclear punctate staining of Ikaros proteins is detected in wild-type but not in Ikaros C$-/-$ thymocytes. In contrast, the nuclear punctate Aiolos staining is observed in both.

(E) Western blot analysis of nuclear extracts prepared from the thymus of 2- to 4-week-old mice revealed that C$-/-$ mice (lane 2) lack wild-type Ikaros isoforms (Ik-1 and Ik-2) and have a truncated Ikaros protein (Ik-M) that is present in a ≥100-fold lower concentration than the wild-type Ikaros isoforms (Ik-1 and Ik-2) produced in $+/+$ mice (lane 1). The antibody used was raised to the N-terminal domain of Ikaros proteins. The two additional bands detected in the C$-/-$ thymocyte lysates (lane 2) are Aiolos proteins (Aio) that cross-react with Ikaros antibodies. Antibodies raised to a specific domain of Aiolos proteins not conserved in Ikaros detected only these two proteins, and not the Ikaros mutant form (lanes 3 and 4).

null proteins made by the mutant locus are unstable and not detected at the cellular level. Thus, this mutation produces a true Ikaros null phenotype. Both fetally derived B-1a and adult-derived conventional B cells are absent from Ikaros C-terminal mutant (C$-/-$) mice. However, fetal- and adult-derived T cell lineages are differentially affected. Throughout gestation and for the first days after birth, the thymus is devoid of thymocytes and any of their identifiable precursors. Definitive thymocytes are detected in the postnatal thymus between days 3 and 5 after birth. These thymocytes expand to reach nearly normal numbers in the adult. T cell progenitors in the Ikaros C$-/-$ neonatal thymus differentiate predominantly into conventional $\alpha\beta$ T cells and give rise to severely reduced numbers of adult-derived $\gamma\delta$ T cells. Consistent with the absence of fetal thymocyte development, no dendritic epidermal Vγ3 T cells can be detected in the Ikaros C$-/-$ mice. NK cells are also absent from these mice, and the numbers of intestinal intraepithelial $\gamma\delta$ T cells are reduced. Thymic dendritic antigen-presenting cells (APCs) are severely reduced, and mice lack lymph nodes. Furthermore, differentiation along the $\alpha\beta$ T lineage is not normal. Thymic T cell profiles are skewed toward $CD4^+CD8^-$ cells and cells in transition to this phenotype. Thymocytes display augmented T cell receptor (TCR)-mediated proliferative responses, and a few weeks after their appearance oligoclonal expansions are detected. In aging homozygotes, monoclonal populations take over the thymus and are exported to the periphery. In spite of the consistent defects in the B and T lymphoid compartments manifested in Ikaros C$-/-$ mice, normal to increased numbers of erythroid and myeloid cells are detected in the bone marrow and in the spleen. The phenotype of this Ikaros null mutation establishes the role of the *Ikaros* gene family in the lymphoid differentiation of fetal and postnatal HSCs.

Results

Deletion of the Last Translated Exon of the *Ikaros* Gene Leads to Its Functional Inactivation

To avoid the dominant-negative effects of the Ikaros proteins generated by the N-terminal DNA-binding domain deletion, we designed a vector to replace a 1.35 kb genomic fragment that contains the major part of the coding region of exon 7 including its 5′ splice donor site with the neomycin resistance expression cassette by homologous recombination (Figure 1A). This deletion disables utilization of exon 7 from the *Ikaros* transcript. Exon 7 encodes the C-terminal zinc finger dimerization domain required for interactions among Ikaros proteins and between Ikaros and Aiolos proteins (Morgan et al., 1996; Sun et al., 1996). The deleted domain also contains a bipartite activation domain essential for the ability of the Ikaros proteins to activate transcription (Sun et al., 1996). In vitro and in vivo studies with mutant Ikaros

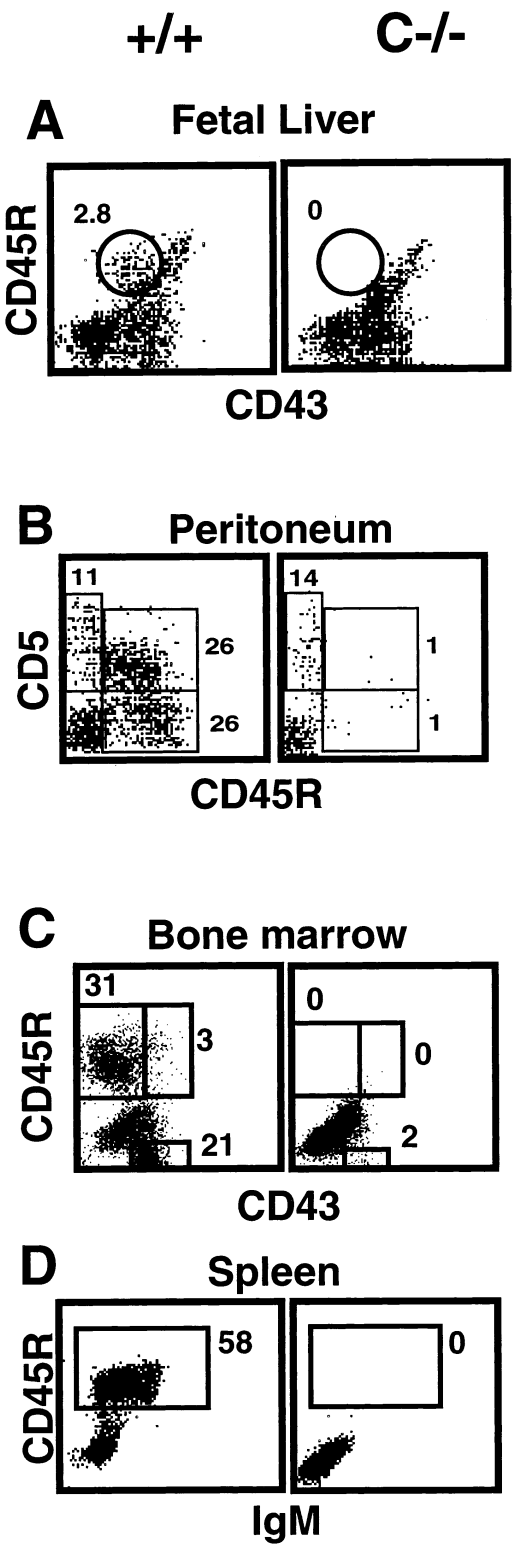


Figure 2. Both Fetal and Adult B Cell Development Are Blocked in Ikaros C −/− Mice

Cells obtained from the embryonic day 16 (E16) fetal liver (A) and peritoneum (B), bone marrow (C), and spleen (D) of 4-week-old Ikaros C−/− mice and wild-type littermates were analyzed with the following combinations of MAbs: anti-CD45R-PE/anti-CD43-FITC (A); anti-CD5-PE/anti-CD45R-FITC (B); anti-CD45R-PE/anti-CD43-FITC (C); anti-CD45R-PE/anti-IgM-FITC (D). Positively stained populations are boxed and percentages are shown. Fetal liver pre-B cell precursors (CD45R$^+$) and their progeny, peritoneal B-1a B cells (CD5$^+$/CD45R$^+$), were absent in Ikaros C−/− mice. Bone marrow pro-B cells and splenic B cells were also missing from all C−/− mice tested.

proteins in which the dimerization domain has been disrupted or which lack the last translated exon have shown that they are transcriptionally inactive and do not display dominant-negative effects on transcription (Sun et al., 1996; unpublished data).

