

T Cell-specific Ablation of Fas Leads to Fas Ligand-mediated Lymphocyte Depletion and Inflammatory Pulmonary Fibrosis

Zhenyue Hao,¹ Brigitte Hampel,¹ Hideo Yagita,³ and Klaus Rajewsky^{1,2}

¹Institute for Genetics, University of Cologne, D-50931 Cologne, Germany

²The CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA 02115

³Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Abstract

To study the role of Fas–Fas ligand (FasL) interaction-mediated apoptosis in lymphocyte homeostasis, we generated a mutant *fas* allele allowing conditional inactivation of the *fas* gene through Cre-mediated recombination. Experiments in which Fas was ablated in T cells, B cells, T and B cells, or in a more generalized manner demonstrated that the development of lymphoproliferative disease as seen in Fas-deficient mice requires Fas ablation in lymphoid and nonlymphoid tissues. Selective inactivation of Fas in T cells led to a severe lymphopenia over time, accompanied by up-regulation of FasL on activated T cells and apoptosis of peripheral lymphocytes. In addition, the mutant animals developed a fatal wasting syndrome caused by massive leukocyte infiltration in the lungs together with increased inflammatory cytokine production and pulmonary fibrosis. Inhibition of Fas–FasL interaction *in vivo* completely prevented the loss of lymphocytes and initial lymphocyte infiltration in the lungs. Thus, FasL-mediated interaction of activated, Fas-deficient T cells with Fas-expressing cells in their environment leads to break down of lymphocyte homeostasis and development of a lung disease strikingly resembling idiopathic pulmonary fibrosis in humans, a common and severe disease for which the mutant mice may serve as a first animal model.

Key words: lymphocyte homeostasis • lymphopenia • apoptosis • lungs • conditional gene targeting

Introduction

Fas (CD95/Apo-1) is a member of the TNF-R family expressed on a variety of cells, including thymocytes, T cells, activated B cells, macrophages, and neutrophils, as well as organs such as liver, lung, and heart (1). Engagement of Fas by Fas ligand (FasL) or agonist anti-Fas antibody induces Fas-positive cells to undergo apoptosis (2). FasL is a TNF-related type II transmembrane molecule predominantly expressed on activated T and natural killer cells, as well as in immune privilege sites such as testis, eye, and brain (1). Fas–FasL interactions down-regulate immune responses through the induction of apoptosis of activated Fas-positive cells during immune response (2). The mutation of Fas in *lpr* or FasL in *gld* mice leads to autoantibody production and lymphoproliferative disease (3, 4) and, in humans, to a severe autoimmune

lymphoproliferative syndrome (ALPS; references 5, 6). The cellular basis of ALPS is not well understood, although T cells clearly play a critical role, given that lymphoproliferation involves a peculiar type of T cell (so-called “double negative” [DN] cells, whose immunophenotype is Thy1⁺ B220⁺CD4[−]CD8[−]) and is prevented by thymectomy (7, 8). Furthermore, transgenic restoration of Fas expression in T cells of *lpr* mice rescues the mice from lymphoproliferative disease (9, 10). B cells also seem to be involved in the *lpr* syndrome because, in B cell-deficient *lpr* mice, lymphoproliferation is largely inhibited (11). However, Fas deletion in lymphocytes *per se* does not seem to be sufficient to cause ALPS because the transfer of BM cells from MRL^{*lpr/lpr*} mice to lethally irradiated congenic wild-type recipients results in a severe wasting syndrome instead, resembling GVHD (12, 13). However, GVHD did not develop when BM from

Address correspondence to Zhenyue Hao at his present address Advanced Medical Discovery Institute, 620 University Ave., Toronto, ON, M5G 2C1, Canada. Phone: (416) 946-4501; Fax: (416) 204-2278; email: zyhao@uhnres.utoronto.ca; or Klaus Rajewsky, The CBR Institute for Biomedical Research, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 278-3132; Fax: (617) 278-3129; email: rajewsky@cbr.med.harvard.edu

Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; BALF, bronchoalveolar lavage fluid; DN, double negative; FasL, Fas ligand; IPF, idiopathic pulmonary fibrosis; MIP-2, macrophage inflammatory protein 2.

MRL mice lacking both Fas and FasL was transferred (14). This suggests that the development of GVHD in this transfer system may be based on the interaction of Fas-deficient T cells with Fas-expressing cells in the environment through Fas–FasL interaction, and that the development of ALPS requires the absence of Fas on lymphocytes and nonlymphoid cells. Paradoxically, tetraparental mice derived from the fusion of *lpr* and wild-type blastocysts were found not to develop a GVHD-like wasting syndrome, but rather the typical *lpr* phenotype characterized by lymphoproliferation and autoantibody production, respectively (15, 16).

To clarify these matters, it seemed desirable to establish a system in which Fas is selectively inactivated in a tissue-specific or inducible manner in the intact mouse, thus avoiding complications from cell transfers and whole-body X-irradiation, which by itself causes cellular damage and makes the animal prone to disease. Therefore, we have generated a mouse strain allowing conditional Fas inactivation upon Cre recombination. We find that, on the C57BL/6 genetic background, mutant animals with Fas inactivation in T cells, B cells, or both types of cells do not develop ALPS, and an attenuated form of the disease develops under these conditions on the autoimmune-prone (C57BL/6×MRL)F1 background, compared with the disease resulting from fas gene inactivation in the germ line. However, T cell-specific Fas inactivation in C57BL/6 mice leads to a FasL-dependent profound T and B cell deficiency over time, together with a chronic inflammatory and fatal lung disease strikingly resembling idiopathic pulmonary fibrosis (IPF) in humans (17).

Materials and Methods

Generation of Conditional fas KO Mice. A clone containing the *fas* genomic locus was provided by S. Nagata (Osaka University Medical School, Osaka, Japan). A gene-targeting construct was generated to flank exon IX coding for the death domain by two *loxP* sites. Through standard cloning, a *loxP* site and a *loxP*-flanked neomycin-resistant cassette were cloned into the upstream and downstream of exon IX, respectively. The thymidine kinase (*tk*) gene was added to the 5' ends of the targeting vector. Bruce-4 embryonic stem cells derived from C57BL/6 mice were cultured, transfected, and selected as described previously (18). The homologous recombinant clones were transiently transfected with a Cre-encoding plasmid to remove the *loxP*-flanked neomycin cassette. Male chimeras were mated with C57BL/6 females to obtain germline transmission of the *fas*-floxed allele (*fas^{fl}*). Deletion of exon IX encoding the death domain would completely abolish the apoptotic function of Fas. *Fas^{fl/fl}* mice were crossed to *CD4-cre* mice provided by C. Wilson (University of Washington, Seattle, WA; reference 19) or *lck-cre* (20) and *CD19-cre* transgenic mice (21) to achieve T and B cell-specific Fas ablation. Mice with Fas inactivation in both T and B cells were obtained by combining the two *cre* alleles with the *fas^{fl}* allele. To establish a system of inducible *fas* inactivation, *fas^{fl/fl}* mice were bred with *Mx-cre* transgenic mice in which the *cre* transgene is under the control of the type I IFN-inducible *Mx* promoter (22). All mice used had been backcrossed to C57BL/6 for 5–10 generations unless otherwise stated. For the study of lymphoproliferation on the (C57BL/6×MRL)F1 background, we crossed tissue-specific Fas KO mice on the C57BL/6 background with MRL^{*lpr/lpr*} mice. All mice used

in this work were housed in a conventional animal facility in the Institute for Genetics, Cologne, Germany. All animal studies were approved by the institutional review board.

Induced Generalized Inactivation of fas. To induce generalized Cre expression and subsequent inactivation of *fas*, 8–10-wk-old mice of the genotype *fas^{fl/fl}* and *fas^{fl/fl}*, *Mx-cre* were given 3 × 400 μg Poly(I) · Poly(C) (Amersham Biosciences) i.p. on days 0, 3, and 6.

