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Transgenic mice ubiquitously expressing human Fas ligand develop a slight form of graft-versus-host-like disease[1]

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KEY WORDS Fas ligand; graft *vs* Host disease; transgenic mice; flow cytometry; reverse transcriptase polymerase chain reaction; antisense DNA; fluorescent antibody technique

ABSTRACT

AIM: To construct transgenic mice bearing human Fas ligand (FasL/CD95L) cDNA, and further explore the physiological effects of ubiquitous expression of FasL on such animals. **METHODS**: Transgenic mice were produced by pronuclei microinjection method. Integration and transmission of transgene were identified by nest-PCR and Southern-blot analysis. Level of FasL mRNA was evaluated by semi-quantitative RT-PCR analysis. FasL protein was detected by immunofluorescence analysis. Morphological alterations in tissues were analyzed by histological examination. The percentage of αβT cells in the spleen was determined by flow cytometry analysis. **RESULTS**: Two independent founder mice bearing human FasL cDNA under the control of CMV promoter were generated healthily. Human FasL was moderately expressed in the majority of tissues examined in F1 heterozygotic mice. Although developing normally, adult transgenic mice exhibited a slight form of graft-versus-host (GVH)-like disease characterized by many morphological abnormalities occurring locally in the spleen, testis, lung and liver. In addition, the percentage of αβT cells in the spleen was respectively decreased approximately by 32 % and 24 % in two independent transgenic lines, relative to wild-type mice. **CONCLUSION**: Ubiquitous expression of Fas ligand can lead to slight GVH-like disease.

INTRODUCTION

Fas ligand (FasL/CD95L) is a M_r 40 000 type II transmembrane protein of the tumor nesrosis factor (TNF) family that induces apoptosis through interaction with Fas. FasL is expressed mainly on populations of T cells, B cells and NK cells[1-3], and its expression is regulated by cyclosporin A[4], retinoids[5], and CD3 stimulation[6]. Fas (CD95/APO-1) is a type I transmembrane protein in the TNF/nerve growth factor (NGF) receptor family that has a broader distribution, and is expressed on activated B and T cells, neutrophils, and macrophages, as well as nonlymphoid tissue including the testis and liver[7,8].

The interaction of FasL with Fas plays an important role in the downregulation of the immune response through several mechanisms that include induction of cell death after T cell activation (AICD)[9]. Altered expression of FasL and Fas has been related to the pathogenesis of many diseases associated with immune regulation. For example, loss of functional Fas/FasL, caused by homozygous expression of the *lpr* mutation of the Fas gene or *gld* mutation of the FasL gene, results in lymphoproliferation and autoimmunity in both humans[10] and mice[11]Constitutive expression of FasL has been reported in patients with large granular lymphocytic leukemia and NK cell leukemia[12]. Levels of both Fas and FasL have been shown to be increased in graft-versus-host (GVH) disease[13,14]. Recently, evidence has been presented for increased Fas expression in sudden infant death syndrome[15], Sjogren's syndrome[16], myositis[17], Down syndrome[18], and in human ageing[19].

Fas-mediated apoptosis has been recently associated with the phenomenon of immune privilege. Constitutive expression of FasL on sertoli cells in the testis, and epithelial cells in the anterior chamber of the eye, is be-

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lieved to be responsible for inducing apoptosis of infiltrating Fas-bearing activated T cells thus mediating immune privilege of these organs[20,21]. Mice genetically deficient in functional FasL do not exhibit immune privilege in the eye or testis, demonstrating the requirement of FasL for this phenomenon[22]. In addition, FasL expression on a subset of tumors may contribute to evasion of immune surveillance[23]. Bellgrau *et al*[24] employed a strategy based on the FasL model of immune privilege to prevent graft rejection successfully, they demonstrated that testicular sertoli cells expressing functional FasL were able to survive transplantation under the kidney capsule. Lau *et al*[25] reported that syngeneic myoblasts engineered to express FasL could delay rejection of pancreatic islet allografts when co-transplanted under the kidney capsule. Hence, down-regulation of immune responses through FasL expression appears to be an attractive approach forwarding maintenance of transplanted grafts. Because co-transplantation of grafts and FasL-expressing cells has a potential limit, and is not effective in large animals[26], many researchers turned to investigate whether direct FasL expression on transplanted grafts could prevent rejection or not. Here we report the establishment of transgenic mice bearing human FasL cDNA under the control of cytomegalovirus (CMV) promoter, and preliminarily characterize the phenotypic alterations in such mice for further study on transplantation.

