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_J Immunol_ 2004; 172:2118-2125;
doi: 10.4049/jimmunol.172.4.2118
http://www.jimmunol.org/content/172/4/2118
Complete Loss of Fas Ligand Gene Causes Massive Lymphoproliferation and Early Death, Indicating a Residual Activity of gld Allele

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To investigate the in vivo function of Fas ligand (Fasl), we produced a mouse strain with a Fasl gene flanked by loxP sequences. Mice with homozygous floxed Fasl gene showed no obvious abnormalities. However, germline deletion of the Fasl gene, obtained after mating with mice expressing ubiquitous Cre recombinase, resulted in an unexpectedly severe phenotype. Fasl-/- mice exhibited an extreme splenomegaly and lymphadenopathy associated with lymphocytic infiltration into multiple organs and autoimmune disease. This severe phenotype led to the premature death at 4 mo of age of >50% of the homozygous mice. It stands in sharp contrast with the milder disease observed in gld (generalized lymphoproliferative disease) mice, indicating that the Fasl allele of these mice encodes a protein still able to bind, albeit at a very low level, the Fas receptor. The Journal of Immunology, 2004, 172: 2118–2125.

Materials and Methods
Generation of Fasl-floxed and Fasl-deficient mice

The targeting vector was constructed by flanking the Fasl gene with loxP sites. A bacterial artificial chromosome containing the Fasl gene was isolated from a C57BL/6 library (Genome Systems, St. Louis, MO). The floxed neo gene was excised from the neoflox8 plasmid (kindly provided by K. Rajewsky, Institute for Genetics, Cologne, Germany) and inserted into a SacI site at the 5’ end of the Fasl gene. The third loxP site was introduced into another SacI site at the 3’ end of the Fasl gene. To allow negative selection against random integration events, the tk gene, excised from the pC19R plasmid, was inserted into a PstI site at the 5’ end of the targeting vector. The targeting construct carries ~2.6 and 3.9 kb of homologous DNA sequences beyond the 5’ and 3’ loxP sites and was transfected into 2 × 107 129/Sv embryonic stem (ES) cells (15). Cells were grown in medium containing 0.418 (300 μg/ml) and ganciclovir (2 μM) for positive and negative selection, respectively. Resistant clones were analyzed for homologous recombination by PCR and Southern blot. At the 3’ end, a nested PCR was used to identify homologous recombinant colonies. A sense primer (primer 1, 5’-GTATGGGCGGAGGCATCTTGGAAC TG-3’) and an antisense primer located outside the targeting vector (primer 2, 5’-GCAGGAACAGAGTGGGTGAGCAGG-3’) were used to amplify a 4-kb fragment using the Advantage-GC Genomic PCR kit (Clontech Laboratories, Palo Alto, CA). Then the 4-kb amplified fragment was used as DNA template to amplify the floxed allele using primer 3 (5’-ATTA ATTCAGTGAAGAGATGG-3’) and primer 4 (5’-GATCTCTTTC TGATAAGGCC-3’). Homologous recombination events at the 5’ end were tested by Southern blot of EcoRI-digested genomic DNA using a 1.2-kb probe located outside the targeting vector. Three ES clones with the targeted allele were microinjected into C57BL/6 blastocysts and transferred to foster mothers to produce male chimeras mice. Mating to C57BL/6 female mice evidenced the contribution of these stem cells to the germline of chimera mice. Fasl-deficient mice were obtained by mating mice heterozygous for the floxed Fasl allele to PGK-Cre transgenic female mice (16). Genotype of the offspring was determined by three-way PCR using primers 3, 4, and 5 (5’-GGAGTTGAACGAGTAGC-3’).
Mice

C57BL/6 wild-type and C57BL/6 gld/gld mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively. All mice were maintained in our animal facilities in specific pathogen-free conditions.

