28. Mitochondria were isolated from transformants precultured in galactose-containing selective medium and then inoculated into selective medium containing 0.3% yeast extract. Mitochondria (3 mg of protein) were suspended in 150 μl of 50 mM Na_2HPO_4, 0.9% NaCl, and 1 mM EDTA and were solubilized by addition of 100 μl of 10% Na-cholate (pH 7.8) for 15 min on ice. After centrifugation at 100,000*g* for 15 min at 4°C, the supernatant was divided into 40-μl aliquots and incubated for 30 min on ice with trypsin (0, 5, 10, 100, or 250 μg/ml). After addition of PMSF to a final concentration of 1.2 mM and incubation for 10 min, 0.45 ml of reducing sample buffer (2×) was added to each aliquot. A 60-μl portion of each sample was analyzed by SDS-PAGE followed by immunoblotting with antisera to Cox subunits. The immunoblot was developed by ^{125}I-labeled protein A and autoradiography.
29. For assessment of the assembly state of the F_1F_0 ATPase, mitochondria isolated from the various transformants (400 μg) were suspended in 40 μl of 0.75 M 6-aminocaproic acid, 50 mM bistris-HCl (pH 7.0), and 1 mM PMSF and were solubilized by addition of 7.5 μl of 10% lauryl maltoside. After centrifugation at 100,000*g* for 15 min at 4°C, the supernatant was mixed with 2.5 μl of 5% Serva blue G and electrophoresed on a 5 to 12% nondenaturing polyacrylamide gel (*22*). After electrophoresis, the gel was incubated for 30 min with 50 mM bistris-HCl and 15 mM tricine-HCl (pH 7.0). The separated proteins were analyzed by immunoblotting with antisera to the α, β, and γ subunits of F_1 ATPase and to mitochondrial hsp60. For quantitation, different amounts of protein were loaded to ensure that measurements were within the linear range; these different samples were prepared from 800, 400, and 200 μg of mitochondrial protein.
30. Supported by grants from the Swiss National Science Foundation (to G.S.), the Human Capital and Mobility Program of the European Union (to G.S. and L.A.G.), EMBO (to M.R. and J.M.v.D.), and the Damon Runyon–Walter Winchell Cancer Research Foundation (to C.K.S.).

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Requirement for Invariant Chain in B Cell Maturation and Function

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Previously the role of invariant chain (Ii) had been described only as a chaperone that facilitates folding and transport of major histocompatability complex class II molecules; here it is shown that Ii is required for B cell development. B cells from mice lacking Ii were found to have a low response to T-independent type II antigen and could not proliferate after the mice were injected with antigen. Study of cell surface markers revealed a developmental arrest that prevented immature virgin B cells from becoming mature B cells in the periphery. This block was independent of major histocompatability complex class II expression and was an intrinsic feature of B cells that correlated with the amount of Ii. Thus, Ii participates by an unknown mechanism in B cell maturation.

Major histocompatability complex (MHC) class II molecules associate with trimers of Ii during biosynthesis. Ii facilitates folding of class II molecules, interferes with their association with peptides, and is involved in MHC class II transport (*1*). Furthermore, elimination of the Ii gene by gene targeting greatly diminishes the ability of antigen-presenting cells (APCs) to present exogenous protein antigen in a class II–restricted fashion and impairs the maturation of $CD4^+$ T cells in the thymus (*2–4*). The assembly, transport, and function of MHC class II have been studied in detail in mice lacking Ii (*2–4*). There has not, however, been a rigorous examination of the functional capability of B cells. We therefore analyzed the function of B cells lacking Ii.

To examine the function of B cells lacking Ii (Ii^-), we measured B cell response both to the type II thymic-independent (TI) antigen NP-Ficoll and to NP-CGG, a thymic-dependent (TD) antigen (Fig. 1). Both TD and TI responses were markedly reduced in the Ii^- mice. The Ii^- mice have reduced numbers of $CD4^+$ T cells, which predicts that these mice should have weak responses to TD antigen. However, unlike the response in class II–deficient mice (*5, 6*), 6 days after immunization, concentrations of immunoglobulin M (IgM) to NP were low, suggesting that the primary response of B cells was also impaired. This observation is consistent with the defective primary antibody response by the Ii^- mice after keyhole limpet hemocyanin (KLH) injection (*2*). In response to NP-Ficoll, B cells lacking Ii produced little IgM both 6 and 14 days after injection. Thus, the B cells in Ii^- mice were unable to respond normally to TI antigen (Fig. 1).