MATERIALS AND METHODS

Generation of transgenic mice Human FasL 865-bp cDNA fragment corresponding nucleotides (nt) 69 – 934 (GenBank accession: U08137), containing entire coding sequences was obtained by RT-PCR using total RNA isolated from peripheral blood lymphocytes according to the method of Takahashi *et al*[27] and confirmed by sequencing analysis. The primers used for PCR were 5′ ACTGAATTCTGACTCACCAGCTGCCATGC3′ (corresponding to nt 69 – 89; underline denotes EcoR I site), and 5′ATCCTCGAGCTATTAGAGCTTATATAAGCCG3′ (complementary to nt 913 – 934; underline denotes Xho I site). The amplified FasL cDNA fragment was cloned into the pcDNA3 vector under the control of CMV promoter/enhancer. The construct was linearized with Nru I and Tth111 I, gel-purified, and then microinjected into the pronuclei of fertilized eggs of C57BL/6J F1 hybrid mice (Jackson laboratory) to produce transgenic mice according to the standard method. F0 newborn mice were screened for genomic integration of transgene as described below. Transgene-positive founder mice and their descendents and wild-type control mice were housed in groups (< 4 mice per cage) in temperature-and humidity-controlled environment with 12-h light/12-h dark cycle.

Genotypic characterization of transgenic mice For nest PCR analysis, genomic DNA was isolated from the tails of 4-week-old F0 newborn mice. The primers described above (P2-P3 indicated in Fig 1a) were employed to specifically amplify FasL cDNA. Another primer (5′TGGAGTTCCGCGTTACATAACTTACG3′, P1 indicated in Fig 1a) was located in the upstream of CMV promoter region. With P2-P3 and P1-P3 primer pairs, amplification was respectively carried out as follows: 94 ℃ for 45 s, 60 ℃ for 1 min, and 72 ℃ for 1 min; 34 cycles.

For Southern-blot analysis, 10 μg genomic DNA extracted from the tails of F1 mice was digested with Nde I and Xho I, fractioned by electrophoresis on 1.0 % gel, and transferred to a nylon membrane (Amersham), followed by hybridization with a randomly ^{32}P-labeled FasL cDNA (EcoR I – Xho I) fragment excised from the transgenic construct (Fig 1a). After hybridization, blots were washed and then exposed against an X-ray film for 24 h.

Semi-quantitative RT-PCR analysis A variety of tissues were removed from 2-month-old wild-type mice and F1 transgenic heterozygotes after cervical dislocation. Whole RNA was extracted with Trizol reagent (GIBCO) according to the manufacturer's instructions, then identified by formaldehyde-electrophoresis. Before reverse transcription, RNA samples were thoroughly treated by incubating with RNA-Free DNase I (5 U/g RNA; Promega) for 45 min at 37 ℃. Using FasL-specific primers (P2-P3), PCR was performed with conditions mentioned above. GAPDH mRNA was co-detected with primers (5′ACGACCCCTTCATTGACC3′; 5′AGACACCAGTAGACTCCACG3′) which matched nt 141 – 344 of murine GAPDH cDNA. Resultant RT-PCR products were referred to normalize the expression level of FasL mRNA. Relative intensities of PCR fragments were estimated with a Molecular Image FX System (Bio-Rad). RT-omitted RNA samples were directly amplified by PCR with FasL-specific primers and 5 fold amounts of the corresponding templates to demonstrate whether the resultant products were derived from mRNA or genomic DNA.

Immunofluorescent detection Two-month-old F1 transgenic heterozygotes and wild-type mice were anesthetized with sodium pentobarbital, and consequently

