

FIGURE 7. B and NK lymphopoiesis in the fetal liver is intact in the mutant males. (A) In vitro differentiation of NK (NK1.1⁺) and B (CD19⁺) cells from fetal liver cells. HPCs (B220⁻, Thyl⁻, DX5⁻, Ter119⁻, Gr-1⁻, CD11b⁻) from the fetal liver of male and female littermates were cultured on OP9 stromal cells in the presence of exogenous cytokines. Numbers in plots indicate the percentages of live cells. Data are representative from two independent experiments. (B) Female or male (Ly5.2⁺) E17.5 fetal liver cells were injected into irradiated *Il2rg*^{-/-} recipients. B (left panel) and NK (right panel) cell populations in the bone marrow were analyzed by FACS 4 wk after transplantation. (C) The percentage of B or NK cells in the E17.5 fetal liver of siblings produced from a mating between a B cell-deficient male mouse and a wild-type female mouse. Fractions A-D (B220⁺ IgM⁻), Fraction E (B220⁺ IgM⁺), NKP (CD122⁺ CD3⁻ CD11b⁻ NK1.1⁻), and iNKP cells (CD122⁺ NK1.1⁺ CD3⁻ CD11b⁻) were analyzed. Data are from two independent experiments with three or four recipient mice per genotype or two independent experiments pooled with five male or four female fetuses.

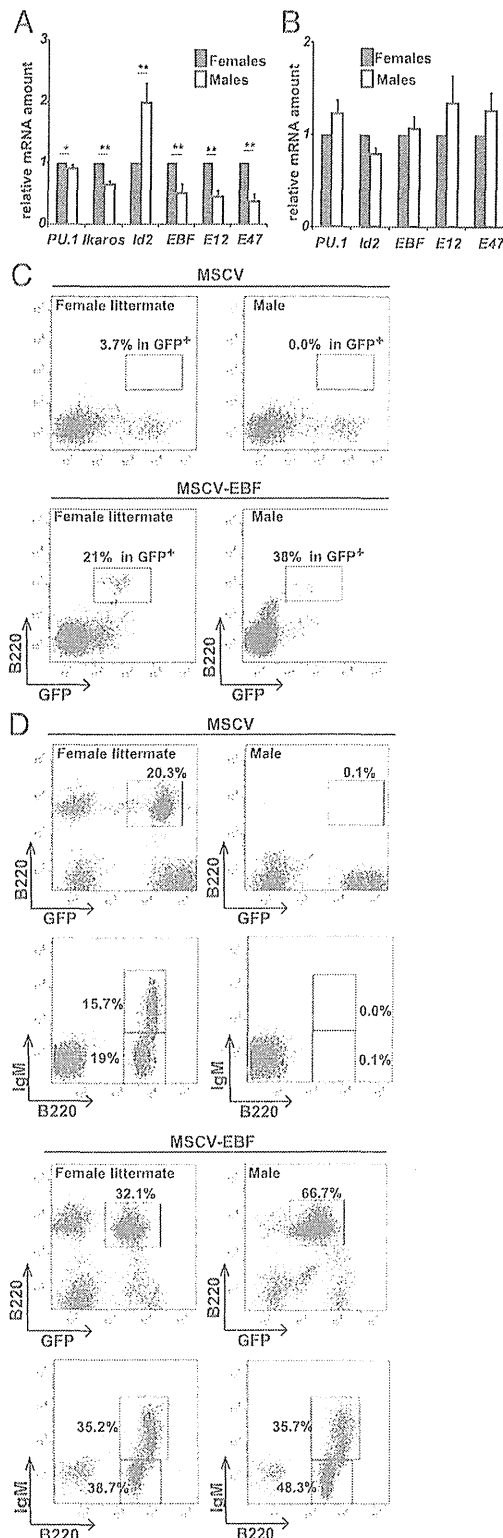


FIGURE 8. EBF restores B cell development in mutant male hematopoietic cells. The expression of genes involved in B cell development was analyzed in male and female littermates by real-time PCR. Hardy fraction A ($B220^{+}CD43^{+}BP-1^{-}CD24^{-}$) cells from adult bone marrow (**A**) or immature B ($B220^{+}IgM^{-}$) cells from the fetal liver (**B**) of male and female littermates were isolated by FACSARIA II (BD Biosciences). Extracted RNA was reverse-transcribed and used for real-time PCR (see *Materials and Methods* for primers). All values were normalized to GAPDH and are presented as the fraction relative to cDNA from control mice (set as 1). Data show three independent experiments, each done in triplicate with

adult bone marrow (Fig. 8A) or immature B cells ($B220^{+}IgM^{-}$) from the fetal liver (Fig. 8B) of male and female littermates. We used real-time RT-PCR to compare mRNA levels of essential transcription factors for B cell development. The transcription factors EBF and E2A (E12 and E47), both of which are essential for B cell development, were significantly decreased in the adult bone marrow (Fig. 8A) but not the fetal liver (Fig. 8B) of males as compared with females. However, there was a 2-fold increase in Id2, which is a transcriptional repressor for EBF and E2A, in the male adult bone marrow, although the fetal liver Id2 levels were comparable in males and females (Fig. 8A, 8B).