The targeting vector was electroporated in the embryonic cell line J1 (Li et al., 1992). Two independent embryonic stem cell lines (whose DNA analyses are shown in Figure 1B) with legitimate homologous recombination events were used to generate mice with germline transmission for the C-terminal deletion. Homozygous C-terminal mutant mice were born with the expected Mendelian frequency and were indistinguishable from their wild-type littermates (Figure 1C). They live up to 4 months, and males can breed. Their longevity is in contrast with the Ikaros DNA-binding deletion mutants, most of which die during the first 3 weeks after birth (Georgopoulos et al., 1994).

Northern blot hybridization of RNA prepared from Ikaros C−/− thymocytes using an N-terminal *Ikaros* cDNA probe revealed decreased levels of a short 700–900 bp message that lacks both the coding and long untranslated region of exon 7 (data not shown). Immunohistochemical analysis of Ikaros C−/− thymocytes using antibodies raised to the N-terminal domain of the Ikaros proteins failed to reveal the characteristic punctate nuclear Ikaros staining or any staining above background (Figure 1D). Nevertheless, Ikaros C−/− thymocytes stained readily with an antibody to the Ikaros homolog Aiolos (Morgan et al., 1996). Wild-type Ikaros proteins (Ik-1 and Ik-2) were not detected in Western blots of C−/− thymocyte lysates. A short mutant variant was detected in Western blots of C−/− thymocyte lysates, but at 100-fold lower concentrations than wild-type Ikaros proteins present in wild-type thymocyte lysates (Figure 1E).

Both Fetal- and Adult-Derived B Cells Are Absent in Ikaros C−/− Mice

B cells and their precursors were absent during both the fetal and postnatal stages of development in Ikaros C−/− mice. Fetal liver B cell precursors (CD45R$^+$) were absent (Figure 2A). These cells normally give rise to B1-a B cells (Hardy et al., 1991, 1994; Kantor et al., 1992). Consistently, B1-a B cells (CD5$^+$/CD45R$^+$) were not detected in the peritoneum of adult homozygotes (Figure 2B). Pro-B cells (CD45R$^+$/CD43$^+$) and pre-B cells (CD45R$^+$) were absent from the bone marrow, and mature B cells (CD45R$^+$/IgM$^+$) were absent from the spleens of adult Ikaros C−/− mice (Figures 2B–2D) (Hardy et al., 1991; Li et al., 1993).

Fetal but Not Postnatal Waves of Thymocyte Differentiation Are Impaired in Ikaros C−/− Mice

The thymus in Ikaros C−/− mice was devoid of identifiable lymphoid precursors throughout fetal life and for

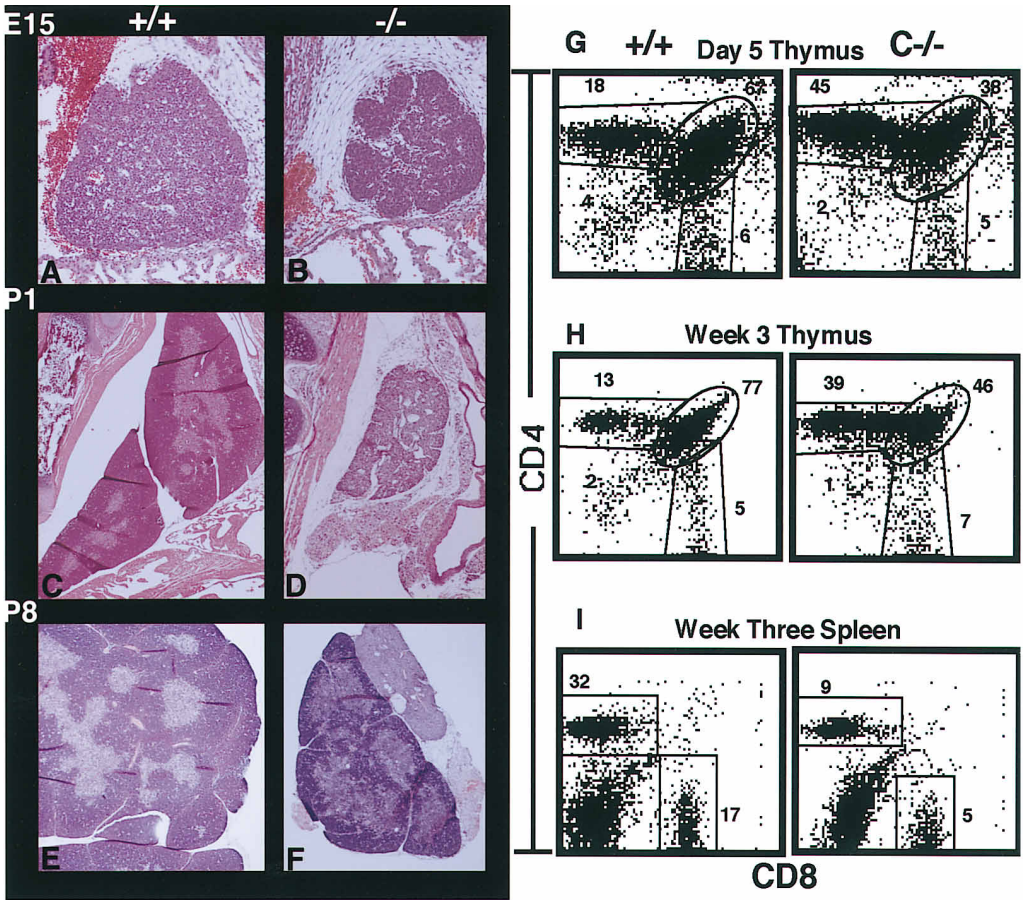


Figure 3. Fetal but Not Postnatal Waves of Thymocyte Differentiation Are Absent in Ikaros C−/− Mice

The structure of wild-type and Ikaros C−/− thymi is revealed by hematoxylin and eosin staining at 2–4× magnification. Thymocyte precursors are detected in the wild-type but not in the mutant thymus at E16 (A and B). A difference in size between the two thymi is already apparent at this early stage of thymocyte development. At birth, the thymus of a wild-type newborn has developed medullary (m) and cortical (c) structures indicative of a proliferating thymocyte compartment (C). In contrast, the Ikaros C−/− thymus remains similar in size to the fetal day 13–14 thymus and consists of epithelial elements (D). Cortical areas indicative of an expanding thymocyte precursor population are detected 1 week after birth in the Ikaros C−/− organ (F). A wild-type thymus at 1 week after birth is shown for comparison (E). In the adult, the Ikaros C−/− thymus can reach nearly normal size. Cytofluorometric analyses of 5-day-old and 3-week-old animals is shown (G and H). At 5 days after birth, Ikaros C−/− thymi contain 100- to 300-fold fewer thymocytes than age-matched wild-type thymi. An increase in the proportion of CD4 single positive thymocytes is detected even at this early stage in thymocyte development (G). The increase in the proportion of CD4 single positive thymocytes persists in the adult C−/−thymus in which the total number of thymocytes has reached nearly normal levels (H). Single positive CD4 and CD8 thymocytes are exported to the spleen (I). Thymocyte and splenic populations were stained with FITC- and PE-conjugated isotype control antibodies (data not shown) or anti-CD4-PE and anti-CD8-FITC. Positively stained populations are boxed and percentages are indicated.

the first few days after birth (Figures 3B and 3D). No cortical or medullary structures were discernible in the thymus of newborn homozygous mutants (Figure 3D). In all respects, the newborn Ikaros C−/− thymus was very similar in appearance to the day 13–14 fetal organ at the beginning of its development as a thymopoietic site. In sharp contrast, the thymus in wild-type newborns had formed elaborate cortical and medullary structures, which are indicative of an actively differentiating thymocyte compartment (Figure 3C).

