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Short-form Ron receptor is required for normal IFN-γ production in concanavalin A-induced acute liver injury

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Departments of [1]Pediatrics, [2]Pathology, and [3]Surgery, University of Cincinnati College of Medicine and [4]Cincinnati Children's Hospital Research Foundation, Cincinnati, Ohio; and [5]Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee

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Wetzel CC, Leonis MA, Dent A, Olson MA, Longmeier AM, Ney PA, Boivin GP, Kader SA, Caldwell CC, Degen SJ, Waltz SE. Short-form Ron receptor is required for normal IFN-γ production in concanavalin A-induced acute liver injury. *Am J Physiol Gastrointest Liver Physiol* 292: G253–G261, 2007. First published September 28, 2006; doi:10.1152/ajpgi.00134.2006.—Abrogation of Ron receptor tyrosine kinase function results in defects in macrophage activation and dysregulated acute inflammatory responses in vivo. Several naturally occurring constitutively active alternative forms of Ron have been identified, including from primary human tumors and tumor cell lines. One of these alternative forms, short-form (SF) Ron, is generated from an alternative start site in intron 10 of the Ron gene that eliminates most of the extracellular portion of the receptor and is overexpressed in several human cancers. To test the physiological significance of SF-Ron in vivo, mice were generated that solely express the full-length form of Ron (FL-Ron). Our results show that elimination of the capacity to express SF-Ron in vivo leads to augmented production of IFN-γ from splenocytes following stimulation ex vivo with either concanavalin A or anti-CD3/T cell receptor monoclonal antibody. Moreover, in a concanavalin A-induced murine model of acute liver injury, FL-Ron mice have increased production of serum INF-γ and serum alanine aminotransferase levels and worsened liver histology and overall survival compared with wild-type control mice. Taken together, these results suggest for the first time that SF-Ron impacts the progression of inflammatory immune responses in vivo and further support a role for the Ron receptor and its various forms in liver pathophysiology.

receptor tyrosine kinases; regulation of cytokine production; animal models of liver injury

RECEPTOR TYROSINE KINASES (RTK) play a critical role in multiple biological functions including immune system regulation. The Ron receptor is a member of a distinct family of multifunctional RTKs that also includes c-Met. Activation of Ron and Met results in pleiotropic biological responses, including a set of properties allowing cells to undergo "invasive growth" (4, 13, 33). In addition, Ron receptor activity is critical for proper modulation of inflammatory responses to toxic insults in vivo, including the necroinflammatory hepatic injury in an endotoxin-mediated murine model of acute liver failure (5, 11, 12, 27).

When mouse Ron (mRon) cDNA was cloned from hematopoietic stem cells, an alternative 1.9-kb short-form Ron (SF-Ron) transcript was identified in addition to the 4.8-kb full-length Ron (FL-Ron) mRNA transcript (9). SF-Ron is expressed in human lung, ovary, and tissues of the gastrointestinal tract and several human cancers and cancer cell lines; however, the tissue-specific expression of SF-Ron has not been fully characterized (1, 7, 9, 22). The transcription start site for SF-Ron mRNA has been localized to intron 10 of the mRon gene (21, 30), and SF-Ron mRNA encodes a truncated Ron protein that possesses a truncated extracellular domain and the entire transmembrane and cytoplasmic tyrosine kinase domains. SF-Ron displays constitutive tyrosine kinase activity (1, 6), and overexpression of SF-Ron results in loss of an epithelial phenotype and aggressive cell behavior (1). Moreover, mouse strains that express SF-Ron transcript in adult bone marrow tissue are susceptible to Friend virus-induced erythroleukemia (21), and SF-Ron kinase activity is necessary for erythropoietin-independent expansion of erythroid progenitors in response to Friend virus (6). These observations suggest that the function of SF-Ron is biologically distinct from that of FL-Ron, which is neither constitutively active nor sufficient for Friend virus infection.

To investigate the physiological roles of the Ron receptor in vivo, gene-targeting strategies have been utilized to generate mice lacking expression of FL-Ron (5, 27). Mice deficient in FL-Ron expression survive to adulthood and are fertile, yet have altered inflammatory responses to a variety of physiological stressors (5, 11, 12, 27). Given the potential for SF-Ron to operate in a ligand-independent manner in vivo based on its inherent constitutive kinase activity, and prior demonstration that SF-Ron kinase activity is physiologically important in vivo (6, 21), we hypothesize that additional important biological functions of the Ron receptor may be attributed to alternative SF-Ron activity.