Assessment of Lymphoproliferation. To evaluate the severity of lymphoproliferative disease, spleens were weighed and analyzed for DN (i.e., Thy1⁺B220⁺CD4⁻CD8⁻) cells by FACS[®]. All enlarged LNs were weighed, and only the biggest LN was analyzed by FACS[®] in mice displaying lymphadenopathy; inguinal and cervical LNs were collected for weight and FACS[®] analysis in animals that did not display unusually big LNs.

Flow Cytometry. 10⁶ lymphoid cells were stained with fluoro-chrome- (FITC, PE, or Cychrome) or biotin-conjugated monoclonal antibodies for flow cytometric analysis. Streptavidin-Cychrome was used to reveal biotinylated antibodies. The following homemade mAbs were used: R33-24-12 (anti-IgM), 1.3-5 (anti-IgD), RA3-6B2 (anti-B220), and Thy1.2 (Cfo 1). mAbs against active caspase-3, CD3, CD4, CD8, CD19, CD25, CD44, CD69, CD62L, annexin V, Fas (Jo2), and FasL (MFL3) were purchased from BD Biosciences. Staining of LN cells with anti-FasL antibody (MFL3) was performed in the presence of a matrix metalloproteinase inhibitor from BD Biosciences. Staining of LN cells for active (cleaved) caspase-3 was performed according to the manufacturer's instruction (BD Biosciences).

Immunohistology. Lungs inflated with 50% OCT in PBS and spleens were embedded in OCT and frozen on dry ice. Frozen sections were cut, air-dried, and fixed with cold (–20°C) acetone. For immunostaining, sections were rehydrated in PBS and stained with monoclonal antibodies against mouse CD3, CD19, Gr1 (BD Biosciences), and F4/80 (Serotec) followed by alkaline phosphatase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories) or streptavidin-peroxidase/streptavidin-alkaline phosphatase (Boehringer) for biotinylated antibody. After washing, bound peroxidase was revealed by 3-amino-9-ethyl carbazole (Sigma-Aldrich), and biotin by alkaline phosphatase substrate kit III/blue (Vector Laboratories). The TUNEL assay was done using the DeadEnd[™] Fluorometric TUNEL System or DeadEnd[™] Colorimetric TUNEL System (Promega). Hematoxylin and eosin staining and elastic fiber staining were performed according to the manufacturer's protocol (Sigma-Aldrich).

Measurement of Cytokines. Serum cytokine levels were measured by ELISA according to the manufacturer's instructions (R&D Systems). In addition, lungs were subjected to intrabronchial lavage (three washes of 500 μl PBS). Cytokine levels in the bronchoalveolar lavage fluid (BALF) were measured in the same way as for the serum.

Application of Anti-FasL Antibody In Vivo. Anti-mouse FasL mAb MFL1 or MFL3 (23), or isotype-matched hamster IgG1 (BD Biosciences) were injected intravenously (0.3 mg/mouse, twice a week) or intraperitoneally (0.5 mg/mouse, twice a week) into 14-wk-old *fas^{fl/fl}* and *fas^{fl/fl}*, *CD4-cre* mice. 8 or 12 wk after administration, animals were killed and analyzed for cellularity in the lymphoid organs and immunohistology of spleens and lungs.

Results

Generation of a Mouse Strain Allowing Tissue-specific Inactivation of Fas. A conditional *fas* allele (*fas^{fl}*) in which the death domain-encoding exon IX flanked by *loxP*-sites (Fig. 1, a and b) was generated to allow conditional inacti-

vation of Fas through cell type-specific expression of Cre recombinase. Fas^{fl/fl} mice showed normal lymphocyte development and subset distribution as determined by FACS[®] analysis (unpublished data). Deletion of the *loxP*-flanked (floxed) *fas* sequence through crossing to the *deleter* strain (24) produced mice carrying an inactive *fas* allele (*fas*^{del}) in all cells of the body. As expected, *fas*^{del/del} mice developed the typical *lpr* phenotype (unpublished data). To delete Fas selectively in T cells, *fas*^{fl/fl} mice were crossed to *CD4-cre* transgenic mice in which the cre coding sequence is under the control of a CD4 minigene (19). Southern blot analysis of DNA isolated from thymus and various cell populations in the spleen of *fas*^{fl/fl}, *CD4-cre* mice demonstrated efficient Cre-mediated recombination in T cells (Fig. 1 c). In the nonlymphocyte population of the spleen, a band characteristic for the *fas*^{del} allele was also detectable, but its intensity (16% of that representing the *fas*^{fl} allele) corresponded to the fraction of contaminating T cells (17%). The absence of Fas expression in thymocytes of the compound mutants was also confirmed by FACS[®] analysis at the protein level (Fig. 1 d). Similarly, efficient Cre-mediated recombination in B cells, and both T and B cells was observed in *fas*^{fl/fl}, *CD19-cre* and *fas*^{fl/fl}, *CD4-cre/CD19-cre* mice, respectively (unpublished data).

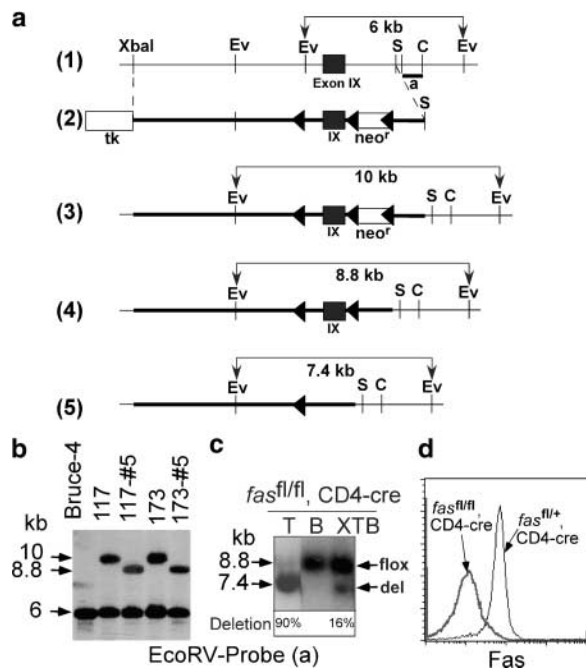


Figure 1. T cell-specific KO of Fas. (a) Targeting scheme. (1) Genomic structure of the *fas* locus surrounding exon IX. (2) Targeting vector construction. The chromosomal locus after homologous recombination is shown in 3. Cre-mediated deletion produces the *fas* floxed (4, *fas*^{fl}) and deleted alleles (5, *fas*^{del}). (triangles) *loxP* sites. Ev, EcoRV; S, SphI; C, ClaI restriction site. (b) Southern blot analysis on DNA from wild-type embryonic stem cells (Bruce-4) and homologous recombinants (clones 117 and 173 and *neo*^r deleted clones 117#5 and 173#5). (c) T cell-specific deletion of Fas. T, thymocytes; B, purified splenic B cells (CD19⁺); XTb, purified nonlymphocyte population. Deletion efficiencies are indicated. (d) FACS[®] analysis showing loss of Fas expression in thymocytes of *fas*^{fl/fl}, *CD4-cre* mice.

Absence of lpr Disease in C57BL/6 Mice Lacking Fas Selectively on Lymphocytes. Splenomegaly and lymphadenopathy were evident in *fas*^{del/del} C57BL/6 mice when they became older than 5 mo. In contrast, there was no evidence for lymphoproliferative disease in 7–9-mo-old mice lacking Fas on T, B, or both T and B lymphocytes (Fig. 2 a). In the LNs of these animals, there were almost no DN (i.e., Thy1⁺B220⁺CD4⁻CD8⁻) T cells, they are typically seen in mice homozygous for the *lpr* mutation. With respect to autoantibody formation, T or B cell-specific deletion of Fas led to a three- to eightfold increase of IgM and IgG antibodies specific for single-stranded DNA, but this was still two- to threefold below the titers seen in *fas*^{del/del} mice (unpublished data). Thus, Fas inactivation in lymphocytes only is insufficient for the *lpr* disease to occur, indicating that Fas-defective nonlymphoid cell types are also critically involved in disease development. To prove this point, inactivation of Fas in multiple cell types, including lymphocytes and nonhematopoietic cells such as liver cells, was induced in *fas*^{fl/fl}, *Mx-cre* mice by injection of Poly(I) · Poly(C) (22). As expected, mice with Fas inactivation in both lymphoid and nonlymphoid cells reconstituted the lymphoproliferative disease seen in *fas*^{del/del} mice (Fig. 2 a).