Because a lack of EBF or E2A causes B cell defects (22–24), the *EBF* or *E2A* gene was retrovirally transduced into mutant HPCs, which were then cultured on OP9-stroma cells under B cell differentiation conditions. Although the *E2A*-transduced male HPCs did not develop into $B220^{+}$ cells (data not shown), forced EBF expression successfully rescued the B cell development (Fig. 8C). *EBF* or a control vector was then retrovirally transduced into HPCs from male or female littermates ($Ly5.2^{+}$), and the HPCs were transplanted into irradiated wild-type mice ($Ly5.1^{+}$) for in vivo reconstitution. $B220^{+}$ B cells were not detected in recipient mice transfused with control vector-transfected HPCs from males, but were clearly reconstituted in recipients transfused with *EBF*-transduced (GFP^{+}) HPCs (Fig. 8D). Control recipients transfused with female HPCs transfected with either *EBF* or control genes showed clear $B220^{+}IgM^{+}$ B cell reconstitution (Fig. 8D). As EBF restricts lymphopoiesis in the B cell lineage by blocking the development of other lymphoid-derived cell pathways (25), *EBF* transfection did not result in the recovery of NK cells (data not shown). Collectively, forced EBF expression was able to rescue impaired B cell development, but not NK cell development, in the male mutant mice.

Discussion

In this study, we describe a novel mouse strain characterized by a male-specific defect of B cells, NK cells, and PPs. T cells, monocytes, granulocytes, and erythrocytes were relatively intact in these male mice. Most notably, this defect was linked to the Y chromosome. Mating the B cell-deficient males to wild-type females for several generations revealed that the B and NK cell deficiencies were inherited as Y chromosomal Mendelian characteristics. A multicolor FISH analysis demonstrated a structural abnormality in the Y chromosome.

We do not know how the Y chromosome abnormality mediates the B and NK cell defects. Because the female littermate mice, which do not have any genes encoded by Y chromosome, are immunologically normal, the immunodeficiency was not caused simply by the defect of a Y chromosome gene. Therefore, there are

pooled bone marrow from six mice or fetal liver from at least three mice per genotype. (**C**) Bone marrow HPCs were retrovirally transfected with a murine stem cell virus (MSCV; control) or MSCV-EBF and cultured on OP9 cells in the presence of SCF, Flt3L, and IL-7. Cells were analyzed by FACS 7 d postinfection to determine the B220 and GFP expression. Numbers in plots indicate the percentage relative to GFP^{+} cells. A representative result from two independent experiments is shown. (**D**) Littermate female or male HPCs ($Ly5.2$) were infected with an MSCV or EBF retrovirus and injected into sublethally irradiated wild-type mice ($Ly5.1$). Bone marrow cells were analyzed for B220, IgM, and GFP expression by FACS. Numbers in plots indicate the percentage relative to $Ly5.2$ cells (*top panel*) or GFP^{+} cells (*bottom panel*). A representative result from two independent experiments with three mice per group is shown. * $p < 0.05$, ** $p < 0.01$.

at least three possible mechanisms: a gene that exists on another chromosome may be inserted into the Y chromosome and be overexpressed or ectopically expressed in B and NK cell precursors due to abnormally controlled gene expression. Another possibility is that a gene from another chromosome is translocated or inserted into the Y chromosome and forms a chimeric gene, which may produce an abnormal chimeric protein affecting B and NK cell differentiation. The third possibility is that a structural abnormality of the Y chromosome itself induces the ectopic expression of a Y chromosome gene in lymphocyte precursor cells, interfering with their differentiation.

To address the first possibility, we performed a multicolor FISH analysis and array-based comparative genomic hybridization to identify gene loci inserted from autosomes or the X chromosome into the Y chromosome. However, FISH analysis failed to show any abnormal signals on the Y chromosome (data not shown). Although we found copy-number abnormalities on three loci on chromosomes 2, 9, and 10, these did not appear to be associated with the Y chromosome (data not shown). To address the second possibility, we used a next-generation sequencer to sequence Y chromosome exons. However, we could not find any significant differences in the nucleotide sequence, including known single nucleotide polymorphisms, between the mutant males and control male mice kept in the same facility (data not shown). Therefore, chimeric gene formation is unlikely. Genomic sequencing of the entire Y chromosome would be necessary to completely rule out this possibility. However, unlike other chromosomal genes, the Y chromosome's many repetitive sequences make it difficult to analyze data from the next-generation sequencer. Thus, a new sequencing method is needed for the Y chromosome. To address the third possibility, we conducted microarray and real-time RT-PCR analyses of the Y chromosome gene expression in the bone marrow immature B cells of mutant and control male mice. These analyses also showed negative results (data not shown). Thus, the genetic mechanism for this novel Y chromosome-linked disease remains unclear.