In this report, mice lacking the capacity to express SF-Ron, but still possessing the ability to express FL-Ron, were generated by utilizing a knock-in targeting vector strategy that eliminated the alternative transcription mRon start site in intron 10. Mice homozygous for the FL-Ron transgene live to adulthood and display differential responses to Friend leukemia viral infection. Moreover, interrogation of FL-Ron mice immune responses, both ex vivo by stimulating isolated splenocytes with concanavalin A (ConA) and in vivo in a ConA-induced model of acute liver injury, suggest that SF-Ron serves distinct nonredundant biological functions relative to FL-Ron in vivo.

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EXPERIMENTAL PROCEDURES

Use of animals. All animals received humane care according to the criteria outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

RT-PCR analyses. Total RNA was isolated from wild-type mouse tissues (50% Sv129/50% Black Swiss) and murine erythroleukemia (MEL) cells by using Trizol reagent (Invitrogen, Carlsbad, CA). Three micrograms of total RNA were reverse transcribed with oligo(dT) primers in a 25-μl reaction, and 2 μl of the RT reaction were used in a subsequent PCR allowing detection of both FL-Ron and SF-Ron mRNA transcripts. To detect SF-Ron, *primers 4* and *5* were used to detect SF-Ron in the RT reaction and were identical to those previously reported to identify SF-Ron (21). The inclusion of *primer 3* (5′-GAAGACCCTATTGTGTTGGACATCAG-3′; nucleotides 7196 to 7221), located in exon 8 of the mRon gene sequence, along with *primers 4* and *5*, allowed for the detection of both the FL-Ron transcript and SF-Ron all in one PCR.

Primers used to amplify mouse β-actin probes (5′-TGACAGCATTGCTTCTGTGTAAATT-3′ and 5′-ATTGGTCTCAAGTCAGTGTACAGGC-3′) were derived from the mouse β-actin mRNA sequence (accession number x03672).

Isolation of splenic T cells. Splenic T cells were isolated by positive selection using anti-CD90 microbeads and separated on an AutoMACS as described by the manufacturer (Miltenyi Biotec, Auburn, CA). The purity of the T cells was determined to greater than 85% by flow cytometry.

Gene targeting of the mouse Ron allele. The mRon gene was used to construct a replacement-type targeting vector (30). A 736-bp *Kpn*I/*Msc*I fragment (nucleotides 6571 to 7357 of the mRon gene), encompassing part of intron 7 through the middle of exon 9, served as the 5′ boundary of homology for the recombination event with the endogenous Ron gene. A plasmid containing the mRon cDNA was digested with *Msc*I and *Bam*HI to obtain a 2.0-kb fragment coding for the middle of exon 9 to the 3′ untranslated region. Both fragments were cloned into the *Kpn*I-*Bam*HI site of pcDNA 4.0 myc/his verA (Invitrogen). The plasmid containing the combined mRon sequences was then digested with *Kpn*I and *Xho*I, to give a 2.8-kb fragment that was directionally cloned into pKO Scrambler 901 (Lexicon Genetics, The Woodlands, TX).

A 4.8-kb *Xba*I/*Apa*I fragment (nucleotides 8232 to 13079 of the mRon gene) was directionally cloned into pcDNA 4.0 myc/his verB (Invitrogen). This plasmid was then digested with *Eco*RI and *Sst*II, and the resulting 4.8-kb Ron fragment was directionally cloned into the pKO Scrambler vector that contained the 5′ region of homology and the cDNA insert described above. This 3′ fragment served as the long arm of homology for recombination with the endogenous Ron gene.

For positive selection, a neomycin cassette was isolated from pKO selectNeo (Lexicon Genetics) and cloned into the unique *Asc*I site of the pKO vector containing both the 5′ and 3′ regions of homology and the cDNA insert. For negative selection, a thymidine kinase (TK) expression cassette was isolated from pKO selectTK (Lexicon Genetics) and cloned into the unique *Rsr*II site in the pKO vector, giving rise to the final targeting vector.