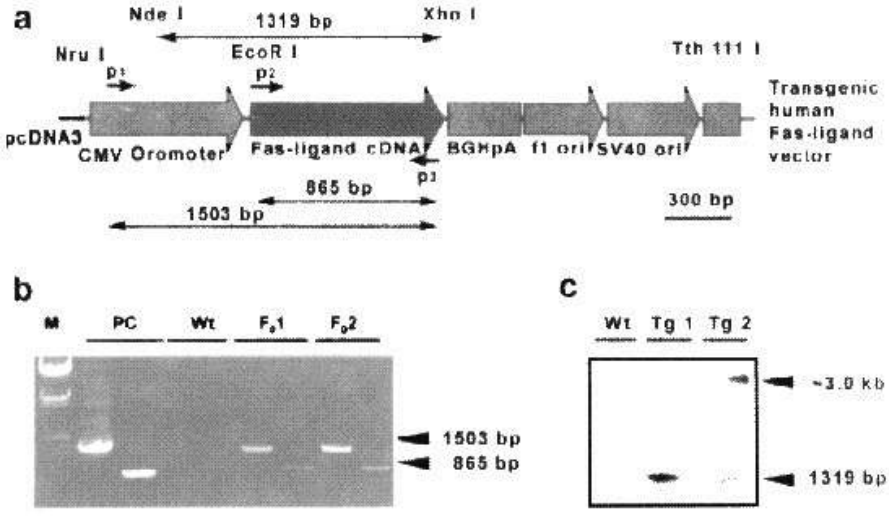

Fig 1. a) Schematic illustration of the human FasL transgene construction. b) Nest PCR identification of the integration and integrity of transgene in two independent founders ($F_0$1 and $F_0$2). Primer positions and predicted size of PCR products are indicated in "a". M λ/Hind III & EcoR I; PC positive control (plasmid); Wt wild-type mouse. c) Southern-blot analysis of the transmission of transgene. Ten μg genomic DNA extracted from tails of F1 mice were digested with Nde I and Xho I, electrophoresed, blotted, and hybridized with ^{32}P-labeled human FasL cDNA. Arrow shows the predicted band, whose location and size are indicated in "a"; arrowhead indicates the approximately 3.0 kb band resulting from asymmetrical cleavage of the tandem copies of transgene by Nde I and Xho I; Tg 1 and Tg 2 indicate F1 heterozygotic mice raised from two independent founders respectively.

perfused intracardially with 95 % ethanol. After perfusion, tissues were surgically removed and then immersed in 30 % sucrose in phosphate-buffered saline (PBS), subsequently frozen in OCT. Cryosections 20 μm thick were cut and air-dried onto the APES-coated slides. In staining procedure, sections were first blocked with 10 % goat serum containing 1 % BSA and 0.2 % Triton X-100 in PBS, and then incubated with anti-human FasL rabbit polyclonal antibody (Sc-957, Santa Cruz) at 4 ℃ overnight, the sections were subsequently exposed to FITC-conjugated anti-rabbit IgG goat polyclonal antibody (Sc-2012, Santa Cruz) at 4 ℃ overnight, and finally coverslipped with PBS-buffered glycerol. Between each step, sections were washed in PBS three times and each time for 10 min. Sections were examined and photographed under Olympus fluorescence microscope.

Histology Animals and perfusion procedure were same as described above except that the fixative was substituted by 4 % paraformaldehyde in 0.1 mol/L phosphate buffer. Tissues were removed and dehydrated through up graded ethanol, and then cleared with xylene, finally embedded in paraffin. 5 μm thick-sections were cut and stained with haematoxylin and eosin according to the standard protocol, then examined and photographed under Leitz microscope.

Flow cytometry analysis Single-cell suspensions of spleen cells from wild-type mice and F1 transgenic heterozygotes were prepared by mechanical isolation and subsequent filtration through a hole density-optimized nylon fiber. Samples were treated with 0.168 mol/L NH_4Cl to lyse erythrocytes. Splenocytes were washed with PBS containing 1 % BSA, and then labeled with optimal concentration of FITC-conjugated anti-mouse TCRβ chain hamster monoclonal antibody (PharMingen) at 4 ℃ for 45 min, followed by intensive washing with PBS for 10 min, finally suspended in 400 μL PBS and analyzed by flow cytometry on FACScan (Becton Dickinson, CA), and the data were processed in a Hewlett-Packard (Palo Alto, CA) computer.

Statistical analysis Mean values and difference were analyzed by unpaired t-test.