B cell development is regulated by a transcriptional network consisting of, but not limited to, the transcription factors PU.1, Ikaros, E2A, EBF, Pax5, and Id2. PU.1 and Ikaros are critical for early lymphoid-lineage specification, whereas E2A, EBF, and Pax5 are essential for the commitment of common lymphoid progenitors to become B cells. Quantitative RT-PCR with prepro-B cells from the male mutant mice showed lower Ikaros, E2A, and EBF expression and higher Id2 expression (Fig. 8). Because Id2 is a physiologically relevant, negative EBF regulator, high Id2 levels may block B cell development in males at the prepro-B stage by suppressing EBF or/and E2A. As expected, deliberately expressing EBF in mutant male HPCs rescued the B cell differentiation, clearly indicating that an insufficient expression of EBF, likely induced by Id2 overexpression, may cause the B cell deficiency seen in the male mice. However, because the T cell Id2 levels were comparable in male and female littermates (data not shown), an *Id2* mutation cannot explain the abnormal Id2 expression in the prepro-B cells in the male mice. Further work is needed to clarify whether and how these genes are associated with B lymphopoiesis.

B and NK development from fetal liver cells was intact, whereas that from bone marrow was severely impaired in the male mice. Few molecules have been reported to act in such a way, with IL-7 and IL-7R being the prototypical examples (26). Because Flt3 (also called FLK2) signal can compensate IL-7 signal in the fetal liver, B lymphopoiesis is independent of IL-7 in the fetal liver, which is indispensable for that in bone marrow (27, 28). However, no study has shown that fetal and adult NK cell development is regulated by distinct cytokine signal or transcription factors. The age-

dependent defect of NK cells in mutant males sheds light on the new aspect of the NK cell development.

IL-7 requirement between B1- and B2-B cell lymphopoiesis is different. As described above, B2-B cell development from bone marrow is dependent on IL-7, whereas IL-7 is indispensable for B1-B cell development, which is rather promoted in IL-7 deficient mice (29). Therefore, although the age-dependent B2-B cell defect in the mutant mice resembles that in IL-7-deficient mice, the defect of both B1- and B2-B cells in the mutant mice cannot be explained by the IL-7/Flt3 compensation mechanism. Thereby, the immunodeficient phenotype of the mutant mice suggests a unknown pathway, which is shared by B1-B, B2-B, and NK lymphopoiesis.

Among immunodeficiencies, it is rare to see a combined B and NK immunodeficiency accompanied by normal T cells. Several common factors are essential for B and NK lymphopoiesis, such as Ikaros, PU.1, and cytokine signals. NK development depends on IL-15, because both *IL15*^{-/-} and IL-15R α -chain-deficient mice (*IL-15R α* ^{-/-}) lack peripheral NK cells and NK cell-mediated cytotoxicity (30, 31). IL-7, which shares a γ -chain with IL-15, is essential for B cell development. However, NKP cells are generated from HSCs independently of IL-15 or any γ c cytokines (32). Therefore, an abnormality in γ -chain cytokines and their intracellular signals might not cause the immunodeficient phenotype in our mice. Interestingly, a previous study reported that mice with a C-terminal Ikaros deletion (Ikaros C-null knockout mice), like our mice, have T cells but are deficient in B and NK cells (33). A combined immunodeficiency syndrome in humans, characterized by adult-onset B and NK cell deficiencies with normal T cells, was recently reported in a female patient (34). However, the T cell responses were aberrantly hyper in both the patient and in Ikaros-mutant mice, whereas the *in vitro* T cell responses in our male mutant mice appeared relatively normal (data not shown). Therefore, the combined immunodeficiency of B and NK cells in our mice is a novel phenotype, suggesting an unknown molecular mechanism shared by B and NK cell development.

The mutant male mice showed a marked reduction of lineage⁻c-Kit^{hi}Sca-1⁻ cell populations in the bone marrow. However, because the absolute number of the late progenitors and mature populations of myeloid and erythroid lineages were normal in the mutant male mice, the mutant mice should have the functional hematopoietic progenitor cells. Similarly, functional common lymphoid progenitor cells may also exist in the male mice because they have a normal number of prepro-B cells. These results suggest that the early progenitors in the bone marrow of the mutant mice cannot be defined by the conventional commonly used marker Sca-1. The possible effect of Sca-1 overexpression on B and NK cell differentiation will be required.

This is the first report, to our knowledge, of a Y-linked immunodeficiency. Identifying the target substrate or affected pathway would help us determine the Y chromosome's exact contribution in lymphocyte development. Males from our novel strain will provide a key experimental model for studying possible common factors that are responsible for B and NK lymphopoiesis but do not contribute to T cell development in adults.

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Disclosures

The authors have no financial conflicts of interest.

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