Between 3 and 6 days after birth, thymocytes were detected in the Ikaros C−/− thymus, but at 100- to 300-fold lower numbers than in the age-matched wild-type organs (0.2×10^6 to 1×10^6 versus 0.5×10^8 to 1×10^8). The thymus in Ikaros C−/− mice began to develop cortical and medullary structures within 1 week after birth (Figure 3F). A dramatic increase in the number of Ikaros C−/− thymocytes was detected between weeks 2 and 6 postpartum. The number of thymocytes ranged from normal to 2- to 5-fold lower than wild type in the 4- to 6-week-old homozygotes.

In spite of their increase in numbers, Ikaros C−/− thymocytes do not follow a normal differentiation pathway. A larger proportion of $CD4^+CD8^-$ single positive cells was present in the Ikaros C−/− thymus than in the wild type (Figures 3G and 3H). CD4 single positive T cells accounted for up to 50% of the thymic population in these young animals, which had 50- to 100-fold fewer total thymocytes than their wild-type siblings (Figure 3G). This increase in the $CD4^+CD8^-/TCR^+$ population

Table 1. Response of T Cells to TCR Stimulation

Population	Phenotype	Cells per Well ($\times 10^5$)	[^{3}H]Thymidine Incorporation (SD)[a]	
			+/+	−/−
Thymocytes	Whole[b]	1.25	9,044 (446)	73,736 (2,766)
		0.63	4,299 (179)	34,743 (1,713)
		0.3	860 (670)	10,474 (2,684)
	$CD4^+CD8^-$[c]	1.25	10,665 (13,481)	127,470 (25,404)
		0.63	8,635 (4,833)	65,617 (6,139)
		0.3	9,461 (5,541)	28,872 (7,105)
	$CD4^-CD8^+$[c]	1.25	36,760 (1,148)	175,189 (26,250)
Spleen Cells	Whole[d]	2.5	51,561 (5,480)	52,874 (5,863)
		1.25	12,716 (822)	18,011 (1,859)
		0.63	3,765 (579)	8,480 (555)

[a] Duplicate samples of thymocytes or spleen cells were stimulated with plate-bound anti-TCR MAb (H57) and irradiated syngeneic APCs for 48 hr, pulsed for 4 hr with [^{3}H]thymidine, harvested, and counted. Background [^{3}H]thymidine incorporation (cells identically cultured with plate-bound hamster immunoglobulin and irradiated APCs) ranged from 100 to 775.
[b] The +/+ thymus contained 8.8% and 3.5% $CD4^+CD8^-$ and $CD4^-CD8^+$ cells, respectively, and the −/− thymus contained 31% and 3.4% $CD4^+CD8^-$ and $CD4^-CD8^+$ cells, respectively.
[c] $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were separated to 95% purity by sorting on a Coulter Elite.
[d] The +/+ and −/− spleens contained 22% and 10% T cells, respectively.
T cell populations were analyzed from 3-week-old animals.

was detected from the early points of thymocyte development to the adult and was accompanied by a concomitant decrease in the proportion of double positive thymocytes (Figures 3G and 3H). The proportion of CD8 single positive cells was similar to that found in wild type (Figures 3G and 3H).

The absolute number of T cells detected in the postnatal spleen of Ikaros C−/− mice was initially low but increased with age. In some cases, especially with animals of the C57BL/6 background, the absolute number of splenic T cells remained significantly reduced compared with wild type. The ratio of splenic CD4 to CD8 T cells varied from being similar to wild type to being increased by 2- to 3-fold (Figure 3I; data not shown).

We compared the ability of Ikaros C−/− and wild-type thymocytes and splenic T cells to proliferate when stimulated through their TCR. Ikaros C−/− thymocytes proliferated more than wild-type thymocytes upon stimulation of their TCR complex (Table 1). Both $CD4^+$ and $CD8^+$ Ikaros C−/− thymocytes displayed a high degree of proliferation.

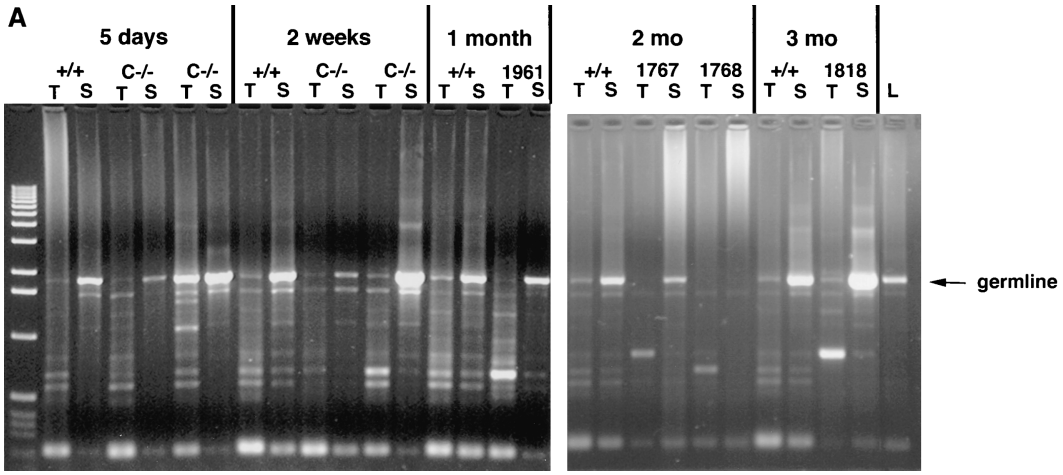
Clonal Populations Are Detected in the Early Ikaros C−/− Thymus and Predominate in the Adult Organ

Given that Ikaros C−/− thymocytes hyperproliferated upon TCR stimulation, we tested for the presence of clonally expanded thymocyte populations in Ikaros C−/− thymi. Dβ to Jβ segment rearrangements of the TCR β chain gene were examined in wild-type and mutant populations (Anderson et al., 1992). Seven Dβ2–Jβ2 rearrangements and one band corresponding to the germline configuration of the β chain gene were detected in the thymi and spleens of wild-type mice (Figure 4A). These results are consistent with the normal polyclonal nature of thymocytes and mature T cells in these organs. In contrast, certain Dβ2–Jβ2 rearrangements showed an increase in intensity in Ikaros mutant thymi as early as 5 days after birth, indicating expansion of certain thymocyte clones. A single but in each case different Dβ2–Jβ2 rearrangement predominated in the thymi of C−/− animals older than 4 weeks, reflecting the presence of an expanding thymocyte clone. These aberrant clonal T cell populations were detected at later timepoints in the spleens of older mutants.

The stage in thymocyte development during which Ikaros C−/− thymocyte clones began to expand was investigated by staining thymocytes with antibodies to CD4 and CD8 and a panel of Vβ-specific monoclonal antibodies (Figure 4B). Thymocyte clones with distinct TCR Vβ usage were seen in the majority of C−/− thymi. Expanded populations of thymocytes expressing a given TCR Vβ were detected in both the double positive ($CD4^+CD8^+$) and single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) windows indicating that their aberrant clonal expansion occurs as early as the immature double positive stage but these cells transit and further proliferate as single positive $CD4^+$ and $CD8^+$ T cells.