RESULTS

Establishment of human FasL transgenic

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mice The human FasL cDNA fragment containing entire coding sequences under the control of CMV promoter/enhancer was introduced into C57BL/6J F1 hybrid mice germ line by microinjection method (Fig 1a). Among 52 newborn mice, 25 died at various stages of development before attaining adulthood, and the remaining 27 grew up normally (Tab 1). To identify the integration and integrity of transgene, nest PCR analysis was carried out (Fig 1b). Using FasL specific primers (P2 P3 indicated in Fig 1a), 2 among 27 newborn mice alive yielded the predicted 865-bp fragment. Further amplification using the primer (P1 indicated in Fig 1a) located in the upstream region of CMV element and the complementary primer (P3) matched FasL cDNA also yielded the expected 1503-bp fragment, demonstrating that the two mice, termed founder mice, bore the intact transgene. Founder mice were bred with normal C57BL/6J mice to generate F1 mice. Southern blot analysis demonstrated that 29 among 54 F1 mice were heterozygous for transgene, which was consistent with the predicted Mendelian ratio. As shown in Fig 1c, transgenic heterozygotes yielded the predicted 1319-bp fragment and another approximately 3.0-kb fragment resulted from asymmetrical digestion of tandem copies of transgene, whereas neither fragment could be detected in wild-type control mice. It indicated that the transgene could be transmitted from both independent founder mice to their offspring.

Tab 1. Generation of human FasL transgenic mice.

	Number
Eggs transferred	665
Pseudopregnant recipients	19
F0 total newborn mice	52
F0 newborn mice died during development	25[1]
Founder mice (F0)	2
F1 newborn mice[2]	54
F1 heterozygotic mice[2]	29

1) Seventeen mice of the 25 were killed and eaten by their surrogate mothers at 1 – 5 d after birth; other 8 mice died at 1 – 3 weeks after birth without any macroscopic injury.
2) Mice were generated by crossbreeding founder mice with normal C57BL/6J mice.

Characterization of the expression of FasL in transgenic mice The expression level of FasL transcript in F1 mice heterozygous for transgene, derived from one founder, was evaluated by semi-quantitative RT-PCR analysis (Fig 2). Using a same primer-pair, endogenous murine FasL mRNA (corresponding to nt 175 – 1023) yielded a 858-bp fragment (in which 10-bp nucleotides were introduced by 5′-terminal mismatched sequences of the primers), whereas transgenic human FasL mRNA (corresponding to nt 69 – 934) yielded a 865-bp fragment (Fig 2a). In a variety of tissues examined, endogenous murine FasL mRNA was detected in the brain, spleen and testis, which was consistent with previous reports[1-3,20,21,28]. However, endogenous FasL mRNA was also detected at a relatively low level in the

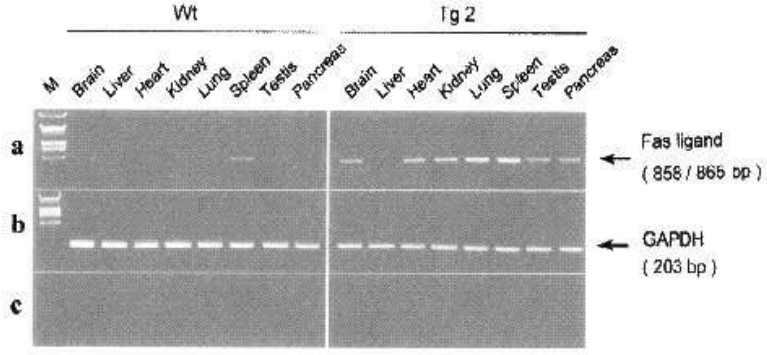

Fig 2. Semi-quantitative RT-PCR analysis of FasL expression in a variety of tissues. a) Using the FasL-specific primers, 858-bp fragments corresponding to endogenous murine FasL were obtained in brain, heart, spleen, testis from both wild-type (Wt) and transgenic mice (Tg 2), whereas 865-bp fragments corresponding to transgenic human FasL could be achieved in all tissues except liver only from transgenic mice. All resultant products were subsequently sequenced to confirm their origin. Note that the two fragments respectively derived from endogene (murine) and transgene (human) can not be discriminated by their mobility here. b) The amplification of GAPDH serving as an internal standard. c) The direct amplification with RT-omitted RNA samples using the primers employed in "a".

heart. The possibility that it resulted from the infiltrating activated lymphocytes (Fas^+) into the heart could be excluded since we did not observe this phenomenon on histological examination. Contrasting sharply with wild-type mice, transgenic human FasL mRNA could be detected in all tissues examined except liver from transgenic mice. It is noteworthy that the endogenous murine FasL was simultaneously expressed in the brain, spleen, testis and heart of the transgenic mice. All resultant PCR products were cloned and subsequently sequenced to confirm their origin (data not shown). Quantitative analysis by normalization to internal GAPDH mRNA (Fig 2b) revealed that transgenic human FasL was moderately expressed with varying levels in various tissues. Additionally, no specific PCR products from the reverse transcription-omitted RNA samples could be obtained (Fig 2c), which convinced that the amplification of FasL was derived from mRNA other than genomic DNA.