Attenuated Lymphoproliferative Disease in (C57BL/6 × MRL)F1 Mice Lacking Fas Selectively on Lymphocytes. The lymphoproliferative disease in *lpr* mice is strongly dependent on the genetic background of the animals (25). To study the effects of cell type-specific Fas inactivation in the context of an autoimmune-prone genetic background, we conditionally inactivated the *fas* gene in (C57BL/6 × MRL)F1 mice, which are as autoimmune-prone as the MRL strain itself (26). In contrast with what we had observed in C57BL/6 mice, Fas inactivation in T cells of the F1 animals led to a 12-fold enlargement of LNs due to the expansion of the DN T cells typical for the *lpr* syndrome (Fig. 2 b). Among five mice analyzed, four had a twofold enlarged spleen and one had a spleen of normal size. A similar enlargement of LNs and spleen (17- and 5-fold, respectively), but no expansion of DN cells was found upon B cell-specific Fas inactivation. However, in both cases, the weight of the enlarged LNs was still two to three hundredfold below that in *fas*^{lpr/del} mice. The lymphoproliferative disease in (C57BL/6 × MRL)F1 mice with Fas inactivation in both T and B cells was not more severe than that seen upon T cell-specific Fas inactivation; among three mice analyzed, only one mouse had mildly enlarged LNs (unpublished data). Thus, in contrast with C57BL/6 mice, the autoimmune-prone animals develop an attenuated lymphoproliferative disease upon lymphocyte-specific Fas inactivation, but in both strains, Fas-defective nonlymphoid cells are critically involved in the full development of ALPS.

Loss of T and B Cells upon T Cell-specific Fas Inactivation. Rather than leading to lymphoproliferation, T cell-specific Fas inactivation resulted in a decline of both T and B cell numbers in the secondary lymphoid organs over time, and finally led to an almost complete lymphopenia in the LNs of the animals (Fig. 3, a and b). The numbers of total cells,

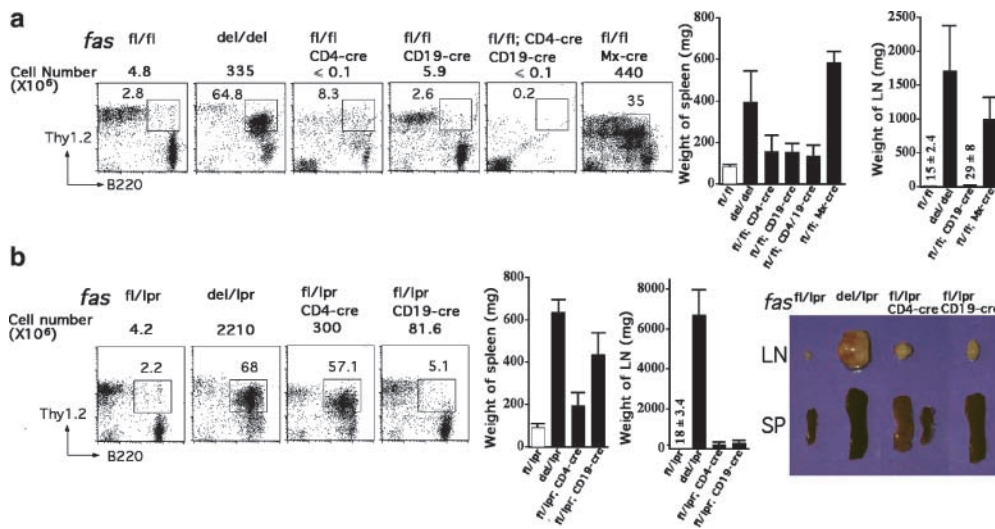


Figure 2. Defective Fas expression in nonlymphoid cells is required for the development of lymphoproliferative disease. FACS[®] analysis of DN T cells in LNs is shown. (a) 7–9-mo-old C57BL/6 mice with Fas deletion in tissue-specific or inducible manner. Mx-cre-mediated deletion was induced 8 mo before analysis. (b) Mild lymphadenopathy and splenomegaly upon T, B, and T plus B cell-specific Fas inactivation in (C57BL/6×MRL) F1 mice at the age of 5–6 mo. Pictures of spleen and LNs are shown. Weights are shown as means ± SD (*n* = 2–6). Cell numbers shown on top of FACS[®] plots indicate total cellularity from the largest LNs of mice suffering from lymphadenopathy or of inguinal or cervical LNs of mice without apparent lymphadenopathy.

T cells, and B cells in the spleens of T cell-specific Fas KO mice were ~160% of the controls at the age of 2 mo. However, T cell and B cell counts in the mutants declined to ~60% of the controls at the age of 5 mo, and further to 23% for T cells and 44% for B cells at the age of 7 mo (Fig. 3 a). As with the spleen, cell numbers in the inguinal LNs of the mutants were comparable to control values at the age of 2 mo (Fig. 3 a). However, at 5 mo of age, the inguinal LNs in 6 out of 8 mutant animals analyzed were atrophic and contained <0.1 million cells. At 7 mo, the inguinal LNs in all of four mutants analyzed were almost empty. Similar to the inguinal LNs, age-dependent cell loss was also observed in other LNs, including mesenteric, axillary, and cervical LNs (unpublished data). Enlargement of the spleen (157 ± 27 mg against 85 ± 25 mg in the controls; *n* = 4) and disruption of splenic architecture characterized by loss of white pulp, sclerosis, and hyalinization became evident in the mutants at the age of 5 mo (Fig. 3 b and not depicted). Flow cytometric analysis showed that 65% of T cells and 64% of B cells in the LNs of 16-wk-old mutants were positive for annexin V, a marker for cells undergoing apoptosis (Fig. 3 c). Similarly, widespread cellular apoptosis was observed in histological sections of the spleens of the mutants, whereas, in the controls, apoptotic (TUNEL positive) cells were rare and exclusively located in the splenic red pulp (Fig. 3 d). These results indicate that cellular apoptosis is the main cause for lymphocyte depletion upon T cell-specific Fas inactivation. A subset of DCs called “myeloid” DCs in mouse spleen is CD4 positive (27) and, thus, it is possible that the *fas* gene was also deleted in these cells. To clarify this point, we bred the *fas*^{fl/fl} mice to *lck-cre* transgenic mice in which Cre recombinase is only expressed in T cells but not DCs (20). Similar to *fas*^{fl/fl}, *CD4-cre* mice, the LNs of *fas*^{fl/fl}, *lck-cre* mice at the age of 7 mo contained <0.1 million cells, confirming that loss of T and B cells is

indeed due to Fas deletion in T cells (unpublished data). The possibility that Cre expression as such is toxic for T cells was excluded by two pieces of evidence as follows: T cell numbers in aged *fas*^{fl/+}, *CD4-cre* mice were comparable to those in *fas*^{fl/fl} controls; and aged *fas*^{del/del}, *CD4-cre* mice had the expected splenomegaly and lymphadenopathy (unpublished data).

The loss of lymphocytes in the peripheral lymphoid organs of the conditional mutants prompted us to analyze lymphocyte development in primary lymphoid organs. The cellularity of the thymus was comparable to that of littermate controls at the age of 2 mo, and declined to ~50 and 10% of the controls at the age of 5 and 7 mo, respectively (unpublished data). Occasionally, the thymi of 7-mo-old mice were completely involuted as judged by cellular composition, and only thymic LNs were detectable in the area where the thymus is normally localized (unpublished data). However, thymic atrophy can only partly account for the severe loss of peripheral T cells, as we have directly demonstrated that in the mutant animals peripheral T and B cells die by apoptosis (Fig. 3, C and D). We also note that conditional recombination-activating gene (*RAG*)-2 knockout mice (18) still have substantial numbers of peripheral T cells in the lymphoid organs 7 mo after induction of *RAG*-2 deletion (~25 and 50% of the controls in LN and spleen, respectively; unpublished data). B cell development in the bone marrow of the mutants was undisturbed, and peripheral B cells were not activated even in old animals (not depicted), indicating that, similar to T cells, the loss of B cells in the secondary lymphoid organs is due to massive apoptosis (Fig. 3, c and d) rather than a B cell developmental defect.