The final targeting vector was linearized with *Sal*I and electroporated into the embryonic stem (ES) cell line E14TG2a as previously described (2, 8). G418 and gancyclovir were added to the ES cell media as positive and negative selection reagents, respectively. After 5–7 days, a total of 300 drug-resistant clones were isolated following two separate electroporation procedures.

ES cell clones that had undergone homologous recombination were identified by PCR analysis of genomic DNA, using *primer 1* (5′-CACAGTCCCAATCTTCTTCCC-3′; nucleotides 6472 to 6492) located in intron 7, and *primer 2* (5′-GACGCTCAGATTCCCTGTTGC-3′; nucleotides 7648 to 7668) located in exon 10 of the mRon gene (Fig. 2*A*) (30). The endogenous Ron gene and recombinant FL-Ron allele generate an 1,197-bp product and 1,055-bp PCR product, respectively. Three positive ES cell clones were identified (146, 182, and 217). Confirmation of successful targeting of these three clones was confirmed by Southern blot analysis of *Bam*HI-digested DNA, using a probe encompassing nucleotides 4975–5873 of the mRon gene, and located outside and 5′ of the targeting vector (Fig. 2*A*).

Generation of FL-Ron gene-targeted mice. Targeted ES cells were injected by the Transgenic Core Facility at Cincinnati Children's Research Foundation into C57BL/6 blastocysts and transferred into pseudopregnant C57BL/6 females. Chimeric founders were crossed to NIH Black Swiss mice (Taconic Laboratory, Germantown, NY) to generate F1 offspring. F1 hemizygous (+/FL-Ron) littermates were crossed with each other to generate F2 offspring. Hematological analyses of blood were determined in the clinical laboratory of Cincinnati Children's Hospital Medical Center.

Genotype analysis of mice. Genomic DNA was isolated as previously described (2). Genotype analysis of the initial mice and their offspring was performed by Southern blot and PCR analysis as described for the identification of positive ES cell clones. Subsequently, mice were genotyped by PCR using *primers 2* and *3*. The endogenous Ron gene and the FL-Ron allele generate a 473-bp and 331-bp PCR product, respectively.

Northern blot analysis. Twenty micrograms of total RNA from testes were analyzed by Northern blot analysis. Membrane-bound RNA was hybridized with a 513-bp ^{32}P-radiolabeled mouse Ron cDNA fragment coding for the 3′ end of the mRNA and subsequently reprobed with a 213-bp ^{32}P-radiolabeled GAPDH PCR fragment, generated by using primers 5′-GCTCCTCTCGCCAAGGTTATTC-3′ and 5′-GCTCTGGGATGACTTTGCCTACAG-3′ of the mouse GAPDH sequence (accession number MMU09964).

Activation of peritoneal macrophages. Adult mouse peritoneal macrophages were isolated by peritoneal lavage as described previ-