To determine whether transgenic mice express human FasL protein, we performed *in situ* immunofluorescent detection with polycolonal antibody raised against a carboxy-terminal peptide (extracellular domain) from human FasL (Santa Cruz). As shown in Fig 3, all tissues examined including lung, spleen and testis from transgenic mice exhibited FasL-immunoreactivity with moderate intensity, which was consistent with the results of RT-PCR analysis. Specific staining of the bronchial epithelial cells but not alveolar cells in the lung possibly reflects the cell-type specific expression pattern of transgene driven by CMV promoter, as previously suggested by other researchers[29]. Unexpectedly, spleen and testis from wild-type mice, in which constitutive expression of FasL was previously suggested[20,21], were also labeled with relatively low intensity, relative to transgenic mice. It can be probably attributed to the fact that the immunoreactivity with FasL of the antibody employed in the present study is not confined to human origin. The cross-reactivity of the antibody produced by Santa Cruz Biotechnologies has also been discovered recently by other investigators[30,31]. However, contrasting sharply with transgenic mice, the lung from wild-type mice, which was demonstrated by RT-PCR analysis to be FasL mRNA-absent, was not labeled, strongly suggesting that the observed immuneoreactivity of the antibody employed was specific to FasL. With all above mentioned results, we conclude that the human FasL is ubiquitously expressed in transgenic mice.

Morphological abnormalities in transgenic mice Transgenic mice ubiquitously expressing human FasL developed normally grossly. However, there was evidence for increased necrosis in some tissues of these mice. As shown in Fig 4, transgenic mice exhibited decreased density of cells and disperse marginal zone in the spleen, atelectasis accompanied by hyperaemia in the lung, and vacuolated spermatogenic epithelium within the seminiferous tubules with expanded lumen in the testis in contrast to wild-type mice. Particularly, increased amount of Kupffer cells with remarkably expanded size associated with focal necrosis, characterized by cytoplasmic condensation and nuclear fragmentation, was observed in the liver of transgenic mice compared with that of wild-type mice. It should be pointed out that these abnormalities were local events, and did not occur throughout the whole region of the related tissues. Most of the morphological alterations described above are considered to be consistent with slight GVH disease features. No apparent morphological changes were observed in other tissues examined including brain, heart, kidney and pancreas.

Reduced αβT cells in the spleen of transgenic mice Single-cell suspensions of spleen cells from unstimulated transgenic mice exhibited a reduced percentage of $\alpha\beta T$ cells, relative to wild-type mice (Fig 5). Statistical analysis revealed that the percentage of αβ TCR-bearing T cells in the spleen was respectively reduced by 32 % in Tg 1 mice and 24 % in Tg 2 mice compared with wild-type mice ($P < 0.05$). Considering the apparent cell depletion in the spleen demonstrated by histology (Fig 4), this result indicated that total T cells in the spleen of transgenic mice decreased in number compared with control, since approximately 95 % peripheral mature T cells express α and β chain.

DISCUSSION

Two independent founder mice and their progenies bearing human FasL cDNA under the control of CMV promoter were viable and fertile. However, approximately half of the F0 newborn mice died during development before attaining adulthood (Tab 1), suggesting that selective pressure operated against these mice. A previous study suggested that excessive FasL production by the transgene might be lethal, even targeted to the T cells[32]. It is also possible that the mice in this study died for this very reason. The opinion is potentially supported by the observation that these transgenic mice established did not express very high levels of human FasL.