Up-Regulation of Activation Markers and FasL on Fas-deficient T Cells. Approximately 50% of the peripheral T cells in the T cell-specific Fas KO mice developed an acti-

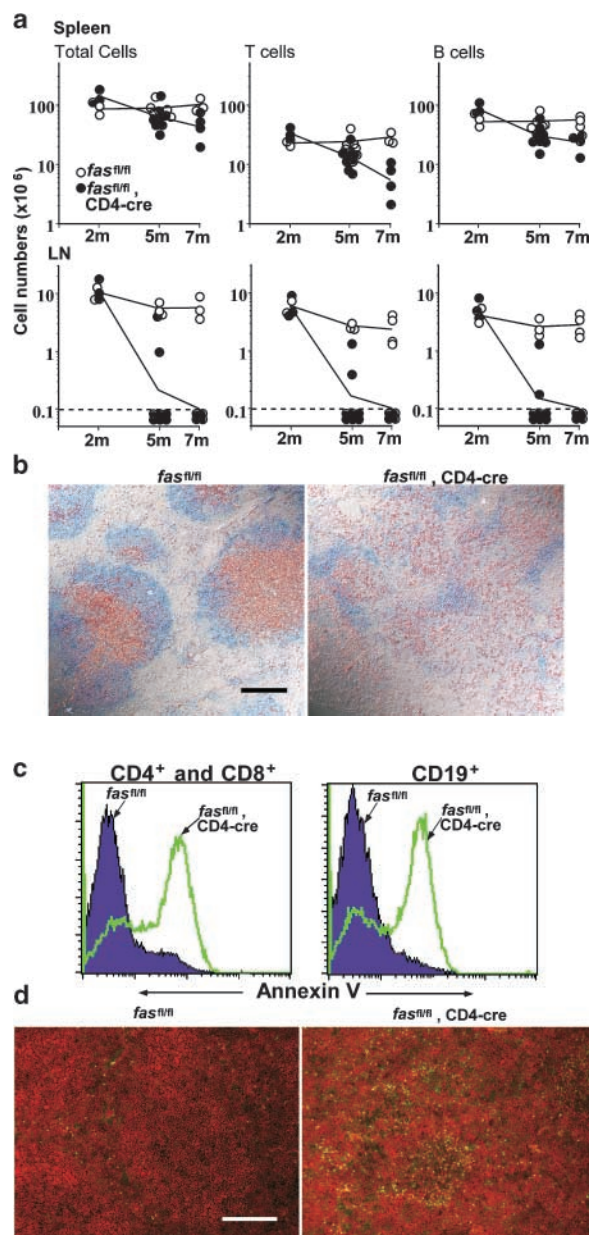


Figure 3. Loss of peripheral lymphocytes upon T cell-specific Fas inactivation. (a) Cellularity in the spleen and inguinal LNs. Each circle represents an individual mouse. Lines connect mean values of each group. (b) Immunohistochemical staining of spleens of 5–6-mo-old mice (four mice per group analyzed) with anti-CD3 (red) and anti-CD19 (blue) antibody. (c) Annexin V staining of T and B cells in LNs (four mice per group analyzed) at the age of 16 wk. (d) TUNEL staining of the splenic sections from animals (four mice per group analyzed) at the age of 16 wk. Green and red staining shows apoptotic cells and nuclei, respectively. (b and d) Bars, 200 μ m.

activated phenotype as was evident from the up-regulation of CD69 at the age of 5 mo, but not 2 mo (Fig. 4 a). Likewise, the proportions of $CD62L^{high}/CD44^{low}$ naive and $CD62L^{low}/CD44^{high}$ activated/memory T cells among the CD4 population in the mutants were comparable to those in the controls at the age of 2 mo, but an increase of the fraction of activated/memory T cells became evident at the

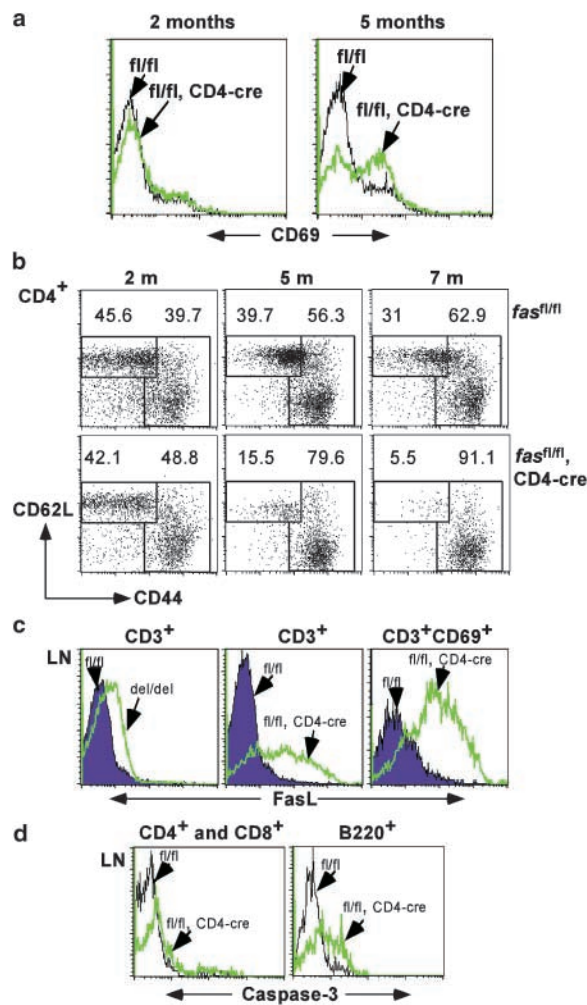


Figure 4. Activation and high levels of FasL expression in Fas-deficient T cells. (a) Expression of the activation marker CD69 on splenic $CD3^+$ T cells. (b) Analysis of CD62L versus CD44 on gated $CD4^+$ splenic cells to determine the proportion of naive ($CD62L^{high}/CD44^{low}$) and activated/memory ($CD62L^{low}/CD44^{high}$) T cells. (c) FasL expression on gated $CD3^+$ or $CD3^+CD69^+$ LN cells from 5-mo-old animals. (d) Active caspase-3 activity in T and B cells of LN cells from 18-wk-old mice. Three to five mice per group were analyzed, and a representative analysis is shown for each group.

age of 5 mo. At 7 mo, there were almost no naive T cells in the spleens of the mutant mice, and 91% of the T cells were activated/memory cells (Fig. 4 b). A similar trend was also seen in the case of CD8 T cells, but it was less dramatic (unpublished data). In parallel, activated T cells from T cell-specific Fas KO mice expressed very high levels of FasL that were far beyond those on T cells from $fas^{del/del}$ mice at the age of 5 mo, but not 2 mo (Fig. 4 c and not depicted). Notably, the loss of lymphocytes in the secondary lymphoid organs correlated with increasing T cell activation. Although not explaining the loss of naive T cells, it appeared possible that the activated T cells appearing in the animals were directly causing the loss of B cells through Fas–FasL interaction. To obtain evidence in this direction, we analyzed the lymphocytes in the mutant animals for the