Equivalent numbers of $B220^+$ B cells were found in the periphery of the control mice, Ii^- mice, and two lines of transgenic Ii mice that express low amounts of one of the two isoforms of Ii, p31 and p41 (designated Ii^{p31lo} and Ii^{p41lo}, respectively) (*7, 8*). To determine the ability of these B cells to respond to antigen in vivo after stimulation, we immunized mice with KLH and examined draining lymph node B cells 9 days later. In the draining lymph nodes of control mice, the $B220^+$ B cell population had proliferated and increased to 54.3 ± 6.8% of the total cells from 13.75%. In the absence of Ii or in the presence of low amounts of p31 or p41 Ii, however, the B cell population expanded to only 26.6 ± 3.2% (*9, 10*). Because in both Ii^{p31lo} and Ii^{p41lo} mice $CD4^+$ T cells are present in normal amounts (*7*), this low proliferation cannot be attributed to the $CD4^+$ T cell deficiency. Thus, the low number of B cells found after immunization with protein antigen could be explained by a defect in the B cell response or by the rapid death of these cells. B cells from Ii^- mice proliferated as well in vitro as did wild-type cells in the presence of lipopolysaccharide (LPS) (*11*).

B cell development occurs independently of MHC class II expression (*5, 6*). To analyze B cell maturation in the absence of Ii, we compared spleen cells from control and Ii knockout mice (Ii^-) using a panel of antibodies to B cell markers. Unlike B cells from control mice, the Ii^- B cells expressed lower

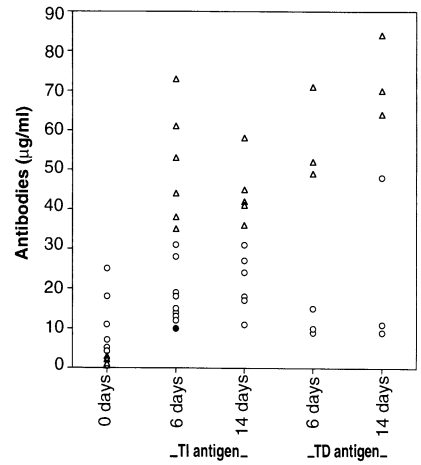

Fig. 1. Immunoglobulin M response to TD and TI antigens. Ii^- (circles) or wild-type littermates (triangles) were challenged with the type II-TI antigen NP_{90}-Ficoll or with the TD antigen NP_{16}-CGG. At the indicated times after injection, blood was drawn and IgM titers were quantitated by enzyme-linked immunosorbent assay (*21*). The concentration of antibodies at time zero in the absence of immunization probably represents basal concentrations of low-affinity IgM, although it is not clear why this amount is higher in the Ii^- mice than in the control mice. Mean titers (micrograms per milliliter) were as follows. Day 0: control, 1.415 ± 0.96 (n = 6); Ii^-, 10.7 ± 7.9 (n = 7). Day 6: control, 50.6 ± 14.6 (n = 6); Ii^-, 17.2 ± 7.06 (n = 10). Day 14: control, 43.8 ± 7.5 (n = 6); Ii^-, 19.85 ± 7.75 (n = 7). The symbol that appears solid represents two Ii^- mice with identical antibody concentrations.