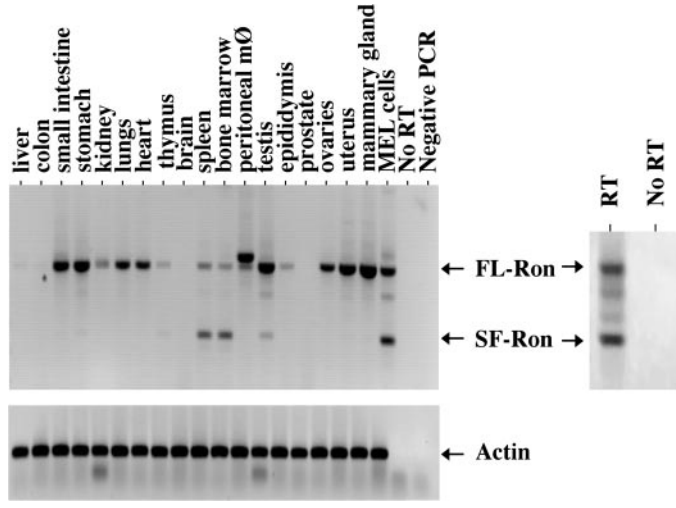

Fig. 1. Tissue-specific expression of short and long forms of Ron receptor mRNA transcripts. *Top left*: tissue-specific expression of murine Ron receptor mRNA was evaluated by RT-PCR using primers specific for full-length (FL)-Ron and short-form (SF)-Ron, as described in EXPERIMENTAL PROCEDURES. RNA isolated from murine erythroleukemia (MEL) cells was used as a positive control. No RT (using MEL cell RNA) and Negative PCR control reactions lack reverse transcriptase or cDNA template, respectively. The FL-Ron (780-bp) and SF-Ron (457-bp) transcript PCR products are noted. The slower migrating band seen in peritoneal macrophages (mØ) is a result of the amplification of genomic DNA. *Bottom left*: RT-PCR analysis using primers specific for actin, which generates a 218-bp PCR product. *Right*: RT-PCR for FL-Ron and SF-Ron transcripts performed on RNA specimens obtained from purified splenic T cells isolated from wild-type (+/+) mice.