Lympocyte isolation, flow cytometry, and Ig production

Cell suspensions were prepared from lymphoid organs and livers of 5- to 18-wk-old mice. Briefly, thymocytes, lymph node cells, and splenocytes were depleted of erythrocytes by treatment with buffer containing 155 mM of ammonium chloride, 13 mM of sodium bicarbonate, and 0.1 mM of EDTA for 3 min on ice. Cells were passed through 100-μm mesh, washed, and then resuspended in staining buffer (PBS, 5% FCS, 0.1% NaN3) for cell surface labeling or in RPMI 1640 medium containing 10% FCS for killing assay. Livers were minced and passed through 100-μm mesh in PBS–5% FCS and depleted of erythrocytes, and lymphocytes were isolated by Percoll gradient (40–80%) centrifugation. Cell surface staining was performed in staining buffer with the appropriate dilutions of CD3 (CD3ε chain), CD4 PE (LyT4), CD8 FITC (Ly-2), and CD45R/B220 (RA3-6B2) Abs (BD PharMingen, San Diego, CA). Data were acquired and analyzed on a Calibur flow cytometer (BD Biosciences, France). Serum Ig level and autoantibodies were measured by ELISA. Total Ig and Ig isotype were quantified using the Cytofluorotyping System–AP (Southern Biotechnology Associates, Birmingham, AL). The anti-dsDNA Abs were detected using mouse anti-dsDNA ELISA kit (Alpha Diagnostic International, San Antonio, TX). For rheumatoid factor (RF), plates were coated with rabbit IgG (Sigma-Aldrich, St. Louis, MO), as described by Kanoh et al. (17). Serum samples were applied at a 1/10,000 dilution, and rabbit anti-mouse IgG coupled to alkaline phosphatase was used as a secondary detection reagent (Sigma-Aldrich).

Histopathology

Different organs from Fast−/−, control littermates, and C57BL/6 gld/gld mice were fixed in Formalin and embedded in paraffin for histological studies. Sections were stained with H&E or trichrome. For immunostaining, kidneys were quick frozen in OCT compound, and kidney sections were performed in triplicates. After coculture with effector cells at 37°C for 5 h, washed four times, and then add in equal number to each well. For each E:T ratio, cultures were performed in triplicates. After coculture with effector cells at 37°C for 18 h, the cells were harvested and counted using beta plate counter (1205 Vallen). Specific cell killing was calculated as follows: percentage of specific killing = (% E/T × 5) /S, where E (experimental) is cpm of retained DNA in the presence of effector cells and S (spontaneous) is cpm of retained DNA in the absence of effector cells. For inhibition of killing, recombinant mouse Fas-Fc chimera was purchased from R&D Systems (Minneapolis, MN).

Results

Generation of FastL-deficient mice

To investigate the in vivo function of FastL, we used gene targeting to introduce loxp sites at the extremities of the mouse FastL gene. Recombination between the two loxp sites mediated by a Cre recombinase can thus lead to the complete deletion of the FastL gene (Fig. 1).

After electroporation into ES cells and double selection, three recombinant clones were identified by PCR and Southern blot (Fig. 1, a and b). Chimeras mice were generated by injecting ES cells from the three clones (193, 197, and 319) into C57BL/6 blastocysts, and three-coat-color chimeras males derived from two clones (193 and 197) transmitted the floxed allele to their offspring. Mice homozygous for this mutation (FastLfl/fl) showed no obvious abnormalities. Moreover, the absence of the abnormal CD3+ B220+ CD4− CD8− T cells, typical of gld mice, indicated a normal function of the floxed allele (data not shown).

Animals heterozygous for the floxed allele were mated with PGK-Cre transgenic mice to generate mice deficient in FastL. As expected, 100% of the mice carrying the floxed allele had undergone Cre-mediated recombination. PCR analysis showed a 300-bp band for wild-type, a 370-bp band for deleted, and a 380-bp band for floxed allele (Fig. 1c). Then heterozygous mice carrying one wild-type and one deleted FastL allele were bred to generate animals homozygous for the mutation, and genotypes of the offspring were determined by PCR, as above. The three genotypes in the F2 animals were represented according to mendelian inheritance. Furthermore, mating between FastL+/− mice produced normal number of offspring and sex ratio, indicating that the null allele of FastL did not affect fecundity.

Mice derived from both clones (193 and 197) were similar regarding the efficiency of Cre-loxP-mediated recombination and the same phenotypes when homozygous for the null allele. Mice derived from clone 193 were characterized in detail.