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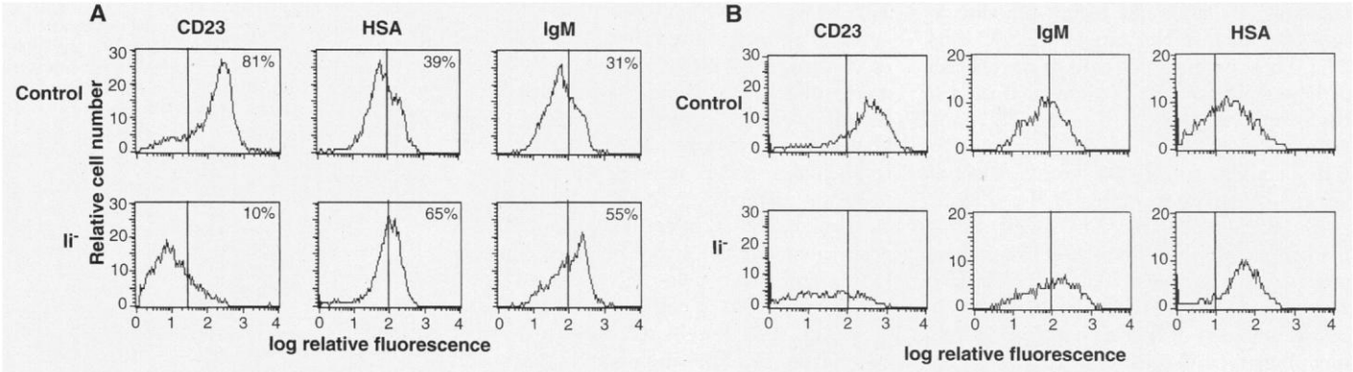

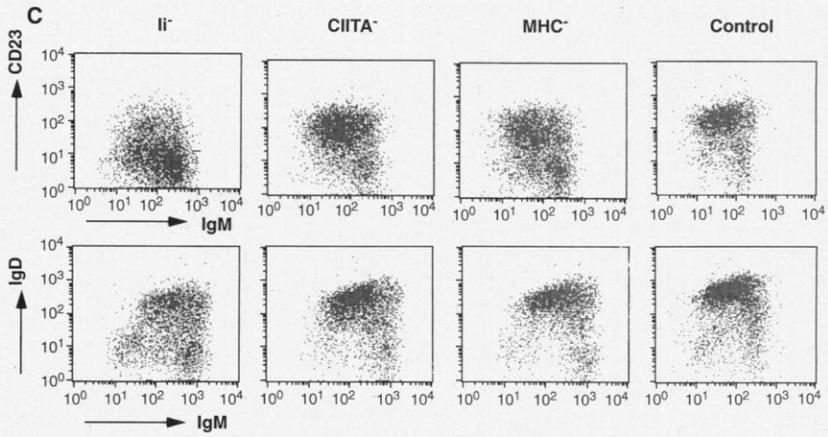

Fig. 2. Cytofluorometric analysis of different maturation markers on B220 cells. (**A**) Control and Ii$^-$ splenocytes were double-stained with antibody to B220 (anti-B220) and anti-IgM, anti-HSA, or anti-CD23. Histograms show expression of these molecules on B220 cells (*19*). (**B**) Nine days after KLH injection, lymph node cells from 6- to 8-week-old control and Ii$^-$ mice were harvested and were stained by FACS for anti-B220 and anti-IgM, anti-HSA, or anti-CD23. FACS analysis of the different markers on B220$^+$ cells is shown (*19, 20*). (**C**) Control, Ii$^-$, MHC$^-$, or CIITA$^-$ mice were triple-stained with anti-B220, anti-IgM, and either anti-CD23 or anti-IgD. The FACS analysis shows the different markers on B220$^+$ cells (*19*). (**D**) Steady state concentrations of Ii. The Ii p31 and p41 isoforms are indicated (*22*). Con, control.