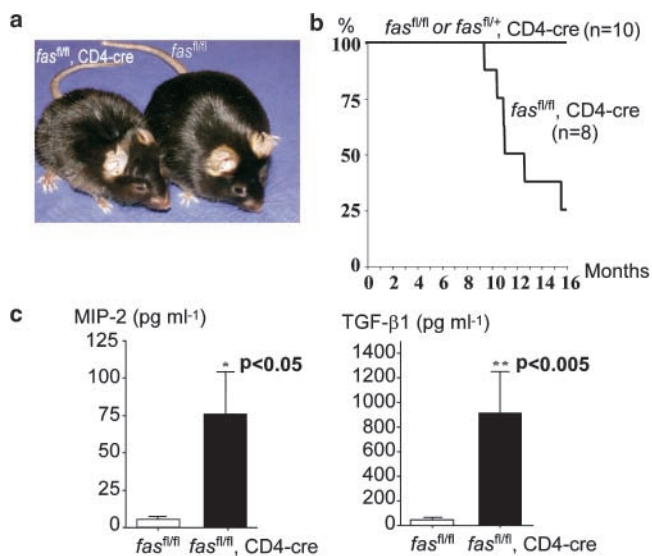


Figure 5. Wasting syndrome and increased inflammatory cytokine production in aged T cell-specific Fas-deficient mice. (a) A mutant and a control mouse at the age of 15 mo. (b) Survival of mutant and control animals. (c) MIP-2 and TGF-β1 levels in the BALF at the age of 14–18 mo. Results shown are means ± SD (*n* = 3–5).

presence of active (cleaved) caspase-3, a marker for cells undergoing apoptosis upon death receptor engagement or stress-induced cytochrome *c* release (2). As shown in Fig. 4 d, ~40% of B cells, but no T cells, from the LNs of the mutants indeed displayed caspase-3 activity.

Development of Inflammatory Pulmonary Fibrosis in Aged *fas^{fl/fl}, CD4-cre* Mice. Accompanying T cell activation and the loss of lymphocytes, T cell-specific Fas KO mice developed a severe wasting syndrome after 10 mo (Fig. 5 a, mouse aged 15 mo). Compared with the controls, the mutant animals lost an average of ~30% body weight, and 75% of the mutant animals died within 10–18 mo (Fig. 5 b and not depicted). Histological analysis of the lungs of 10-mo-old diseased mutants revealed severe pulmonary fibrosis as shown by massive accumulation of elastic fibers, and intense alveolitis with accumulation of inflammatory cells including lymphocytes, neutrophils, and activated macrophages in the lung parenchyma (Fig. 6 a). Mild to moderate cellular infiltrations were also seen in the liver and kidney, and occasionally in the pancreas and colon, but not in the heart and stomach of the animals (unpublished data). However, the lung disease was likely the main reason for the ultimate death of the animals.

The lung disease in the mutant animals started with interstitial infiltrates of T and B cells predominantly around arteries and bronchi when the animals reached the age of 5 mo (Fig. 6 b), indicating that lymphocyte infiltration initiates pulmonary inflammation. This was accompanied by massive apoptotic cell death, as indicated by the presence of large numbers of TUNEL-positive cells around the arteries and bronchi, with a distribution fitting that of the infiltrating T cells (Fig. 6 b). This suggests a critical role of the latter cells in the initial lung injury. At later stages of the dis-

ease, TUNEL-positive cells were found scattered in the lung parenchyma, and there was no longer an apparent colocalization with T cell infiltrates (Fig. 6 c).

What causes the severe pulmonary disease developing upon T cell-specific inactivation of Fas? Despite extensive efforts, we were unable to obtain any evidence for an infectious process involving bacteria, viruses, or intracellular parasites such as pneumocystis carinii (unpublished data). This suggests that, rather than being due to immunodeficiency, the disease is likely caused by inflammation initiated by the interaction of the Fas-deficient T cells with their Fas-proficient environment. To better understand this interaction and the ensuing inflammatory response, we determined the levels of various cytokines in the blood and the BALF in the mutants at the ages of 5 mo and 14–18 mo. The levels of TGF-β1, TGF-β2, macrophage inflammatory protein 2 (MIP-2), TNF-α, IFN-γ, IL-1α, IL-1β, IL-6, KC, and soluble FasL in the sera were not significantly different between mutants and controls at all time points,

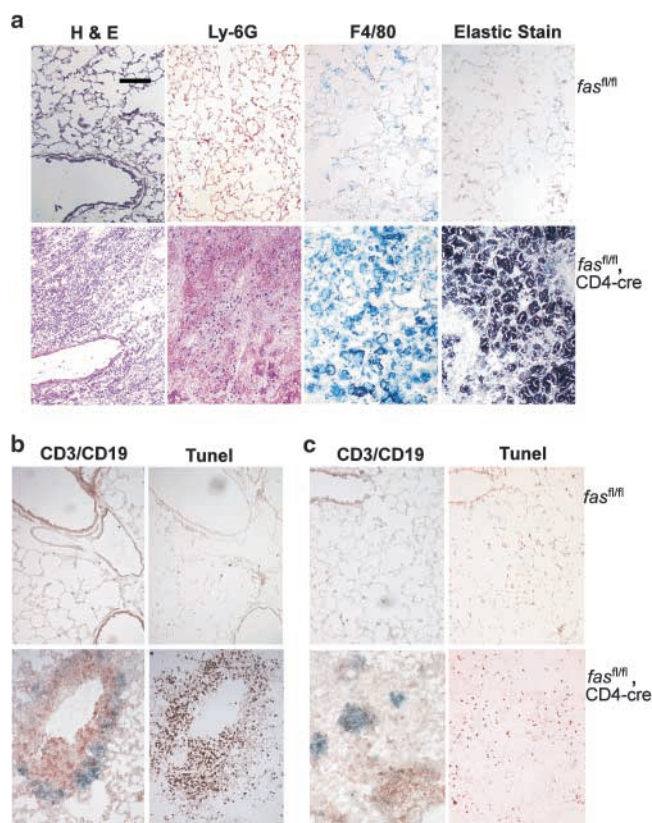


Figure 6. Leukocyte infiltration in the lungs and development of pulmonary fibrosis upon T cell-specific Fas inactivation. (a) Histological analysis of the lungs of mice at the age of 10 mo. H & E, hematoxylin and eosin staining; Ly-6G, staining for neutrophils (red); F4/80, staining for macrophages (blue); Elastic Stain, staining of elastic fibers (black blue) and cell nuclei (black). (b) Immunohistochemical staining of the lungs of 5–6-mo-old mice with anti-CD3 (red) and anti-CD19 (blue) antibody, and TUNEL (brown) on serial lung sections. (c) The staining was the same as in b, except the sections were from mice at the age of 15 mo. Three mice per group were analyzed, and representative results are shown. Bar, 100 μm.

and this was also the case for TGF- β 2, KC, IL-4, IL-13, and soluble FasL levels in the BALF of the lungs. However, the level of the chemokine MIP-2, a functional homologue of human IL-8 in the mouse (28), was significantly elevated in the BALF of the mutants at the ages of 14–18 mo, but not 5 mo (Fig. 5 c). Thus, the neutrophil infiltration in the lungs of the diseased animals could be caused by the chemokine activity of MIP-2 (28). Similarly, the level of the active form of TGF- β 1 in the BALF of the mutants was drastically increased (Fig. 5 c). Because TGF- β 1 has been shown to play a central role in the development of pulmonary fibrosis in other contexts (29–31), this result suggests that TGF- β 1 is critically involved in the development of lung fibrosis in the animals selectively lacking Fas in T cells.