FastL-deficient mice show severe lymphoproliferation

Female and male FastL−/− mice developed splenomegaly and lymphadenopathy in age-dependent manner. Splenomegaly was evident at 6–7 wk of age. Beyond 16 wk of age, the average weight of the spleen was 15 and 10 times higher than those of control littermates and C57BL/6 gld/gld mice, respectively (Fig. 2A). Similarly, the lymph nodes of 3- to 5-mo-old FastL−/− mice were considerably enlarged when compared with those of age-matched C57BL/6 gld/gld mice. As shown in Fig. 2B, the average weight of an axillary lymph node and the total number of recovered lymphoid cells were 3-fold higher in FastL−/− mice than in C57BL/6 gld/gld mice. These results indicate that the splenomegaly and lymphadenopathy of FastL−/− mice were accelerated and more pronounced than in gld mice.

Dominance of the CD3+ B220+ CD4− CD8− abnormal population in the periphery of FastL−/− mice

Abnormal CD3+ B220+ CD4− CD8− T cells were observed in the peripheral lymphoid organs of lpr and gld mice (2). We thus searched for this population by flow cytometry analysis. Results from Fig. 3 indicated that 70–87% of the cells that accumulated in the spleen and lymph nodes of FastL−/− mice at 12–13 wk of age were these CD3+ B220+ CD4− CD8− abnormal T cells. In gld mice, these abnormal T lymphocytes represented only 18–25% in the spleen and lymph nodes, while in wild-type and heterozygous littermate mice they were extremely rare. Fig. 3 also showed that although the proportion of normal T and B lymphocytes was reduced in FastL−/− mice compared with wild-type or heterozygous mice, their absolute number was increased, considering the very high cellularity of the lymphoid organs of these mice (Fig. 2). In fact, in extreme cases, the total number of lymphoid cells recovered from FastL−/− mice at 13 wk of age can reach 1.36 × 109 in the spleen and 594 × 109 per lymph node. These results indicated that the spleen and lymph nodes of FastL−/− mice were expanded not only with abnormal CD4− CD8− T cells, but also with normal T and B lymphocytes.

Despite the hyperplasia of peripheral lymphoid organs, the thymus of FastL−/− mice was of normal size as in gld, lpr, or FastL−/− mice (2, 4). Moreover, flow cytometry analysis showed an equal proportion of CD4+ CD8− double-positive and CD4+ CD8+ or CD4− CD8− single-positive cells in the thymus of 5-wk-old FastL−/− and FastL+/+ littermate mice (Fig. 3).

Hygammaglobulinemia and autoantibodies in FastL−/− mice

The pronounced phenotype of FastL−/− mice was accompanied by an increased Ig production. At 6–7 wk of age, levels of serum Ig

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in FasL−/− mice were 2–3 times higher than control littermates. These levels increased gradually, and mice beyond 16 wk of age produced 5 times more Ig than C57BL/6 gld/gld mice (Fig. 4A). In addition, the various IgG subclasses were elevated and significantly higher than in control littermates and C57BL/6 gld/gld mice (Fig. 4B).

Hypergammaglobulinemia is encountered in autoimmune prone mice that develop Abs against nuclear proteins (18, 19). Therefore, we examined the presence of Abs against dsDNA and IgG (RF) in the sera of 4- to 6-mo-old FasL−/−, control litters, and C57BL/6 gld/gld mice. Notably, circulating anti-dsDNA Abs were detected at high levels in FasL−/− mice compared with C57BL/6 gld/gld (Fig. 4C). However, the level of RF in FasL−/− mice, including mice that were backcrossed five times on C57BL/6 strain (FasL−/− BC5), was slightly higher than in C57BL/6 gld/gld, while in the sera of control litters, the levels of these Abs were very low (Fig. 4D).