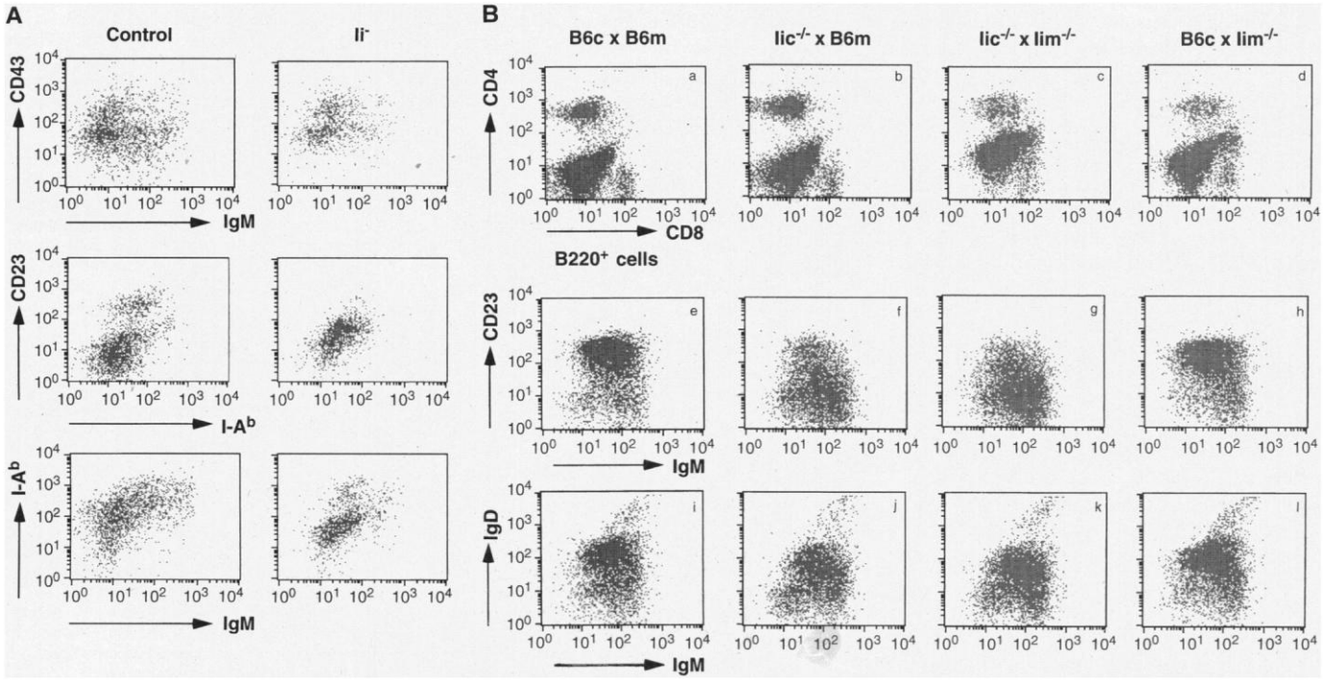

Fig. 3. (**A**) Cytofluorometric analysis of different maturation markers on B220$^+$ cells in bone marrow. Bone marrow cells from control and Ii$^-$ animals were triple-stained with anti-B220, anti-IgM, anti-CD23, anti-CD43, or anti–I-A^b. The FACS analysis shows the different markers on B220$^+$ cells (*19*). (**B**) T cell (top row) and B cell (middle and bottom rows) profiles from chimeric mice after fetal liver cell transfer. B6 (B6c) (panels a, e, i, d, h, and l) or Ii$^-$ (Iic$^-$) (panels b, f, j, c, g, and k) fetal liver cells were transferred to B6 (B6m) (panels a, b, e, f, i, and j) or Ii$^-$ (lim$^-$) (panels c, d, g, h, k, and l) irradiated mice (*23*). Lymph node T cells (panels a to d) and B cells (panels e to l) were prepared and stained with antibodies to B220, CD4, CD8, IgM, IgD, and CD23.

amounts of CD23 and higher amounts of IgM and heat-stable antigen (HSA) (Fig. 2A). Thus, peripheral B cells in the absence of Ii were immature. Analysis of B cells in the periphery of the Ii^{p31lo} and Ii^{p41lo} transgenic mice revealed that the low amounts of Ii that could restore T cell–positive selection (*7*, *12*) could not mediate full B cell maturation in the periphery (*13*). Cell surface markers expressed on these B cells remained unchanged 9 days after KLH injection (Fig. 2B). Thus, B cells in the absence or presence of low amounts of Ii remained of an immature phenotype. B cells that could proliferate in vitro after LPS stimulation remained immature as well (*14*).