Prevention of Loss of Lymphocytes and Initial Lymphocyte Infiltration in the Lungs upon Blockade of Fas–FasL Interaction. Together, our results reveal two major consequences of Fas inactivation selectively in T cells as follows: the loss of peripheral lymphocytes and the development of a fatal inflammatory pulmonary fibrosis. Because it has been reported that the DN (Thy1⁺B220⁺CD4⁻CD8⁻) T cells from *lpr* mice are cytotoxic against Fas-expressing cells on the basis of up-regulated FasL expression (32, 33), and the activated T cells from T cell-specific Fas KO mice abundantly expressed FasL on their surface (Fig. 4 c), we reasoned that both the loss of lymphocytes and the pulmonary disease might be due to FasL-mediated interactions of the mutant T cells with Fas-expressing cells in their environment. With respect to lymphocyte loss, the abundant expression of FasL on T cells could lead to the direct killing of the (Fas proficient) B cells, and Fas–FasL interactions could also directly or indirectly be involved in the eventual depletion of T cells in the mutant animals. If this general notion were correct, application of a neutralizing anti-FasL antibody and, hence, inhibition of Fas–FasL interaction should prevent both lymphopenia and pulmonary disease in these mice. Based on the fact that T and B cells were lost rapidly between 2 and 5 mo of age (Fig. 4 a), 14-wk-old *fas^{fl/fl}, CD4-cre* mice were chosen for treatment with the neutralizing anti-FasL antibody MFL-1/3 (23). LN T cells from the mutant animals expressed high levels of FasL at this age (unpublished data). *fas^{fl/fl}* mice either unmanipulated or treated with anti-FasL antibody or *fas^{fl/fl}, CD4-cre* mice treated with isotype-matched hamster IgG1 served as controls. As shown in Fig. 7 a, loss of T and B cells in the inguinal LNs from two *fas^{fl/fl}, CD4-cre* mice 8 wk after the initiation of anti-FasL treatment was completely prevented. In a single animal analyzed 12 wk after treatment, the cell number in the inguinal LNs was reduced to half a million, but was increased threefold in the spleen as compared with controls (unpublished data). Substantial numbers of cells resembling those undergoing lymphoproliferation in *lpr* mice (Thy1⁺B220⁺CD4⁻CD8⁻) were generated in the anti-FasL antibody-treated mutants (Fig. 7 a). As expected, *fas^{fl/fl}, CD4-cre* mice treated with isotype-matched IgG1 lost their lymph node cells over the time of treatment. In agreement with these data, immunohistological analysis on

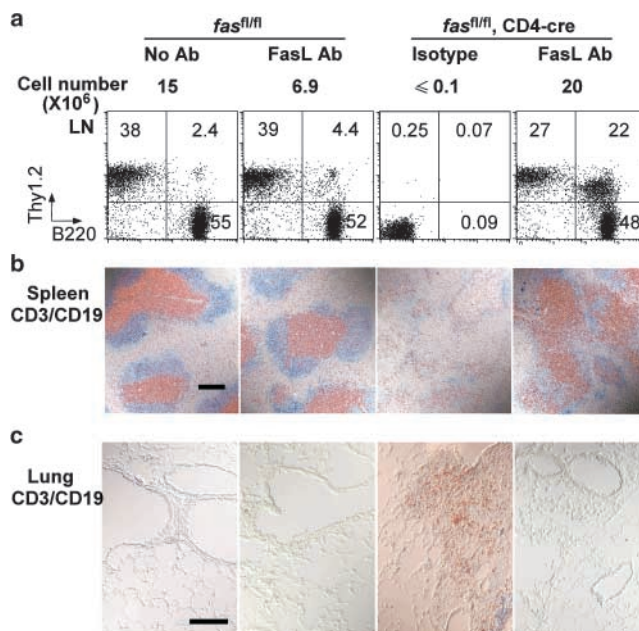


Figure 7. Prevention of lymphopenia and cell infiltration in the lungs upon application of anti-FasL antibody. Groups of three mutant and three control mice were treated with anti-FasL antibody or isotype-matched hamster IgG1. Two mice from each group were analyzed 8 wk after antibody application, and one mouse from each group was analyzed at 12 wk. (a) FACS[®] analysis of lymphocytes in inguinal LNs. (b and c) Coimmunostaining of the spleen (b) and lung (c) sections with anti-CD3 (red) and anti-CD19 (blue) antibody. Shown is the analysis of one set of mice treated with antibody for 8 wk. (b and c) Bars, 200 μ m.

the same set of animals showed that the blockade of Fas–FasL interaction in the mutants also largely prevented the alteration of splenic architecture and the development of splenic sclerosis (Fig. 7 b and not depicted). Strikingly, the anti-FasL antibody treatment also blocked lymphocyte infiltration in the lung (Fig. 7 c). Collectively, these results show that the entire pathology developing in the animals lacking Fas on T cells is due to abnormal Fas–FasL interaction, likely reflecting the interaction of the Fas-deficient T cells with Fas-proficient cells in the environment.

Discussion

A critical role for T and B cells in the development of ALPS in *lpr* mice has been deduced from the finding that both lymphadenopathy and autoimmune disease were suppressed by thymectomy (7, 8) or ablation of B cell development (11). Furthermore, several groups have reported that restoration of Fas expression in T cells of *lpr* mice can correct the lymphoproliferative disease (9, 10). Here, we show that Fas inactivation in T, B, or T plus B cells of C57BL/6 mice is insufficient for the pathogenesis of lymphoproliferative disease. Similarly, autoantibody formation was diminished in the T or B cell-specific Fas KO mice as compared with Fas-deficient mice, although the former animals did have elevated levels of anti-DNA antibodies in the blood as compared with wild-type controls (unpublished data). The

attenuated lymphoproliferative disease seen upon lymphocyte-specific Fas inactivation on the autoimmune-prone (C57BL/6×MRL)F1 background further demonstrates that Fas inactivation in lymphocytes is not sufficient for full ALPS development, and, in addition, defective Fas expression in nonlymphoid cell types is required. Along these lines, it has been reported that the liver may be the initial site of DN T cell proliferation (34). Such an initial expansion of the FasL-expressing DN T cells may be incompatible with Fas expression on the surrounding cells in this organ, and it will be interesting to see whether Fas inactivation in both T and liver cells reproduces the development of ALPS in *lpr* mice.

Mice with T cell-specific Fas inactivation developed a profound lymphopenia over time. In accord with the earlier cell transfer studies (12, 13), this indicates that the interaction between Fas-deficient, activated T cells and Fas-expressing, nonlymphoid cells through Fas–FasL interaction prevents the development of ALPS as it is seen in the *lpr* mouse and human Fas-deficient patients. Absence of Fas in T cells would have been sufficient for the development of lymphoproliferative disease if loss of Fas expression were not accompanied by a dramatic up-regulation of FasL in these cells. This is supported by the appearance of DN T cells in the mutant animals upon application of anti-FasL antibody, which blocks the interaction between the FasL-expressing T cells and Fas-expressing non-T cells. In turn, in *lpr* mice, the expression of FasL by the DN T cells (32, 33) is compatible with ALPS development, as there are no Fas-competent cells in the environment with which these T cells could interact.

In contrast with the rapid development of a fatal wasting syndrome developing in irradiated MRL mice upon transfer of Fas-deficient congenic BM cells (12, 13), T cell-specific Fas inactivation in intact mice led to a situation in which the animals appeared perfectly healthy over the first months of life. This was true not only for C57BL/6, but also (MRL×C57BL/6)F1 mice (unpublished data). We think that this dramatic difference in the onset of disease is due to the different experimental conditions in that in the cell transfer system, which involves X-irradiation, the Fas-deficient T cells are confronted with a (Fas proficient) environment that may promote a rapid general T cell activation. Also, it cannot be excluded that other Fas-deficient hematopoietic cells developing in these animals contribute to the disease phenotype. In contradistinction, the system of Cre-mediated Fas inactivation in T cells used in the present work reconstructs what happens when Fas-deficient T cells lose their Fas–FasL-based homeostatic control under quasiphysiological conditions. Our results suggest that the loss of this homeostatic control, accompanied by FasL up-regulation in concert with chronic T cell activation, is critical for the gradual loss of lymphocytes in the mutant animals and their ultimate fatal disease. The activated cells, arising because of their disturbed homeostatic control, may directly kill the B cells in the mutant animals through Fas–FasL interaction. This is in line with the pres-

ence of activated caspase-3 in a substantial fraction of those cells. The unexpected concomitant loss of naive T cells becomes less surprising, considering that experimental evidence that enforced transgenic expression of FasL in a variety of tissues does not confer immune privilege, but instead leads to rapid tissue destruction (35–37). Fas expression on the target cells is dispensable for this to occur, but is required on the infiltrating cells (36, 38). This implies a cell death signal that is dependent on Fas–FasL interaction, but is not mediated by Fas in a cell-autonomous manner.