Lymphocyte infiltration of the liver and salivary glands of FasL−/− mice

Examination of the FasL−/− mice indicated that the liver was larger (1.802 ± 0.448 g) than that of wild-type or heterozygous littermate mice (1.537 ± 0.233 g). This hepatomegaly was consistent with that described in Fas−/− mice (4). Histological examination of the enlarged liver of 14- to 17-wk-old FasL−/− mice revealed a frequent (8 of 10) lymphocytic infiltration ranging from moderate to severe. Small or large nodular lymphoid deposits were seen predominantly in the portal tract with some sinusoidal permeation, and, sometimes, lymphoid infiltration was disseminated within the hepatic parenchyma (Fig. 5A, a and b). This infiltration was never observed in wild-type litters (data not shown) or in age-matched gld mice (Fig. 5Ac). In addition to the liver, the salivary glands were also infiltrated with variable number of lymphoid cells, from small foci to moderate lymphoid deposits (Fig. 5A). In addition, the normal histology of the gld (Fig. 5Bf) or FasL−/−/+ mice (data not shown).

The total number of lymphoid cells recovered from the liver of FasL−/− mice was 3 times higher (6.7 ± 2.7 × 10⁶, n = 10) than in gld mice (2.5 ± 0.55 × 10⁶, n = 5) or FasL−/−/+ littermate mice (1.57 ± 0.36 × 10⁶, n = 10). Flow cytometry analysis of the intrahepatic lymphocytes of FasL−/− and gld mice indicated the presence of the abnormal CD3−B220−CD4−CD8− population (Fig. 6). However, the frequencies of these abnormal cells as well as that of normal CD4+ and CD8+ cells were higher in FasL−/− than in gld mice. Strikingly, the normal B220+ and CD4−CD8− cells were weakly represented in the liver of FasL−/− mice.

FasL−/− mice show histological signs of glomerulonephritis

We examined 15 FasL−/− mice aged from 3.5 to 6 mo, including FasL−/− BC5 mice, and 13 mice showed histological abnormalities. As shown in Fig. 5Cg, the kidney sections from FasL−/− BC5 mice indicated a marked increase in glomerular size and cellularity, accounted for by an increase in the number of mesangial cells.
and by the presence of inflammatory cells. Some of them had morphologically detectable deposits and microthrombi. It is worthy of note that this mouse had specially elevated serum level of RF (Fig. 4D). Immunofluorescence staining of the kidney revealed IgG deposition (Fig. 5C). In contrast, all the C57BL/6 gld/gld mice revealed glomeruli with normal size (Fig. 5Ch). Consistent with the kidney pathology, the majority of FasL−/− mice showed a significant proteinuria. Of 10 examined FasL−/− mice, 5 (1 from FasL−/− BC5) had urine protein levels greater than 100 mg/dl and 3 mice had proteinuria greater than 30 mg/dl. Only 2 FasL−/− mice showed low (<30 mg/dl) urine protein levels found in control littermates and C57BL/6 gld/gld mice.

The massive lymphoproliferation and autoimmune disease resulted in decreased survival of FasL−/− mice when compared with wild-type littermates. By 4 mo of age, >50% of mice died, and at 7 mo most animals (17 of 20) were dead, while 95% of wild-type littermates survived (Fig. 7). Although the life span of gld mice is known to be reduced compared with normal mice (18), it was not different from that of wild-type mice during a 1-year follow-up period (data not shown).

**Residual activity of FasL in gld mice**

In contrast to gld mice, which express a mutated protein (3), FasL−/− mice are devoid of FasL. Thus, the milder phenotype

**FIGURE 2.** FasL−/− mice develop severe lymphoproliferation. A, Splenomegaly of FasL−/− mice. Ten to 12 FasL+/+, FasL+/−, and FasL−/− mice, and 6 C57BL/6 gld/gld mice were sacrificed at the age of 6–7 and beyond 16 wk (4.5–6 mo), and the spleen was weighted. ——, Indicates the average weight. B, Lymphadenopathy of FasL−/− mice. Eight 3- to 5-mo-old FasL−/− and C57BL/6 gld/gld mice were sacrificed, and the axillary lymph nodes were weighted and the recovered cells were counted using the trypan blue exclusion method. ——, Indicates the average weight.