Selective Defects in γδ T Cells in Ikaros C−/− Mice

The number and distribution of γδ T cell subsets was of particular interest because of the lack of fetal thymocyte development in Ikaros C−/− mice. Dendritic epidermal T cells (DETC) expressing γδ TCR, which were readily identified in wild-type epidermis, were not detected in Ikaros C−/− mice (Figure 5A). No DETC were found in the epidermis of eight mutant mice analyzed even when probed for Thy-1 antigen expression (Figure 5A). In contrast, vaginal epithelial γδ T cells were present in a normal distribution and density in eight of eight mice examined (Figure 5B). Whereas thymic γδ T cells were present, albeit in decreased numbers, no significant population of γδ T cells was detected in the spleen of the adult Ikaros C−/− mice (Figures 5C and 5D). Intestinal intraepithelial lymphocytes (IELs) bearing γδ TCRs and expressing the CD8 αα coreceptor were absent or significantly reduced in six mice analyzed (Figure 5E). The number of $CD8^-$ γδ IELs was also drastically reduced



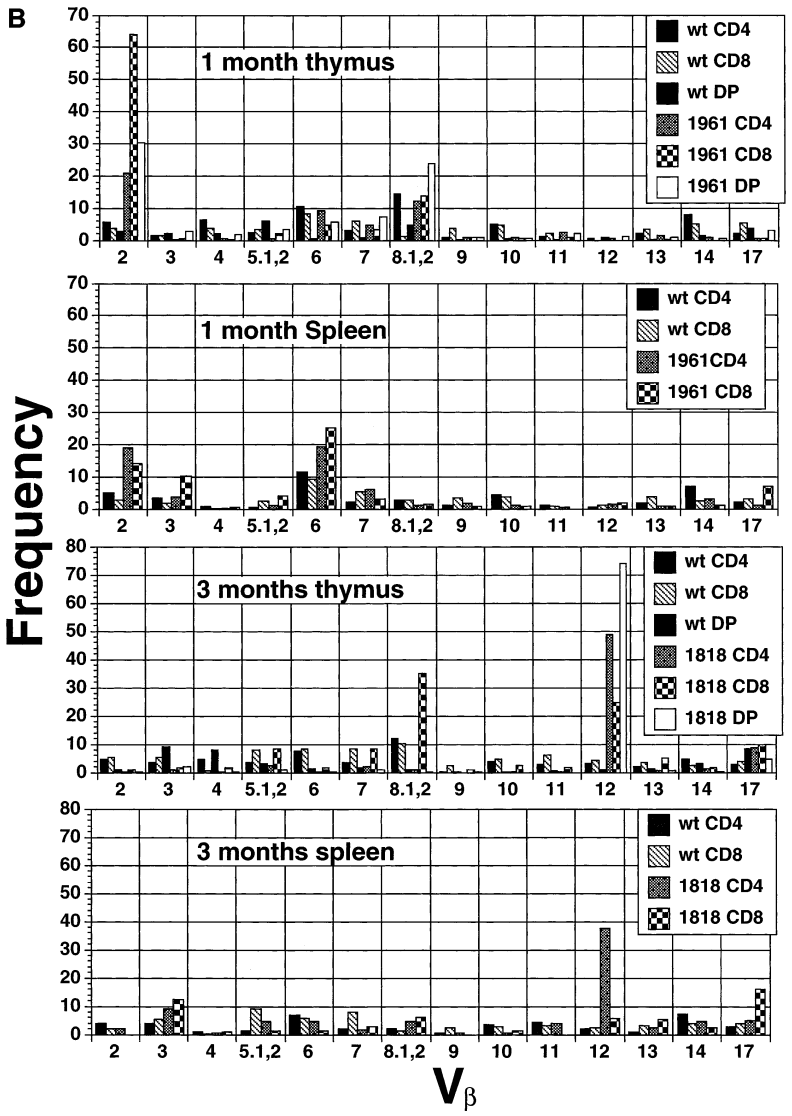


Figure 4. Clonal Expansion in the Maturing Ikaros C−/− Thymocyte Compartment

(A) PCR analysis for Dβ2–Jβ2 rearrangements using genomic DNAs. DNAs analyzed were from the thymus and spleen of wild-type (lanes marked with +/+) and mutant (lanes marked with C−/− or with numbers) animals harvested at 5 days to 3 months. S, spleen; T, thymus; L, wild-type liver. The increased intensity of the band corresponding to the germline configuration at the β chain locus in the spleen corresponds to the presence of non–T cell populations present in this organ. Molecular weight markers on the left are 1 kb DNA Ladder (GIBCO BRL).

(B) Flow cytometric analysis of Vβ usage in $CD4^+/CD8^+$ and $CD4^+$ and $CD8^+$ thymocyte and splenic populations of wild-type and Ikaros C−/− homozygotes.

(Figure 5E). In sharp contrast, the number of αβ IELs was similar in wild-type and C−/− mice (Figure 5E; data not shown). Epidermal and vaginal Langerhans cells identified by their class II expression were present in normal number and distribution in Ikaros C−/− mice (data not shown).

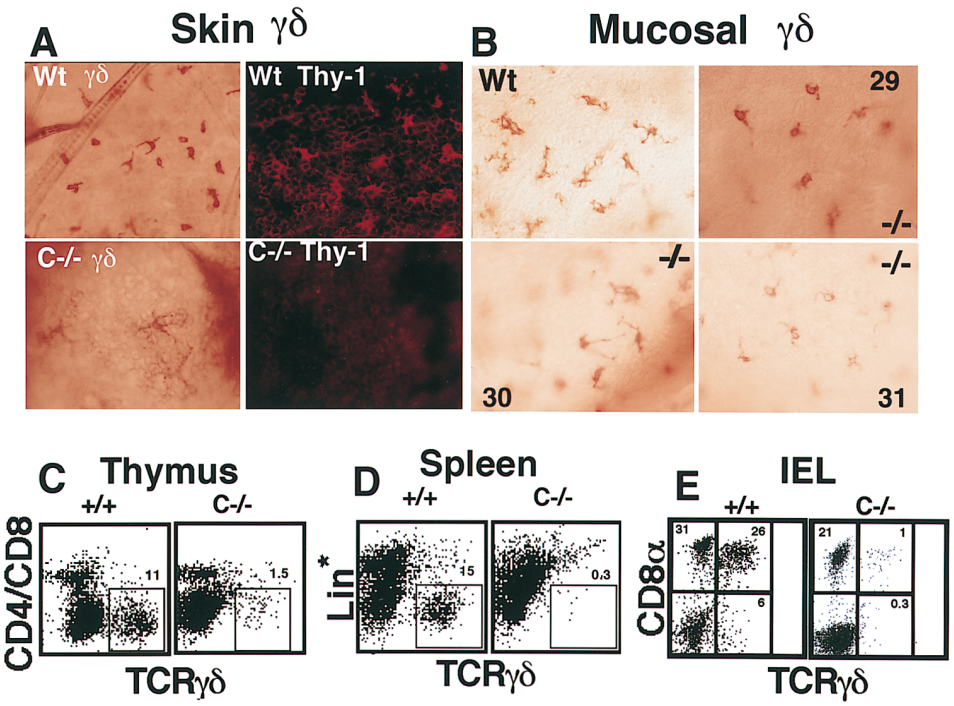


Figure 5. Selective Defects in the Development of γδ T Cells in Ikaros C−/− Mice

Epidermal (A) and vaginal (B) sheets of normal or Ikaros mutant mice were immunoperoxidase stained with anti-γδ MAb and examined by light microscopy or stained with PE-conjugated Thy-1 MAb and examined by epifluorescence microscopy. Skin γδ T cells were absent and mucosal γδ T cells were present in all of the mutant animals analyzed. Thymocyte (C) and splenic populations (D) depleted of $CD4^+$ and $CD8^+$ cells and lineage cells, respectively, were analyzed for their γδ T cell content. γδ T cells were detected in the thymus of mutant mice but at significantly reduced levels. They were not present in the spleen in any appreciable number. Intestinal intraepithelial lymphocytes (E) were analyzed for their αβ and γδ T cell composition. The great majority of Ikaros C−/− IELs were αβ T cells. No appreciable numbers of γδ T cells were present.