How can FasL expression on activated, Fas-deficient T cells mediate the loss of naive T cells? An obvious possibility is that the FasL-expressing cells destroy the natural habitat of naive T and probably also B cells by attacking cells in the environment. Targets for such an attack could be, for example, endothelial cells in lymph and blood vessels, known to be sensitive to Fas-mediated apoptosis in response to inflammation (39). Furthermore, the interaction of the activated T cells with DCs and macrophages is expected to lead to the activation of the latter through Fas engagement (40, 41). This could result in the killing of the Fas-deficient T cells through cytokines such as TNF to which the mutant cells may be more sensitive than wild-type cells. Although we have been unable to obtain experimental support for the latter mechanism in that we have neither observed a preferential killing of Fas-deficient T cells in cocultures with activated DCs, nor an enhanced sensitivity of the mutant T cells to TNF- α (Nguyen, K., personal communication), our experiments strongly support the concept that the activated T cells generate a “hostile” environment in which naive lymphocytes cannot survive. Thus, the splenic microarchitecture was profoundly disturbed in the mutant animals, and there was massive cell death in the spleen *in situ*, as well as in the areas of the initial T cell infiltration in the lungs. However, most significantly, B cell loss was observed also in a situation in which both T and B cells were deficient for Fas (i.e., the B cells could not be a direct target of the FasL-expressing activated T cells). The prediction that in this situation the B cells, similar to the naive T cells would not contain activated caspase-3, could not be tested because the double mutant animals were no longer available. The small fraction of naive T cells that had escaped Cre-mediated Fas inactivation in the T cell-specific KO mice were also lost in the animals over time (unpublished data), but in this case the loss could be due to a direct attack by Fas-deficient activated companion T cells. Other factors may also contribute to the loss of naive lymphocytes in the mutant animals. Thus, the observed thymic atrophy, perhaps itself mediated by an attack of activated T cells on the thymic microenvironment, likely restricts the output of naive T cells over time. Finally, the Fas signaling pathway has been shown to control not only T cell death, but also T cell activation and proliferation (42–46). This may also contribute to the loss of naive T cells in the mutant animals.

The fatal pulmonary disease developing in the mutant animals seems to be initiated by lymphocytes infiltrating the lungs and could thus also be due to an attack of acti-

vated, Fas-deficient T cells against Fas-expressing cells in their environment. It is not surprising that the lung is preferentially targeted by such an attack, given that it is constantly exposed to antigenic stimuli such that activated T cells could easily be recruited into the resulting immune reactions. That the infiltrating T cells in the lungs of the mutant animals indeed kill cells in their environment, likely including alveolar epithelial or endothelial cells, is suggested by the colocalization of TUNEL positive cells and T cell infiltrates in the histological sections (Fig. 6 b). In the present scenario, this is expected to lead to a vicious cycle of inflammatory processes for a variety of reasons. Thus, it is known that FasL expression in various tissues, aside from targeting them for rapid destruction, induces neutrophil infiltration and local inflammation (35–37). The former could result from the observed MIP-2 production (Fig. 5 c) by activated macrophages recruited into the lesions, injured lung epithelial cells, and also extravasated neutrophils (28). Activated macrophages upon engagement of apoptotic lung epithelial cells (47) and the apoptotic cells themselves (48) produce TGF- β 1 (Fig. 5 c, elevated in the BALF) that suppresses the inflammation (49, 50), inhibits FasL expression (51) and neutrophil activation (52), and causes pulmonary fibrosis (29–31). In turn, massive TGF- β 1 production enhances Fas-mediated apoptosis of lung epithelial cells (53) and, thus, increases the inflammatory reaction additionally.

Phenotypically, the lung disease developing in the animals resembles human IPF, a fatal chronic diffuse interstitial pneumonia of unknown cause and characterized histopathologically by inflammation and fibrosis of the lung parenchyma (17). Despite extensive efforts, no animal model of persistent and progressive pulmonary fibrosis was so far available (17). Human IPF and the disease developing in our animals resemble each other in onset at advanced age, chronic progression, massive infiltration of similar types of inflammatory cells, increased production of IL-8/MIP-2 and TGF- β 1, and the development of pulmonary fibrosis. The wasting syndrome seen in the mouse model but not the human disease is possibly due to massive tissue destruction by FasL-expressing T cells in the former. Although it is interesting to note that in mice, Fas–FasL interaction plays a critical role in the development of acute bleomycin-induced pulmonary fibrosis (54) and silica-induced pulmonary silicosis (55), its possible role in IPF remains speculative at this point. In its limits, the present experimental system may address this issue and provide a mouse model of IPF in the human, highlighting in parallel the potentially dramatic impact of somatic mutation on cellular homeostasis through dysregulated gene expression.

We thank A. Leinhaas, A. Egert, S. Willms, C. Uthoff-Hachenberg, and C. Göttlinger for excellent technical help; S. Casola, A. Waisman, M. Pasparakis, M. Alimzhanov, and K. Nguyen for stimulating discussion; S. Nagata for the *fas* genomic clone; and C. Wilson for the *CD4-cre* transgenic mouse. Z. Hao thanks W. Ma for help on graphic work and Y. Wang for constant support.

This work was supported by the Juvenile Diabetes Foundation, the Deutsche Forschungsgemeinschaft through SFB 243, the Land

Nordrhein-Westfalen, the Körber Foundation, and a National Institutes of Health grant (PO1-AI052343-02) to K. Rajewsky.

Submitted: 18 December 2003

Accepted: 1 April 2004

References

1. Krammer, P.H. 1999. CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv. Immunol.* 71:163–210.
2. Krammer, P.H. 2000. CD95's deadly mission in the immune system. *Nature.* 407:789–795.
3. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature.* 356:314–317.
4. Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell.* 76:969–976.
5. Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science.* 268:1347–1349.
6. Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell.* 81:935–946.
7. Steinberg, A.D., J.B. Roths, E.D. Murphy, R.T. Steinberg, and E.S. Raveche. 1980. Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. *J. Immunol.* 125:871–873.
8. Hang, L., A.N. Theofilopoulos, R.S. Balderas, S.J. Francis, and F.J. Dixon. 1984. The effect of thymectomy on lupus-prone mice. *J. Immunol.* 132:1809–1813.
9. Wu, J., T. Zhou, J. Zhang, J. He, W.C. Gause, and J.D. Mountz. 1994. Correction of accelerated autoimmune disease by early replacement of the mutated *lpr* gene with the normal Fas apoptosis gene in the T cells of transgenic MRL-lpr/lpr mice. *Proc. Natl. Acad. Sci. USA.* 91:2344–2348.
10. Fukuyama, H., M. Adachi, S. Suematsu, K. Miwa, T. Suda, N. Yoshida, and S. Nagata. 1998. Transgenic expression of Fas in T cells blocks lymphoproliferation but not autoimmune disease in MRL-lpr mice. *J. Immunol.* 160:3805–3811.
11. Shlomchik, M.J., M.P. Madaio, D. Ni, M. Trunstein, and D. Huszar. 1994. The role of B cells in *lpr/lpr*-induced autoimmunity. *J. Exp. Med.* 180:1295–1306.
12. Theofilopoulos, A.N., R.S. Balderas, Y. Gozes, M.T. Aguado, L.M. Hang, P.R. Morrow, and F.J. Dixon. 1985. Association of *lpr* gene with graft-vs.-host disease-like syndrome. *J. Exp. Med.* 162:1–18.
13. Fujiwara, M., and A. Kariyone. 1984. One-way occurrence of graft-versus-host disease in bone marrow chimaeras between congenic MRL mice. *Immunology.* 53:251–256.
14. Zhu, B., B.C. Beaudette, I.R. Rifkin, and A. Marshak-Rothstein. 2000. Double mutant MRL-lpr/lpr-gld/gld cells fail to trigger *lpr*-graft-versus-host disease in syngeneic wild-type recipient mice, but can induce wild-type B cells to make autoantibody. *Eur. J. Immunol.* 30:1778–1784.
15. Nemazee, D., C. Guet, K. Buerki, and A. Marshak-Rothstein. 1991. B lymphocytes from the autoimmune-prone mouse strain MLR/lpr manifest an intrinsic defect in tetra-