**FIGURE 3.** Flow cytometry analysis of lymphocytes from the spleen, lymph node, and thymus. Lymphocytes from spleen and lymph nodes of 12- to 13-wk-old FasL−/+, FasL+/−, FasL−/− littermates, and gld mice were stained with PE-conjugated anti-B220 and biotinylated anti-CD3 Abs, followed by allophycocyanin-conjugated streptavidin. Thymocytes were prepared from 5-wk-old FasL+/− and FasL−/− littermate mice and stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 Abs. Data are from one representative experiment of three.
FIGURE 4. Hypergammaglobulinemia of FasL<sup>−/−</sup> mice. A, Total Ig levels were assayed in FasL<sup>−/−</sup>, littermate controls, and C57BL/6 gld/gld mice by ELISA (n = 6). B, Ig isotypes were assayed in over 4- to 5-mo-old mice (n = 6). C and D, Autoantibody production. Anti-dsDNA Abs and RF were assayed by ELISA, as described in Materials and Methods, in 4- to 5-mo-old FasL<sup>−/−</sup>, littermate wild-type controls, and C57BL/6 gld/gld mice (n = 6).

FIGURE 5. Histopathology of FasL<sup>−/−</sup> mice. H&E-stained liver (A), salivary gland (B), and kidney (C). Heavy (a) and mild (b) liver infiltration of FasL<sup>−/−</sup> mice (×100), and c, represents normal hepatic parenchyma from gld mice (×100). B, Salivary gland infiltration. Heavy (d) to mild (e) infiltration (×100); f, represents normal salivary gland from C57BL/6 gld/gld mice (×100). Inserts, Show higher magnification of infiltrating cells (×400) that are indicated by large arrows. C, Kidney pathology. Trichrome-stained kidney sections from FasL<sup>−/−</sup> BC5 mice (g) and C57BL/6 gld/gld mice (h). IgG deposition in glomeruli of FasL<sup>−/−</sup> BC5 mice (i) and C57BL/6 gld/gld glomeruli (j).
observed in gld mice could be associated with a low residual FasL function. To test this hypothesis, we compared the ability of Con A-activated splenocytes from gld, FasL−/−, and wild-type littermates to kill target cells through the Fas/FasL pathway. The mouse Fas-transfected T cell line L1210 (L1210-Fas) was used as a target in a cytotoxicity assay (20). As shown in Fig. 8, activated splenocytes from FasL−/− mice were unable to kill L1210-Fas cells. In gld mice, although reduced compared with that of wild-type mice, the cytotoxic activity of activated splenocytes was significantly higher than that of FasL−/− mice (p = 0.02). To further determine the specific contribution of FasL on gld splenocyte-mediated killing of Fas+ target cells, FasL was blocked with Fas-Fc fusion protein before coincubation with target cells. As shown in the insert of Fig. 8, the killing ability of gld-activated splenocytes was significantly inhibited when FasL was blocked with increased concentration of Fas-Fc fusion protein. These results showed that on gld cells the expressed FasL has a residual function.

Discussion

FasL−/− mice developed splenomegaly and lymphadenopathy that were markedly accelerated and more pronounced than in gld mice, leading to the premature death of >50% of homozygous mice. In these mice, B lymphocytes are hyperactivated and the Ig production was dramatically increased. However, 2- to 6-mo-old FasL−/− mice did not show spontaneous B cell malignant transformation (data not shown), as assessed by Southern blot analysis using J chain probe (21). Therefore, the severe glomerulonephritis seen in most of FasL−/− mice, to add to the extreme enlargement of lymphoid organs, might be the principal cause of the accelerated death. However, we have noticed an internal bleeding and ascite formation, in some cases.

The degree of lymphoproliferation in mice carrying the gld or lpr mutation depends on the mouse strain (19, 22). In FasL−/− mice, which have a mixed genetic background, the splenomegaly and lymphadenopathy were considerably more important than in all mouse strains carrying the gld mutation described to date (18, 21, 23). For instance, the weight of the spleen and a single lymph node reached 6 and 1 g, respectively. Interestingly, FasL−/− mice that have been backcrossed five times on the C57BL/6 genetic background showed glomerulonephritis and proteinuria, while gld mice develop kidney disease only on MRL genetic background (19).