NK Cells and Thymic Dendritic APCs Are Absent or Significantly Reduced in C −/− Mice

A common lymphoid progenitor ($CD4^{lo}$/c-kit^+/$CD44^+$) present in the adult thymus may give rise to thymic dendritic APCs, NK cells, and the conventional αβ and γδ T cells (Ardavin et al., 1993; Matsuzaki et al., 1993; Rodewald et al., 1992; Wu et al., 1991a, 1991b, 1995). Thymic dendritic APCs as well as NK cells are derived from the earliest steps in the differentiation of this multipotent lymphoid progenitor. An alternative hypothesis is that these lineages arise from distinct thymic precursors that share a similar surface phenotype (Shortman and Wu, 1996). The presence of thymic dendritic APCs that express class II and CD11c antigens was examined in wild-type as well as in Ikaros C−/− thymi. After lineage depletion, thymic APCs ($CD11c^+$/class $II^{int/high}$) were highly enriched (51%) in the wild-type thymus (Figure 6A). In contrast, no cells with the $CD11c^+$/class II^{int} and very few cells (3%) with the $CD11c^+$/class II^{high} surface phenotypes were detected in the lineage-depleted mutant thymus (Figure 6A).

The presence of NK cells in wild-type and Ikaros C−/− mice was evaluated using an antibody to the NK1.1 marker on lineage-depleted splenocytes. A small population of NK1.1 cells was present among wild-type splenocytes (2%–5%, determined on the SV129 × C57BL/6 background). Cells that expressed NK 1.1 were not present among Ikaros C−/− splenocytes (Figure 6B). A functional assay was also used to address conclusively the existence of NK cells. Spleen cells from wild-type control mice effectively lysed chromium-labeled NK cell targets (Yac-1) over a wide range of effector to target ratios (Figure 6C). However, spleen cells from the Ikaros C−/− mice were unable to lyse NK targets even at the highest effector to target cell ratio (Figure 6C).

Development of thymic dendritic APCs and NK cells, which derive from the earliest branch point in the T cell differentiation pathway, is impaired in the absence of Ikaros activity. Ikaros C−/− mice also lack peripheral lymphatic centers. Inguinal, cervical, axial and mesenteric lymph nodes, Peyer's patches, and lymphoid follicles in the gastrointestinal tract were absent. Lymph nodes that appear during late fetal life rely on dendritic APCs for their proper development. Absence of an intact dendritic APC compartment in the Ikaros C −/− mice may account for the block in the development of their peripheral lymphatic centers.

Erythropoiesis and Myeloid Differentiation Are Relatively Unaffected during Both Fetal and Adult Development

Fetal liver HSCs give rise predominantly to cells of the erythroid and myeloid lineage. Both of these cell types were present in normal numbers in the mid- to late-gestation liver of the Ikaros C−/− fetus (Figure 7A). The spleen is a late fetal hematopoietic site that postnatally becomes populated with T and B lymphocytes but retains some of its hematopoietic potential throughout

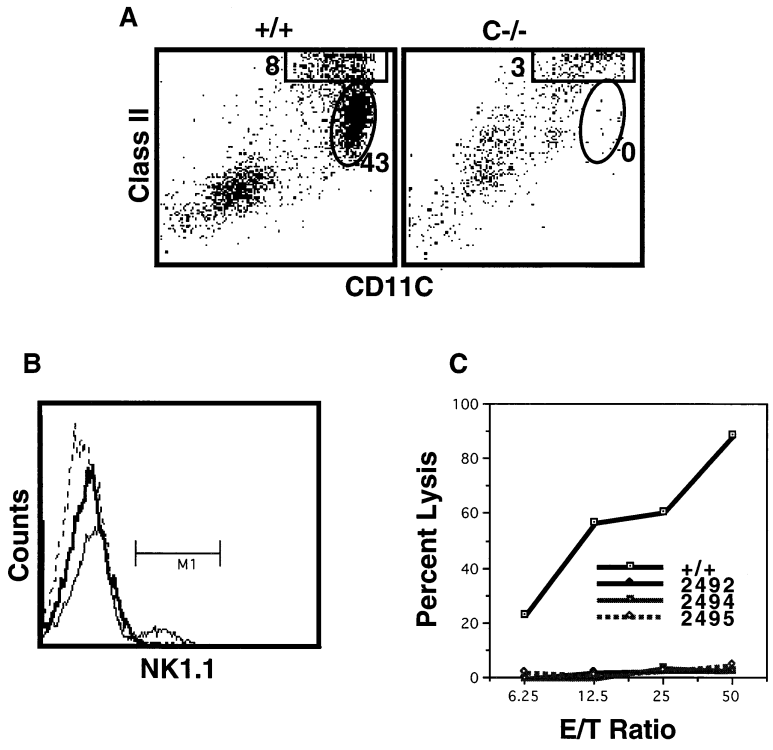


Figure 6. Ikaros C−/− Mice Lack NK Cells and Have a Defect in the Development of Thymic Dendritic APCs

Lineage-depleted splenocytes from Ikaros C−/− and wild-type mice were stained with an antibody to NK1.1, which is expressed on mature NK cells of the C57BL/6 background (A). Among Lin^- splenocytes in the wild type, 3%–5% were $NK1.1^+$. No $NK1.1^+$ cells were detected in the Ikaros C−/− spleen. The broken line histogram indicates the isotype control, whereas simple and bold line histograms show NK1.1 staining of the respective wild-type and Ikaros C−/− splenocytes. NK cell function in Ikaros C−/− and wild-type controls was tested by culturing splenocytes for 4 days in the presence of 500 U/ml of IL-2 (B). In wild-type mice, these conditions are known to generate activated NK cells that can readily lyse Yac-1 targets. Spleen cells from wild-type mice lysed chromium-labeled Yac-1 over a wide range of effector to target cell ratios. In contrast, spleen cells from Ikaros C−/− mice were unable to lyse NK targets even at the highest effector to target cell ratio. Lineage-depleted thymocytes from Ikaros C−/− and wild-type mice were stained with antibodies to class II and CD11c antigens expressed on mature dendritic APCs (C). $CD11c^+$/class $II^{int/high}$ APCs were absent from the Ikaros C−/− spleen. Interestingly $CD11c^+$/class II^{high} cells were present. These cells may represent a distinct class or state of maturation of APCs.

adult life. The major hematopoietic site in the adult is the bone marrow, where erythroid and myeloid precursors are generated. In both young and adult Ikaros C−/− mice, erythroid and myeloid precursors constitute the majority of the bone marrow and spleen populations. These myeloid and erythroid cells range in absolute numbers from normal to significantly elevated (Figure 7B). Red blood cell counts and hematocrits were within physiological range (data not shown). Among myeloid cells, coexpression of Mac-1 with high levels of Gr-1 identifies terminally differentiated, mature neutrophils (Figure 7B). Whereas fetal liver granulocyte populations were similar to wild type in Ikaros C−/− mice, a lower than normal number of granulocytes was detected in the bone marrow of young mutant homozygotes (Figures 7A and 7B).