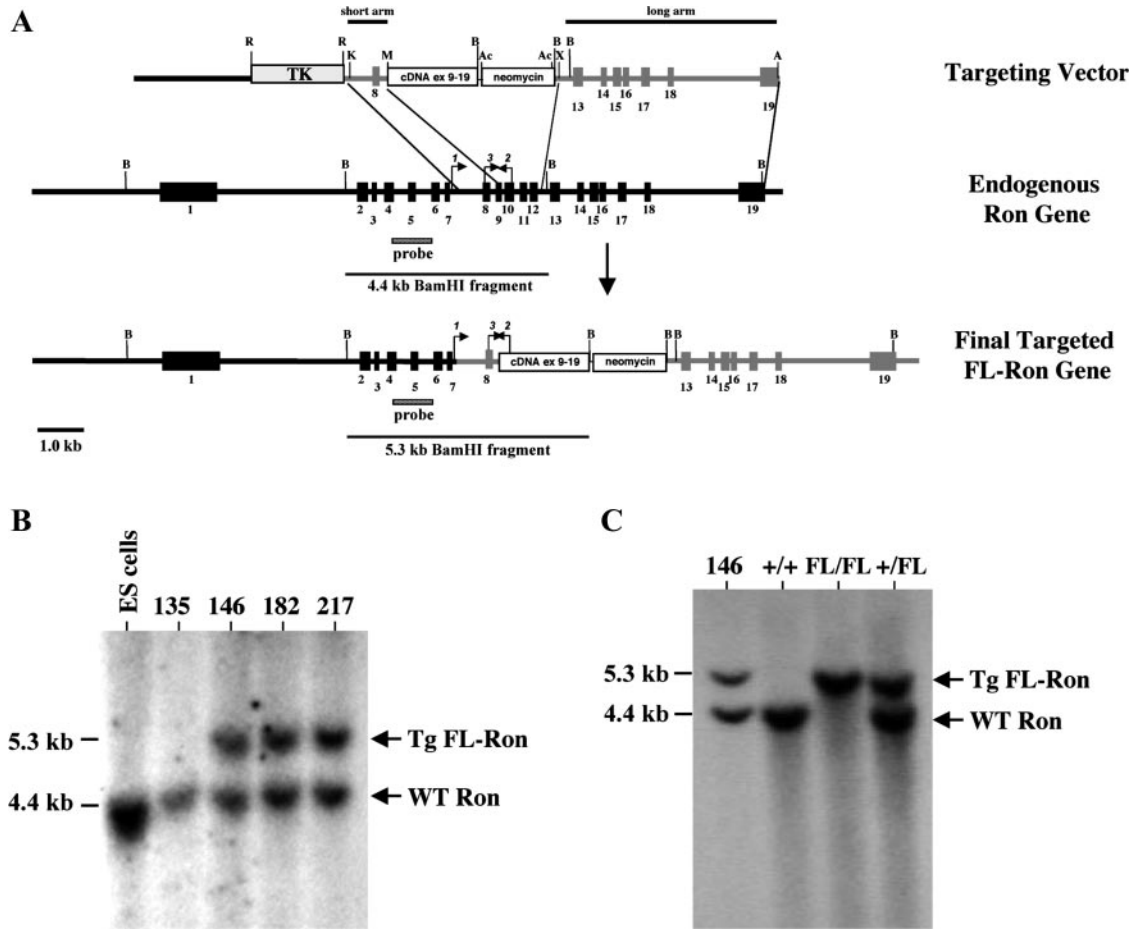

Fig. 2. Targeting of the mouse FL-Ron gene construct into embryonic stem cells and mice. *A*: schematic representation and partial restriction enzyme map of the targeting vector (*top* line) designed to eliminate the expression of the short form of Ron from the endogenous mouse Ron gene (*middle* line). A schematic representation of the knocked-in FL-Ron gene following homologous recombination is shown (*bottom* line). A 786-bp region (i.e., exon 8 and its flanking sequences) and a 4.8-kb region (i.e., exons 13–19 and associated intervening sequences) of the mRon gene were used as the short and long region of homology, respectively (gray lines). The targeting vector also contains part of the mRon cDNA coding for exons 9–19 (open box; 2.0 kb). Positive and negative selection cassettes are shown [neomycin cassette, open box; herpes simplex virus thymidine kinase (TK), shaded box]. Endogenous Ron gene exons are numbered 1–19 below the line. Primers used for genotyping are indicated with arrows numbered 1, 2, and 3 above the lines. Select restriction enzymes shown: B, *Bam*HI; X, *Xba*I; M, *Msc*I; K, *Kpn*I; A, *Apa*I; R, *Rsr*II; Ac, *Asc*I. *B* and *C*: Southern blot analysis of *Bam*HI-digested DNA isolated from selection-resistant embryonic stem (ES) cell clones (*B*) and progeny of an intercrossing of mice heterozygous for the targeted FL-Ron allele (*C*). For both panels, filters were hybridized to an 899 bp Ron genomic DNA fragment used for generating probe, as shown in *A*. The endogenous wild-type Ron allele (WT-Ron) and targeted FL-Ron knock-in allele (Tg-FL-Ron) give rise to 4.4-kb and 5.3-kb *Bam*HI fragments, respectively. *B*: ES cells before electroporation (ES cells) and four selection-resistant candidate ES cell clones (135, 146, 182, and 217) are shown. *C*: DNA obtained from ES *clone 146* (146, *far left* lane) and from mice derived from ES *clone 146*. +/+, wild-type mice; +/FL and FL/FL, mice heterozygous and homozygous, respectively, for the FL-Ron allele.

ously (29). Macrophages were treated for 3 h at 37°C supplemented with or without 400 ng of recombinant human hepatocyte growth factor-like protein (HGFL; R&D Systems, Minneapolis, MN) in DMEM containing 2 mM L-glutamine and 50 μg/ml gentamicin. Macrophages were scored as activated if the cells displayed a compact, spindle-shaped morphology with elongated cytoplasmic processes (27).

Friend virus infection. Wild-type (+/+) and homozygous littermate FL-Ron/FL-Ron (FL/FL) mice, the progeny of heterozygous breeding, that were backcrossed to FVBN mice for three generations were injected with FVP, a strain of Friend virus that induces marked splenic enlargement as a result of proliferating erythroblasts found in splenic foci postinfection, at 5 mo of age. Spleens were isolated from mice 14 days following infection, as previously described (21).

Activation of agonist-stimulated splenocytes. For leukocyte phenotype and cytokine analyses, mice were backcrossed 6 generations onto an FVBN background. Splenocytes were isolated from each genotype and cultured for 24 or 48 h in the presence of 5 μg/ml ConA or with plate-bound anti-CD3/TCR MAb (clone 145–2C11, BD Pharmingen, San Diego, CA), essentially as previously described (3). Briefly, 4×10^6 cells were seeded per well in 24-well plates in 2 ml of RPMI containing 5% FCS. Supernatants were collected and assayed for cytokine production by ELISA analyses as previously described (11). Flow cytometric analysis of splenocytes was performed using a BD LSR flow cytometer, using FACS research software and CellQuest programs (BD Biosciences, Mountain View, CA). Naive T cells were characterized as being $CD3\epsilon^+$ and $CD62L^{high}$, while nonnaive T cells were characterized as being $CD3\epsilon^+$ and $CD62L^{low}$, using peridinin chlorophyll protein-conjugated Armenian hamster anti-mouse CD3ε (clone 145–2C11, BD Pharmingen) and R-phycoerythin-conjugated rat anti-mouse CD62L (clone MEL-14, BD Pharmingen) as primary detection antibody. Resident (F4/80 positive) macrophages were characterized by using Alexa 488-conjugated rat anti-mouse F4/80 (clone A3–1, CALTAG, Burlingame, CA) (19).