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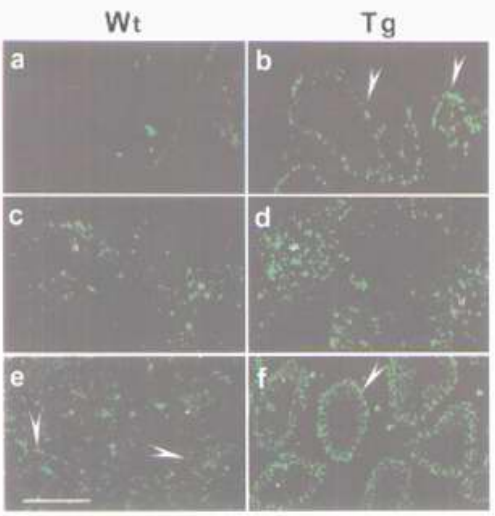

Fig 3. Immunofluorescent detection of FasL protein in the lung (a, d), spleen (b, e) and testis (c, f) from wild-type (Wt, left column) and transgenic mice (Tg 2, right column). Arrowheads in d indicate the bronchial epithelial cells; asterisks in b and c indicate the white pulp; arrowheads in e and f point to the specifically stained cells within seminiferous tubules. Scale bar represents 100 μm. 20 μm-thick cryosections.

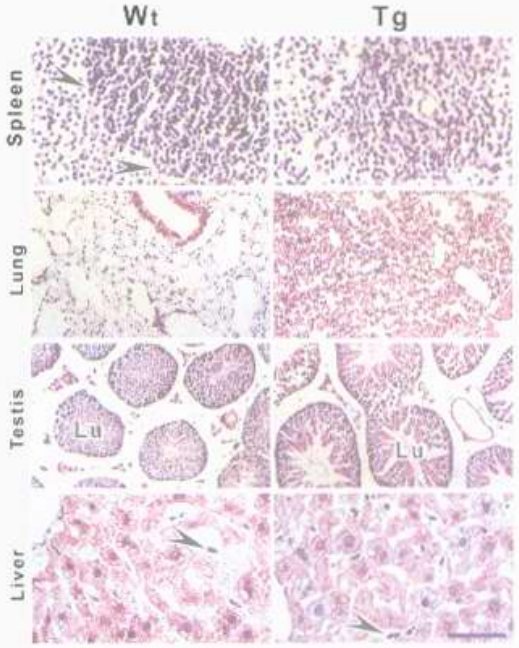

Fig 4. Histological appearance of the spleen, lung, testis, and liver from wild-type (Wt, left column) and transgenic mice (Tg, right column). Note the morphological alternations in Tg mice in contrast to Wt mice, which include decreased density of cells and the disperse marginal zone (indicated by arrowheads in Wt) in the spleen, atelectasis accompanied by hyperaemia in the lung, striking vacuolization in the spermatogenic epithelium of seminiferous tubules with expanded lumen (Lu) in the testis, increased amount of Kupffer cells (indicated by arrowheads) with remarkably expanded size associated with necrosis (discriminated by abnormal color-staining) featuring cytoplasmic condensation and nuclear fragmentation in the liver. Scale bar represents 100 μm in lung and testis, 50 μm in spleen and liver, respectively. Haematoxylin and eosin staining of 5 μm-thick paraffin-embedded sections.

Unfortunately, characterization of the integration as well as expression of the transgene in these dead mice was not performed for some unidentified reason. GVH disease associated with immune cell depletion and damage of liver and lung has been attributed to excessive production of FasL[12,33,34]. Consistent with these studies, our mice heterozygous for human FasL developed slight GVH-like disease. In addition to the depletion of immune cells in the spleen, these transgenic mice exhibited increased necrosis in the lung, testis and liver, relative to wild-type mice. However, the damages were local events within these organs, potentially evidenced by the fact that these mice remained healthy during development. Cheng *et al*[32] previously demonstrated that transgenic mice overexpressing murine FasL specifically in αβT cells could lead to a mild form of GVH disease associated with evidently increased apoptosis through Fas/FasL system. The damage observed in their mice was similar to that in our mice, but seemed relatively severe. It is known that human FasL possesses 76.9 % similarity in amino acid sequences with murine FasL, furthermore, cross-interaction between human Fas/FasL and murine Fas/FasL induces apoptosis at the same efficiency[27]. Hence, damage occurring in our mice possibly resulted from enhanced apoptosis through Fas/FasL pathway as a consequence of human FasL widespread-expression. Interestingly, increased amount of Kupffer cells was observed to be associated with the necrosis in the liver of transgenic mice. In view of the previous study that endotoxin-induced liver injury is mediated by Fas/FasL pathway following induction of Fas by interleukin (IL)-18, secreted by Kupffer cells[35], it may hint at the involvement of Fas-mediated apoptosis in the damage observed in our mice. However, surprisingly, we could not detect the expression of human FasL in the liver of transgenicmice. On the other side, some other organs expressing transgene appeared to