Discussion

Elucidating the molecular mechanisms that control cell fate decisions at the level of a HSC is central to our understanding of how the blood and immune systems develop. The regulatory gene *Ikaros* expressed from the pluripotent stem cell compartment through various multipotent progenitors to mature lymphocytes was identified as a central regulator of lymphocyte specification (Georgopoulos et al., 1994; Morgan et al., 1996). An N-terminal deletion in the DNA-binding domain of the *Ikaros* gene (DN−/−) resulted in an early and complete block in the development of all lymphocytes in the fetal and adult hematopoietic system. In mice heterozygous for this N-terminal mutation, proteins generated by the mutant *Ikaros* locus display a dominant interference effect toward wild-type Ikaros isoforms, causing the rapid development of leukemias and lymphomas (Sun et al., 1996; Winandy et al., 1995). The severe lymphoid defects detected in mice homozygous and heterozygous for this N-terminal deletion may be due to the combined lack of Ikaros activity as well as a dominant interference from its mutant proteins toward other factors with which Ikaros interacts to specify lymphoid identity and maintain homeostasis (Morgan et al., 1996).

To examine this possibility and determine the direct effect of the loss of Ikaros activity in the hemo-lymphoid system, we deleted its last translated exon. This exon includes a zinc finger dimerization domain and an activation domain shared by all of the Ikaros proteins. Without these domains, the Ikaros proteins are functionally inactive and do not display dominant-negative effects on transcription (Sun et al., 1996). Moreover, these truncated forms are unstable and rapidly degraded in cells in which they are produced. Therefore, mice homozygous for this C-terminal *Ikaros* deletion are virtually null for any Ikaros protein. In contrast with the DN−/− mice, which lack all fetal and adult lymphoid lineages, Ikaros C−/− mice display selective defects in their fetal and adult lymphoid compartments. These results provide direct evidence that Ikaros proteins are an absolute requirement for the development or differentiation of fetal HSCs into lymphoid lineages, but their action is partially redundant for the development or differentiation of adult HSCs into some lymphoid lineages.

Fetal and postnatal B cell development was completely blocked in Ikaros C−/− mice. B cell precursors

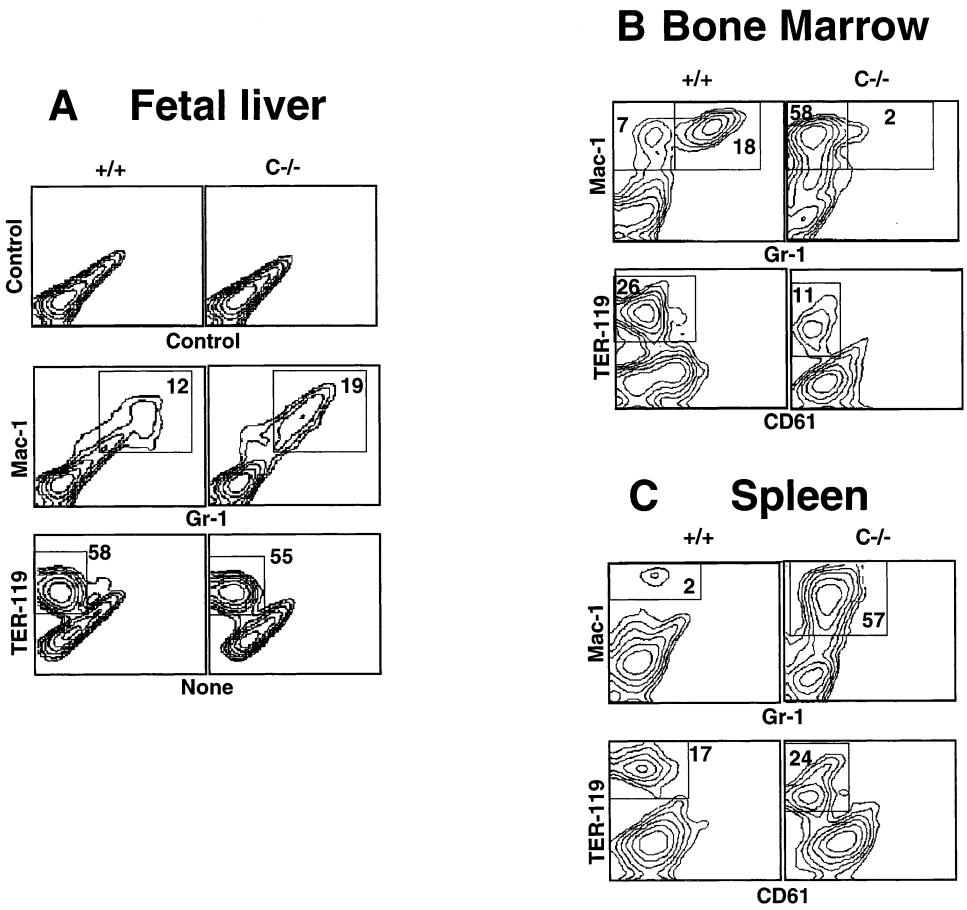


Figure 7. Fetal and Postnatal Waves of Myeloid and Erythroid Differentiation in Ikaros C−/− Mice

Cells obtained from the E16 fetal liver (A) and from the bone marrow (B) and spleen (C) of 3-week-old wild-type and Ikaros C−/− mice were stained with anti-Mac-1-PE/anti-Gr-1-FITC, anti-TER-119-PE, and anti-TER-119-PE/anti-CD61-FITC, respectively. Positively stained populations are boxed and percentages are shown. Similar percentages of granulocyte (Mac-1$^+$/Gr-1$^+$) cells were detected in the fetal liver of wild-type and Ikaros C−/− mice. The granulocyte population was significantly decreased in the bone marrow of Ikaros mutant mice. The percentage of Mac-1$^+$/Gr-1$^-$ cells (which includes committed myeloid precursors, mature monocytes, and macrophage) was significantly increased in both the spleen and bone marrow of Ikaros mutant mice. The percentages of committed erythroid precursors (TER-119$^+$) were similar in fetal and adult hematopoietic sites of Ikaros C−/− and wild-type mice.

normally detected in the mid-gestation fetal liver were absent from the Ikaros C−/− hematopoietic organs (Hardy et al., 1991; Hardy and Hayakawa, 1991). In spite of their ability to give rise to T cell progenitors, postnatal HSCs in Ikaros C−/− mutant mice were unable to generate even the earliest pro-B cells, normally found in the bone marrow of the wild-type animals. Therefore, B lymphocyte differentiation is completely blocked at the level of both fetal and postnatal HSCs.