To investigate whether the absence of conventional MHC class II was responsible for this B cell maturation defect, we examined maturation markers on B cells from class II–deficient mice. B cells from mice lacking MHC class II (MHC^-) (*5*, *6*) and from mice lacking the MHC class II transactivator ($CIITA^-$) (*15*) had only slightly lower numbers of $CD23^{hi}$, IgM^{int}, and IgD^{hi} than did cells from wild-type mice (Fig. 2C). Therefore, the low numbers of $CD23^{hi}$, IgD^{hi}, and IgM^{int} cells in the periphery of the Ii^- B cells were not due to less conventional MHC class II expression or $CD4^+$ T cells, but to the lack of Ii itself or perhaps a secondary consequence of a lack of Ii. Spleen cell lysates from the different mice were analyzed for Ii chain, and the p31 and p41 transgenic mice expressed low amounts of Ii (*7*). However, because CIITA deficiency only partially reduced Ii mRNA levels, the $CIITA^-$ cells expressed about 80% of the Ii of cells from control mice (Fig. 2D). Therefore, the invariant chain, rather than MHC class II, determined the degree of maturation of virgin B cells to mature cells.

To determine the role of Ii in early B cell development, we examined bone marrow from Ii^- and control mice cytofluorometrically for the expression of surface antigens that correlate with specific stages in B cell ontogeny. The percentage of $B220^+$ cells appeared to be slightly lower and the relative proportion of $CD43^+B220^+$ cells slightly higher in bone marrow of Ii^- mice. As expected from their absence in the periphery, $B220^+CD23^+$ cells were absent from Ii^- bone marrow. This $CD23^+$ population might be cells that recirculate from the periphery to this compartment. This places the developmental block between the immature $CD23^{lo}$, IgM^{hi}, IgD^{lo}, HSA^{hi} stage and the mature $CD23^{hi}$, IgM^{int}, IgD^{hi}, HSA^{int} stages (Fig. 3A).

To determine if the lack of maturation was due to an intrinsic B cell defect or if the bone marrow epithelial or peripheral stromal cells failed to provide the signals required for B cell maturation, we isolated day 17 fetal liver cells from either control or Ii^- embryos and transferred them into irradiated control or Ii^- recipients. In control chimeric mice in which the recipients and the donors were Ii^+, peripheral $CD4^+$ and $CD8^+$ T cells were reconstituted at normal levels and a mature normal B cell population was detected as expected (Fig. 3B, panels a, e, and i). In contrast, the irradiated Ii^- recipient mice that received control (Ii^+) fetal liver cells reconstituted low amounts of $CD4^+$ T cells (Fig. 3B, panel d). However, their B cells recovered a mature phenotype (Fig. 3B, panels h and l). Normal mice that were reconstituted with Ii^- cells showed a normal $CD4^+$ T cell population (*16*, *17*) (Fig. 3B, panel b), but their B cell population remained immature (Fig. 3B, panels f and j), showing the same phenotype as B cells of Ii^- mice (Fig. 3B, panels g and k) (*10*). Thus, unlike T cells, the inability of Ii^- B cells to mature is an intrinsic feature. Furthermore, B cells from CD4-deficient or interleukin-4 (IL-4)–deficient mice matured normally. Therefore, B cell maturation could progress in the absence of $CD4^+$ T cells and IL-4, suggesting that these features do not explain the B cell defect seen in Ii^- mice (*18*).

Thus, we have shown that Ii is critical for B cell maturation. Until now Ii was characterized as a chaperone that participates in antigen processing by allowing MHC class II folding, maturation, and transport. We now show that the development from immature to mature B cells is a controlled process that needs a signal to occur. This step is controlled by Ii, and in the absence of this chain, B cells cannot mature or participate efficiently in the immune response.