- parental MRL/lpr in equilibrium DBA/2 chimeras. *J. Immunol.* 147:2536–2539.
16. Katagiri, T., S. Azuma, Y. Toyoda, S. Mori, K. Kano, P.L. Cohen, and R.A. Eisenberg. 1992. Tetraparental mice reveal complex cellular interactions of the mutant, autoimmunity-inducing lpr gene. *J. Immunol.* 148:430–438.
 17. Crystal, R.G., P.B. Bitterman, B. Mossman, M.I. Schwarz, D. Sheppard, L. Almasy, H.A. Chapman, S.L. Friedman, T.E. King, Jr., L.A. Leinwand, et al. 2002. Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. *Am. J. Respir. Crit. Care Med.* 166:236–246.
 18. Hao, Z., and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J. Exp. Med.* 194:1151–1164.
 19. Wolfer, A., T. Bakker, A. Wilson, M. Nicolas, V. Ioannidis, D.R. Littman, P.P. Lee, C.B. Wilson, W. Held, H.R. MacDonald, and F. Radtke. 2001. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat. Immunol.* 2:235–241.
 20. Orban, P.C., D. Chui, and J.D. Marth. 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 89:6861–6865.
 21. Rickert, R.C., J. Roes, and K. Rajewsky. 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res.* 25:1317–1318.
 22. Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science.* 269:1427–1429.
 23. Kayagaki, N., N. Yamaguchi, F. Nagao, S. Matsuo, H. Maeda, K. Okumura, and H. Yagita. 1997. Polymorphism of murine Fas ligand that affects the biological activity. *Proc. Natl. Acad. Sci. USA.* 94:3914–3919.
 24. Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* 23:5080–5081.
 25. Cohen, P.L., and R.A. Eisenberg. 1991. lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
 26. Vidal, S., D.H. Kono, and A.N. Theofilopoulos. 1998. Loci predisposing to autoimmunity in MRL-Fas lpr and C57BL/6-Faslpr mice. *J. Clin. Invest.* 101:696–702.
 27. Shortman, K., and Y.J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2:151–161.
 28. Driscoll, K.E. 1994. Macrophage inflammatory proteins: biology and role in pulmonary inflammation. *Exp. Lung Res.* 20:473–490.
 29. Broekelmann, T.J., A.H. Limper, T.V. Colby, and J.A. McDonald. 1991. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc. Natl. Acad. Sci. USA.* 88:6642–6646.
 30. Sime, P.J., Z. Xing, F.L. Graham, K.G. Csaky, and J. Gauldie. 1997. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J. Clin. Invest.* 100:768–776.
 31. Munger, J.S., X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, J.F. Pittet, N. Kaminski, C. Garat, M.A. Matthay, et al. 1999. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell.* 96:319–328.
 32. Chu, J.L., P. Ramos, A. Rosendorff, J. Nikolic-Zugic, E. Lacy, A. Matsuzawa, and K.B. Elkon. 1995. Massive upregulation of the Fas ligand in lpr and gld mice: implications for Fas regulation and the graft-versus-host disease-like wasting syndrome. *J. Exp. Med.* 181:393–398.
 33. Watanabe, D., T. Suda, H. Hashimoto, and S. Nagata. 1995. Constitutive activation of the Fas ligand gene in mouse lymphoproliferative disorders. *EMBO J.* 14:12–18.
 34. Ohteki, T., S. Seki, T. Abo, and K. Kumagai. 1990. Liver is a possible site for the proliferation of abnormal CD3⁺4⁺8⁻ double-negative lymphocytes in autoimmune MRL-lpr/lpr mice. *J. Exp. Med.* 172:7–12.
 35. Seino, K., N. Kayagaki, K. Okumura, and H. Yagita. 1997. Antitumor effect of locally produced CD95 ligand. *Nat. Med.* 3:165–170.
 36. Kang, S.M., D.B. Schneider, Z. Lin, D. Hanahan, D.A. Dichek, P.G. Stock, and S. Baekkeskov. 1997. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat. Med.* 3:738–743.
 37. Allison, J., H.M. Georgiou, A. Strasser, and D.L. Vaux. 1997. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc. Natl. Acad. Sci. USA.* 94:3943–3947.
 38. Seino, K., N. Kayagaki, K. Fukao, K. Okumura, and H. Yagita. 1997. Rejection of Fas ligand-expressing grafts. *Transplant. Proc.* 29:1092–1093.
 39. Sata, M., and K. Walsh. 1998. Oxidized LDL activates fas-mediated endothelial cell apoptosis. *J. Clin. Invest.* 102:1682–1689.
 40. Rescigno, M., V. Piguet, B. Valzasina, S. Lens, R. Zubler, L. French, V. Kindler, J. Tschopp, and P. Ricciardi-Castagnoli. 2000. Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1 β , and the production of interferon γ in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses. *J. Exp. Med.* 192:1661–1668.
 41. Hohlbaum, A.M., M.S. Gregory, S.T. Ju, and A. Marshak-Rothstein. 2001. Fas ligand engagement of resident peritoneal macrophages in vivo induces apoptosis and the production of neutrophil chemotactic factors. *J. Immunol.* 167:6217–6224.
 42. Alderson, M.R., R.J. Armitage, E. Maraskovsky, T.W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D.H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J. Exp. Med.* 178:2231–2235.
 43. Zhang, J., D. Cado, A. Chen, N.H. Kabra, and A. Winoto. 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature.* 392:296–300.
 44. Kennedy, N.J., T. Kataoka, J. Tschopp, and R.C. Budd. 1999. Caspase activation is required for T cell proliferation. *J. Exp. Med.* 190:1891–1896.
 45. Chun, H.J., L. Zheng, M. Ahmad, J. Wang, C.K. Speirs, R.M. Siegel, J.K. Dale, J. Puck, J. Davis, C.G. Hall, et al. 2002. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature.* 419:395–399.
 46. Salmena, L., B. Lemmers, A. Hakem, E. Matysiak-Zablocki, K. Murakami, P.Y. Au, D.M. Berry, L. Tamblyn, A. Shehabeldin, E. Migon, et al. 2003. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev.* 17:883–895.
 47. Fadok, V.A., D.L. Bratton, D.M. Rose, A. Pearson, R.A. Ezekewitz, and P.M. Henson. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature.* 405:85–90.

48. Hodge, S., G. Hodge, R. Flower, P.N. Reynolds, R. Scicchitano, and M. Holmes. 2002. Up-regulation of production of TGF-beta and IL-4 and down-regulation of IL-6 by apoptotic human bronchial epithelial cells. *Immunol. Cell Biol.* 80: 537–543.
49. Shull, M.M., I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature.* 359:693–699.
50. Chen, W., M.E. Frank, W. Jin, and S.M. Wahl. 2001. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity.* 14:715–725.
51. Genestier, L., S. Kasibhata, T. Brunner, and D.R. Green. 1999. Transforming growth factor β 1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via down-regulation of *c-Myc*. *J. Exp. Med.* 189:231–239.
52. Chen, J.J., Y. Sun, and G.J. Nabel. 1998. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science.* 282: 1714–1717.
53. Hagimoto, N., K. Kuwano, I. Inoshima, M. Yoshimi, N. Nakamura, M. Fujita, T. Maeyama, and N. Hara. 2002. TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. *J. Immunol.* 168:6470–6478.
54. Kuwano, K., N. Hagimoto, M. Kawasaki, T. Yatomi, N. Nakamura, S. Nagata, T. Suda, R. Kunitake, T. Maeyama, H. Miyazaki, and N. Hara. 1999. Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. *J. Clin. Invest.* 104:13–19.
55. Borges, V.M., H. Falcao, J.H. Leite-Junior, L. Alvim, G.P. Teixeira, M. Russo, A.F. Nobrega, M.F. Lopes, P.M. Rocco, W.F. Davidson, et al. 2001. Fas ligand triggers pulmonary silicosis. *J. Exp. Med.* 194:155–164.