During fetal development, HSCs and their immediate progeny, originating from the aorta gonad mesonephros areas or from the liver primordium, colonize the thymus (Dieterlen-Lievre et al., 1994; Dzierzak and Medvinsky, 1995). The expansion of lymphoid precursors in the normal fetal thymus occurs in waves (Jotereau et al., 1987). The first wave enters the fetal thymus by day 13 and gives rise to two successive populations of fetal thymocytes (reviewed by Allison, 1993; Asnarnow et al., 1988; Havran and Allison, 1988, 1990; Ito et al., 1989; Itohara et al., 1990; Koning et al., 1987). The first population expresses the Vγ3 TCR and comprises the majority of TCR-expressing thymocytes between days 14 and 16. These cells migrate to the skin and constitute the Vγ3 DETC compartment (Havran and Allison, 1990; Koning et al., 1987). The second population expresses the Vγ4 TCR and comprise the majority of TCR-expressing thymocytes between days 16 and 19 of gestation (Asnarnow et al., 1988; Havran and Allison, 1988, 1990; Ito et al., 1989; Itohara et al., 1990). They migrate to the mucosal epithelia of the female reproductive tract and tongue. No thymocytes expressing either Vγ3 or Vγ4 TCR are evident in the fetal thymus after day 19, and they are virtually absent in the adult organ (Havran and Allison, 1988). The second wave of lymphoid precursors enters the fetal thymus after day 15. They give rise to the majority of $\alpha\beta$ TCR–expressing thymocytes in the late fetal and early postnatal thymus. Late fetal and postnatal thymic progenitors give rise to $\gamma\delta$ T cells that populate the spleen and lymph nodes. These $\gamma\delta$ T cells preferentially express the Vγ2 TCR. They constitute a minute percentage (0.5%–2%) of splenic and lymph node T cells (Ito et al., 1989). A fourth type of $\gamma\delta$ T cells of

potential extrathymic origin is generated postnatally (Lefrancois, 1991; Lefrancois et al., 1990). These γδ T cells express the Vγ5 TCR and CD8 αα coreceptor and reside only in the intestinal epithelium (Guy et al., 1991).

The first and second waves of fetal thymocyte immigration or expansion do not occur in Ikaros C−/− mice. Therefore, the thymus of these mutant mice is devoid of a lymphoid compartment throughout fetal life and for the first few days after birth. In contrast with the early and complete block in lymphoid differentiation manifested in the fetus, thymocyte precursors are detected in the Ikaros C−/− thymus starting a few days after birth. These thymocyte populations reach nearly normal numbers in 1-month-old Ikaros mutants.

Postnatal αβ T cell precursors give rise to $CD4^+CD8^-$ and $CD4^-CD8^+$ αβ T cells in Ikaros C−/− mice. However, a 2- to 3-fold increase in the proportion of $CD4^+$/αβ T cells was detected from the onset of T cell differentiation. These results suggest that in the absence of Ikaros, deregulation in CD4 versus CD8 lineage commitment occurs, presumably at the level of double positive thymocytes. In addition to the deregulated production of $CD4^+$/αβ thymocytes, both $CD4^+$/αβ and $CD8^+$/αβ thymocytes in Ikaros C−/− mice proliferated significantly more than their wild-type counterparts when triggered through their TCR (Table 1). Deregulated expansion of double and single positive thymocytes with the same Vβ/TCR specificity may occur after engagement of the pre-TCR or TCR complexes. Oligoclonal and monoclonal thymocyte populations predominate in the thymi of older Ikaros C−/− mice. Augmented T cell proliferative responses followed by the rapid development of leukemias and lymphomas were also observed in mice heterozygous for the Ikaros DNA-binding mutation (Winandy et al., 1995). In these mutant mice, dominant-negative Ikaros proteins can interfere with the activity of wild-type isoforms, but also with other factors resulting in deregulation of T cell homeostasis (Sun et al., 1996; Morgan et al., 1996). Comparison of the hyperproliferative phenotype of the two distinct *Ikaros* mutations (DN−/− and C−/−) suggests that a profound decrease or lack of Ikaros activity leads to T cell hyperproliferation, aberrant expansion of thymic clones, and T cell neoplasia. These results indisputably establish *Ikaros* as a tumor suppressor gene essential in both differentiating and mature T cells. In the absence of Ikaros, thymocytes and mature T cells undergo aberrant expansion, possibly after engagement of their TCR complex. The transition of such proliferating mutant T cells to a neoplastic state is either concomitant with or rapidly follows TCR signaling.

Some γδ T cells were found in the early Ikaros C−/− thymus, indicating that this branch point in T cell differentiation is partly intact. Since Vγ3 T cells that give rise to DETC arise only in the context of fetal progenitors and a fetal thymic microenvironment (Ikuta et al., 1990, 1992), no DETC were found in the epidermis of mutant mice. Thymocyte progenitors that mature in the Ikaros C−/− thymus postnatally do not give rise to DETC. In sharp contrast, vaginal epithelial γδ T cells (Vγ4), which are proposed to be predominantly derived from the early wave of fetal T cell progenitors, were present in a normal distribution and density in the Ikaros mutant mice. Therefore, Vγ4 T cells can be readily generated from postnatal T cell progenitors (Ikuta and Weissman, 1991). Although γδ T cells were detected in the thymus, albeit in reduced numbers, no significant γδ T cell population was seen in the spleen of the adult Ikaros C−/− mice. In addition, extrathymically derived IELs bearing γδ TCRs and expressing the CD8 αα coreceptor were absent or significantly reduced. The number of $CD8^-$ γδ IELs was also drastically reduced. These studies support the existence of distinct migration and expansion requirements for distinct types of γδ T cells.

In the wild-type thymus, the early $CD44^+$/$c\text{-}kit^+$/$CD4^{lo/-}$ T cell progenitor has been proposed to give rise to NK cells and thymic dendritic APCs (Wu et al., 1995). NK cells and thymic dendritic APCs were absent or significantly reduced in Ikaros C−/− mice, suggesting that the early branch points into these two pathways were blocked in the absence of Ikaros.

In spite of the multiple defects observed in the lymphoid compartment of the Ikaros mutant mice, both fetal and postnatal HSCs differentiate along the erythroid and myeloid pathways and give rise to some of their mature progeny. However, the percentage of granulocytes was lower in Ikaros C−/− bone marrow than in the wild type, suggesting that the Ikaros mutation may have an effect on later stages of the myeloid pathway. This effect may reflect a block in granulocyte maturation caused by lack of growth factors normally provided by an intact microenvironment. Absence of lymphocytes in the bone marrow may be responsible for this myeloid lineage maturation defect in young homozygotes. Alternatively, lack of Ikaros activity in postnatal myeloid progenitors may directly affect granulocyte differentiation. The lack of mature granulocytes in Ikaros C−/− bone marrow does not appear to be caused by their early release. Mature granulocytes were not found in the spleen or peripheral blood of Ikaros C−/− mice (Figure 7C; data not shown).

We recently described *Aiolos*, a gene with strong structural and functional similarities to *Ikaros* (Morgan et al., 1996). Its encoded protein dimerizes with Ikaros isoforms through a highly conserved C-terminal zinc finger domain and modulates their activity in transcription (Morgan et al., 1996). *Aiolos* expression is first detected in the thymus during late gestation and persists at high levels in T and B lymphocytes and their immediate precursors in the adult organism. Within the bone marrow–derived stem cell compartment, Aiolos is more restricted than Ikaros in the more committed and lymphoid-enriched multipotent progenitors ($Sca\text{-}1^+$/$c\text{-}kit^+$/$Sca\text{-}2^+$) (Okada et al., 1992; Spangrude et al., 1988). *Aiolos* expression is strongly up-regulated when these progenitors become definitive T and B lymphocyte precursors (Morgan et al., 1996). Aiolos activity in postnatal C−/− hemo-lymphoid progenitors that lack any functional Ikaros proteins may rescue their differentiation into the T but not the B cell lineage. Functional differences between Aiolos and the Ikaros proteins may prevent the proper differentiation and homeostasis of T cell precursors and their progeny.