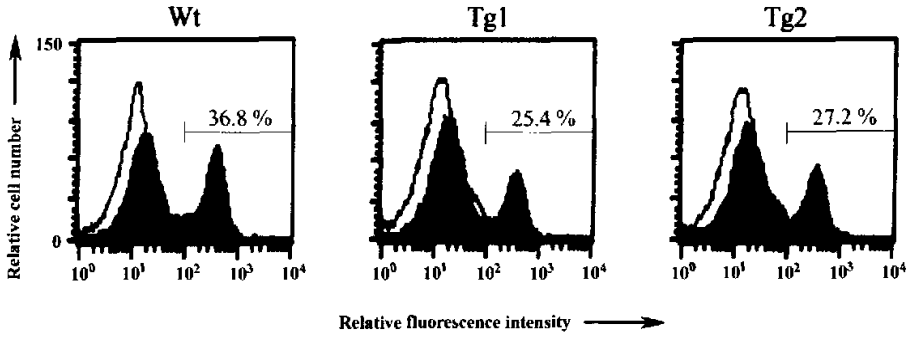

Fig 5. Flow cytometry analysis of αβT cells in the spleen of wild-type (Wt) and two independent lines of transgenic mice (Tg 1, Tg2). Shown are representative samples. Kidney cells served as negative control (closed area under the solid line). The percentage of αβ TCR-bearing T cells in the total spleen cells (black areas) is indicated by the cursor. $n = 4$ mice individually analyzed in two separate experiments for each group.

develop normally. Additionally, the percentage of αβT cells in the spleen of transgenic mice was found to be reduced compared with wild-type mice, but the underlying mechanism and its correlation with the tissue damage remains unknown. Such issues warrant further study.

Studies on transplantation employing FasL-based strategies have been conflicting ever since they were proposed as an attractive idea. Some investigators suggested that co-transplanted carrier cells genetically engineered to express FasL could prevent allograft rejection[24,25,36]. However, other researchers demonstrated that FasL expression in grafts did not confer immune privilege and instead targeted them for rapid rejection[26,37-40]. They claimed that FasL expression in grafts evoked neutrophilic infiltration resulting in their destruction through an as yet poorly understood mechanism. Similarly, transgenic mice specifically expressing FasL in pancreatic β cells developed massive neutrophilic infiltrates and consequent diabetes at a young age[26]. It is noteworthy that no apparent neutrophilic infiltration was observed in all tissues examined in our mice. Recently, several lines of transgenic animals expressing FasL with a tissue-specific pattern in various tissues have been generated[26,32,37,41-43], and some of them were employed in the study on transplantation. Unfortunately, no study has been proved to be clinically useful for transplantation so far. It is obviously beneficial to assess transgenic lines ubiquitously expressing FasL such as our mice in relation to transplantation studies.

In summary, transgenic mice ubiquitously expressing human FasL were generated in this study and these mice developed normally but exhibited a slight form of GVH-like disease.

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广泛表达人 Fas 配体的转基因小鼠发生轻微类移植物抗宿主疾病[1]

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关键词 Fas 配体；移植物抗宿主病；转基因小鼠；流式细胞术；逆转录聚合酶链反应；反义 DNA；荧光抗体技术

目的：建立 Fas 配体(FasL/CD95L)转基因小鼠并研究 FasL 广泛表达的生理效应. **方法**：原核显微注射法产生转基因小鼠，巢式 PCR 和 Southern-blot 鉴定转基因的整合及其完整性，半定量 RT-PCR 分析 FasL mRNA 水平，免疫荧光检测 FasL 蛋白质的表达，组织切片染色检查形态学变化，流式细胞仪检测脾脏 αβT 细胞百分比. **结果**：获得 2 只含置于 CMV 启动子控制下人 FasL cDNA 的建立者小鼠. F1 代杂合体小鼠的大部分组织可检测到人 FasL 中等程度的表达. 转基因小鼠发育正常，但脾、睾丸、肺和肝局部区域形态异常，具有轻微类移植物抗宿主疾病特征. 与野生型对照相比，来源于二个建立者的转基因小鼠 αβT 细胞在脾脏的百分比分别减少了约32 %和24 %. **结论**：广泛表达 FasL 可导致轻微类移植物抗宿主疾病.

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