In FasL−/− mice, the massive lymphoproliferation also gave rise to the frequent infiltration of lymphocytes in the liver, salivary glands, and kidney. Liver infiltration was seen in mice deficient in Fas or transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) receptors (4, 24). Remarkably, the lymphohcytic infiltration of salivary glands was a constant feature, which revealed a novel phenotype resulting from the complete loss of FasL function. None of these organs was affected in C57BL/6 gld/gld mice. These results suggested that the cells accumulating in FasL−/− mice might have acquired the capacity to migrate into some organs. Another possible explanation is that the FasL deficiency has conferred to these tissues an increased susceptibility to
lymphocytic infiltration. The biochemical parameters such as aspartate amino transferase and alanine amino transferase serum levels appeared normal (data not shown), indicating that the hepatocytes are not damaged by the infiltrate. We do not know whether the absence of toxic effect of these infiltrating cells is associated with their FasL deficiency or, alternatively, because of their phenotype and activation state. In the liver of TACI-deficient mice, the invasive cells are mainly malignant B lymphocytes (24). Interestingly, FACS analysis demonstrated that B lymphocytes are weakly represented in the intrahepatic lymphocytes of Fasl−/− mice. In addition, although the CD3+ B220+ CD4− CD8− population is highly represented in Fasl−/− mice, the CD4+ CD8− population is lower than in C57BL/6 gld/gld mice or wild-type control littermates, indicating that in these Fasl−/− mice a subpopulation of CD4+ CD8− lymphocytes is missing (Fig. 6). Studies to further characterize these infiltrating cells are underway.

Ligands of the TNF family interacting with death domain-containing proteins have usually more than one receptor, and disruption of the genes coding for the ligand or its receptor produces different phenotypes, as in the case of, for instance, the TNF-α and its p55 receptor (25, 26). The similarity of the phenotype of Fasl−/− mice (3, 4, 27) and our Fasl−/− mice confirmed the fact that, unlike the other TNF family members, FasL binds Fas receptor exclusively and that it is its only ligand. However, in humans, the existence of another ligand, yet undefined, for the Fas receptor could explain the lack of FasL mutations in patients with lymphoproliferative syndrome (28). Another explanation for this unexpected absence of FasL mutations is the presence of a putative (but sustaining a vital function) other human Fas that could lead to a lethal phenotype in case of FasL deficiency. Defects of the Fas pathway were indeed identified in patients few years ago, and the syndrome was named autoimmune lymphoproliferative syndrome. As in the case of the natural mouse mutations, autoimmune lymphoproliferative syndrome patients show splenomegaly, lymphadenopathy, and the abnormal CD3+ CD4− CD8− lymphocytes. The majority of patients exhibited partial defect of Fas function associated with normal or reduced Fas expression (29–31).

The similarity of the phenotype associated with the two null alleles of Fas and Fasl genes revealed also that they differ from their respective natural mutation, lpr and gld, because mice carrying either mutation show a milder phenotype. The lpr, which is an insertion of a transposon into intron 2 of Fas gene, allows the expression of minute amounts of functional protein (32). In contrast, gld, which is a point mutation in the extracellular region of Fasl that affects its binding to Fas, has, to date, been considered as a null allele (3). However, the severe phenotype of our Fasl−/− mice led us to think that the gld allele could, indeed, encode a weakly interacting protein still able to deliver, at a very low level, a death signal to Fas receptor. This hypothesis explains the weak Fas-Fc binding, observed by Ramsdell et al. (33), on the surface of activated gld T cells and is strengthened by our observation of a specific Fas-dependent cytotoxic activity in gld splenocytes.

Fasl−/− mice that we produced showed no obvious abnormalities. Lymphocyte subpopulations were normally distributed, consistent with normal expression and function of the floxed allele throughout development. This novel mouse strain will therefore provide an invaluable tool to investigate the role of Fasl in different pathology, such as tumor escape or autoimmune diseases, by selectively deleting the Fasl gene in the target organ.

Acknowledgments

We thank Chantal Becourt for technical assistance at the beginning of the project. We warmly acknowledge F. Valette and M. García for expert assistance with mouse experiments, and Dr. Y. Lallemand for the gift of Cre transgenic mouse. We are grateful to Drs. H. J. Gachon and A. Tafuri for critical reading of this manuscript.

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