The lymphoid defects manifested in Ikaros null mice provide us with a unique insight into the complex regulatory network that differentially controls lymphocyte differentiation in the fetal and adult hematopoietic system.

The *Ikaros* gene is essential for the specification of all lymphoid lineages during fetal hematopoiesis, but is partially redundant in adult T lymphocyte development. In its absence, other factors that normally work in concert with Ikaros may substitute for some but not all of its functions in lymphoid lineage specification, differentiation, and homeostasis.

Experimental Procedures

Recombination Constructs and Targeting of ES Cells

The recombination vector described in Figure 1A was constructed with *Ikaros* genomic fragments and neomycin and thymidine kinase expression cassettes and was targeted into J1 embryonic stem (ES) cells as previously described (Georgopoulos et al., 1994; Li et al., 1992). DNA was prepared, digested with KpnI and EcoRV, and analyzed by Southern blotting using a DNA probe from outside the homologous recombination area (probe A). Single integration events were scored using a probe derived from the neomycin gene (probe B). Two distinct ES cell lines heterozygous for this mutation were used in separate blastocyst injections to rule out phenotypes that result from cell line mutations. To explore potential phenotype variability on distinct genetic backgrounds, the mutant ES cells were injected in blastocysts from C57BL/6 and Balb/c mice. The genotype of F1–F3 mice was determined by Southern blot and by polymerase chain reaction (PCR) analysis of tail DNA using either probe A or the appropriate primers designed from the neomycin (*Neo1*) and the *Ikaros* gene (*Int-7F* and *Ex7R*): *Int-7F*, GGG CCT TTG GGG ACA TCG AAG GTC; *Ex7R*, CAT AGG GCA TGT CTG ACA GGC ACT TGT; *Neo1*, CCA GCC TCT GAG CCC AGA AAG CGA.

Immunohistochemical Analysis of Ikaros C−/− and +/+ Thymocytes

Expression of Ikaros and Aiolos proteins in wild-type and mutant thymocytes was performed as previously described (Sun et al., 1996). N-terminal Ikaros and Aiolos antibodies were used at 1:300 dilution. Stained cells were visualized with a Leica confocal epifluorescence microscope with a 100× objective lenses.

Western Blot Analysis of Ikaros C−/− and +/+ Thymocytes

Nuclear and cytoplasmic extracts were prepared from Ikaros C−/− and wild-type thymocytes as previously described (Molnar and Georgopoulos, 1994). These extracts (20 μg) were run on a 12% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with anti-Ikaros and anti-Aiolos polyclonal antibodies (Molnar and Georgopoulos, 1994; Sun et al., 1996). A peroxidase-conjugated goat anti–rabbit immunoglobulin G (IgG) secondary antibody was used for the second step. The ECL kit (Amersham) was used to detect the hybridizing Ikaros/Aiolos proteins.

Histological Analysis of Ikaros C−/− Mice

Tissues harvested from euthanized wild-type and Ikaros C−/− mice were fixed in 4% buffered formalin for 1–2 days. They were then processed and embedded in paraffin. Sections were cut at 5 μm thickness, mounted, and stained with hematoxylin and eosin. Light microscopy was performed at 2–40× magnification on an Olympus BMax-50 microscope.

Cytofluorometry of Hematopoietic Cell Populations

Ikaros C−/− mice were analyzed in parallel with age-matched wild-type siblings. At least 20 groups of animals were studied on each mixed background (SV129 × C57BL/6 and SV129 × Balb/c). Single cell suspensions of thymus, spleen, or bone marrow cells were prepared and analyzed for their lymphoid, myeloid, and erythroid populations on a FACScan as previously described (Georgopoulos et al., 1994; Winandy et al., 1995). Monoclonal antibodies (MAbs) conjugated with phycoerythrin (PE), fluorescein isothiocyanate (FITC), or cychrome were used in two- or three-color cytofluorometric analyses.

PCR Analysis of TCR Gene Structure

DNAs were prepared from thymocytes and splenocytes as previously described (Winandy et al., 1995). Sequences of synthetic oligonucleotides (5′ to 3′) used as primers (Dβ2.1 and Jβ2.7) and as an internal probe (DβINT) for Southern analysis are as follows: Dβ2.1, GTA GGC ACC TGT GGG GAA GAA ACT; Jβ2.7, TGA GAG CTG TCT CCT ACT ATC GAT T; DβINT, ATT GTG GGG ACT GGG GGG GC.

Enumeration of Dendritic Epidermal and Mucosal γδ T Cells and Cut IELs

Ammonium thiocyanate–separated epidermal or vaginal sheets were incubated with a 1:20 dilution of goat serum and then stained with MAb GL3 (specific for γδ TCR), followed by biotin-conjugated goat anti–hamster immunoglobulin, avidin–biotin complexes (Vectastain Peroxidase Standard ABC Kit) and developed with 3-amino-9-ethylcarbazole. (Bigby et al., 1987; Juhlin and Shelly, 1977). Positively stained dendritic cells were identified by light microscopy. Separate sheets were stained with PE-conjugated Thy-1 MAb (53-2.1) or unconjugated MAb M5/114 (specific for class II antigen), followed by FITC-conjugated goat anti–rat antibody as described, and evaluated by immunofluorescence microscopy. Positively stained dendritic cells were identified by epifluorescence microscopy. Hamster immunoglobulin, a PE-conjugated rat IgG_{2a}, and unconjugated rat IgG_{2b} control antibodies were used for GL3, Thy-1, and M5/114, respectively.

Enumeration of Thymic and Splenic γδ T Cells and NK Cells

To enrich thymi or spleens for γδ T cells and NK cells, single cell suspensions were depleted by coating them with a mixture of MAbs (CD4 and CD8 for thymi; CD4, CD8, B220, Mac-1, Gr-1, and Terr119 for spleens) and depleting the coated cells with anti–rat immunoglobulin-coated magnetic beads (Ardavin et al., 1993; Bigby et al., 1993; Lynch and Shevach, 1992; Vremec et al., 1992; Wu et al., 1991a, 1991b). Enriched thymus were stained for two-color fluorescence analysis as described above.

Thymic Dendritic APCs

To enrich thymi for dendritic cells, pooled minced thymi were digested with collagenase and treated with EDTA. Light density cells were collected by density centrifugation and nondendritic cell lineage cells were depleted by coating them with a mixture of MAb and depleting the coated cells with anti–rat immunoglobulin-coated magnetic beads (Ardavin et al., 1993; Vremec et al., 1992; Wu et al., 1991a, 1995). Enriched thymus cell suspensions were stained for two-color fluorescence analysis as described above.

NK Activity Assay

Spleen cells were stimulated for 4 days in vitro with 500 U/ml of recombinant IL-2. The ability of stimulated cells to lyse Yac-1 targets was measured in a standard 4 hr chromium release assay (Garni-Wagner et al., 1990).

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