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8. The Ii^{p31lo} and Ii^{p41lo} mice were obtained by crossing transgenic mice expressing low amounts of one Ii isoform with Ii^- mice to generate Ii^- mice expressing exclusively p31 or p41. $B220^+$ B cells were examined in spleen and lymph nodes in the different mice. Lymph node and spleen cells from wild-type, Ii^-, Ii^{p31lo}, and Ii^{p41lo} mice were stained with antibody specific for $B220^+$ and analyzed by fluorescence-activated cell sorting (FACS) (*19*). Percentage of B cells in spleen were as follows: control, 46.5 ± 8.2; Ii^-, 47 ± 10.9; Ii^{p31lo}, 43.22 ± 9.1; and Ii^{p41lo}, 43.42 ± 6.07. Percentage of lymph node cells were as follows: control 13.75 ± 5.6; Ii^-, 15.07 ± 5.5; Ii^{p31lo}, 12 ± 5.3; and Ii^{p41lo}, 9 ± 1.
9. Lymph node cells from 6- to 8-week-old control, Ii^-, Ii^{p31lo}, or Ii^{p41lo} mice were harvested and were stained by FACS for $B220^+$ expression 9 days after KLH injection (*19*, *20*).
10. After the fetal liver adoptive-transfer experiment (Fig. 3B), we immunized mice with KLH (*20*) and found lower amounts of $B220^+$ B cells when Ii^- cells were transferred, suggesting that the lower amounts of $B220^+$ cells are due to an intrinsic property of these B cells.
11. Purified B cells from control or Ii^- mice were cultured at a density of 2×10^5 cells per 100 μl in Bruff's medium supplemented with 5% fetal bovine serum (FBS), penicillin-streptomycin (100 U/ml), and several concentrations of LPS. DNA synthesis was assayed by pulsing the cultures with 1.0 μCi [^{3}H]thymidine per well at 48 hours and then incubating them for an additional 12 hours, after which the cells were harvested and counted on a scintillation counter. Assays were done in triplicate. Proliferation was determined at 48 hours by [^{3}H]thymidine incorporation for 12 hours.
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13. For cytofluorometric analysis of different maturation markers on $B220^+$ cells, splenocytes from Ii^{p31lo} and Ii^{p41lo} mice were double-stained with anti-B220 and anti-IgM, anti-HSA, or anti-CD23 (*19*). The markers expressed on the B cells from these mice were almost identical to those expressed on splenocytes from Ii^- mice.
14. Purified B lymphocytes from wild-type or Ii^- mice were cultured in the presence of different concentrations of LPS. Cells were then double-stained with anti-B220 and anti-IgM, anti-HSA, or anti-CD23. B cells from Ii^- mice expressed immature markers after LPS stimulation.
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18. Control, Ii^-, $CD4^-$, or $IL\text{-}4^-$ mice were triple-stained with anti-B220, anti-IgM, or anti-CD23 (*19*). The FACS analysis showed that B cells from the $CD4^-$ or $IL\text{-}4^-$ mice expressed almost identical amounts of the markers as did B cells from wild-type mice.
19. The following antibodies used in experiments were purchased from Pharmingen: AF6-120.1 for $I\text{-}A^b$; H129.1.9 for CD4, 53-6.7 for CD8, M1/69 for heat-stable antigen (HSA), S7 for CD43, B3B4 for CD23, 14.8 for CD45RA (B220), R6-60.2 for IgM, and AMS 9.1 for IgD. Bone marrow, lymph nodes, and spleen cell suspensions were prepared in Bruff's medium, and the spleen erythrocytes were lysed by hypotonic shock. Cells were resuspended in cold phosphate-buffered saline (PBS) supplemented with 1% FBS. Staining was performed in the same buffer.
20. Mice were injected in the hind footpads with 100 μg of KLH emulsified in complete Freund's adjuvant. Draining lymph nodes were collected 9 days later.
21. Control and experimental animals 6 to 8 weeks of age were injected intraperitoneally with 25 μg of NP_{90}-Ficoll (NP-AECM-ficoll, Biosearch) in 0.1 ml of 0.85% NaCl. Immunization with NP_{16}-CGG was with 100 μg of Alum-precipitated NP-CGG in 0.1 ml of 0.85% NaCl.
22. Splenocytes from wild-type, Ii^-, or $CIITA^-$ mice were incubated in digitonin (50 μg/ml). The pellet was then lysed in 0.5% Triton X-100, 300 mM NaCl, 50 mM tris (pH 7.4), 1 mM polymethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), pepstatin (10 μg/ml), chymostatin (10 μg/ml), and 20 mM *N*-ethyl-maleimide. Nuclei and debris were eliminated by centrifugation. Lysates were separated on 12% (w/v) SDS–polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose, which was blocked and then incubated with IN1 (monoclonal antibody to the Ii cytoplasmic tail) followed by horseradish peroxidase–conjugated goat antibody to rat IgG.
23. Recipient animals were irradiated with 9.2 Gy delivered by a cesium source. Fetal liver cells from mice after 16 days of gestation were prepared and 5×10^6 cells were injected intravenously.

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