Assessment of liver injury and survival in Con A-treated mice. For the following experiments, all mice were backcrossed eight genera-

tions onto an FVBN background. Eight- to 12-wk-old male mice were injected intravenously with ConA (20 mg/kg). At 6 and 15 h postinjection, mice were killed and venous blood was obtained for analysis. Serum alanine aminotransferase levels were determined in the clinical laboratory of Children's Hospital Medical Center. Cytokine production was determined on serum by ELISA as previously described (27) Liver was preserved for routine histology by fixation in 10% neutral buffered formalin, paraffin embedded, sectioned to 4 μm, and stained with hematoxylin and eosin (11).

For the survival analysis, female mice were injected with ConA (40 mg/kg) and monitored for death at least every 6 h, or more frequently if death appeared imminent, up to 48 h postinjection.

Assessment of the effect of anti-IFN-γ antibody on the progression of ConA-induced liver injury was performed by pretreating male mice with anti-IFN-γ antibody (clone XMG1.2 from BD Pharmingen, San Diego, CA; 1 mg/mouse in a volume of 200 μl) administered intraperitoneally 1 h before injection of ConA (20 mg/kg).

Statistical analyses were performed by use of StatView and ANOVA unpaired *t*-tests.

RESULTS

Expression pattern of SF-Ron. To determine the expression pattern of SF-Ron in wild-type mice, RT-PCR analysis was performed. SF-Ron was detected in the spleen, bone marrow, and testis and was not apparent in the other tissues tested (Fig. 1*A*). This distribution of SF-Ron expression suggests that SF-Ron may be involved in immune cell biology and is further supported by the detection of SF-Ron in purified splenic T cells (Fig. 1*B*).

Disruption of the mouse Ron gene. To define the role of SF-Ron in vivo, we generated mice that were incapable of producing SF-Ron but could still express FL-Ron. To accomplish this, a targeting vector possessing a Ron transgene that eliminates the alternative SF-Ron transcriptional start site located in intron 10, yet still allows for expression of FL-Ron mRNA transcripts, was generated (Fig. 2). Homologous recombination of the FL-Ron knock-in transgene into clonal ES cell populations was initially identified by PCR analysis and confirmed by Southern blot analysis using a Ron probe originating from outside the targeted region (probe, Fig. 2*A*). Homologous recombination of the FL-Ron transgene occurred in 3 of 300 ES cell clones analyzed (Fig. 2*B*). All three ES cell lines obtained were injected into blastocysts, but only one (ES *clone 146*) led to transmission of the targeted FL-Ron allele to offspring (Fig. 2*C*).

Table 1. *Genotype distribution of offspring from +/FL Ron intercrossings*

Genotype	+/+	+/FL	FL/FL
Number %	136 (24%)	310 (54%)	128 (22%)

$N = 574$ total offspring. Genotypes were determined by Southern hybridization or PCR analysis, as described in EXPERIMENTAL PROCEDURES. FL, full-length Ron allele. χ^2 comparison of the genotypes had a P value = 0.1.

Characterization of mice homozygous for the FL-Ron knock-in transgene. To examine the consequences of knocking in the FL-Ron gene at the site of the endogenous Ron alleles on survival and fertility, hemizygous siblings (+/FL-Ron) were intercrossed and genotyped (Fig. 2*C*). The transmission of the FL-Ron allele followed a Mendelian pattern of inheritance (Table 1), showing that there were no lethal effects of the targeted FL-Ron transgene.

Homozygous FL-Ron (FL/FL) mice produced fertile males and females, with females carrying pregnancies to term and with comparable litter size to wild-type controls. No differences were observed with regard to appearance, weight gain, or histological appearance of major organs between FL/FL and wild-type mice (data not shown), nor were there observed differences in the number or composition of circulating leukocytes, red blood cells, or platelets. Furthermore, the number of resident peritoneal macrophages did not differ between wild-type and FL/FL mice (data not shown).

Analysis of alternative Ron receptor mRNA expression in FL-Ron knock-in transgenic mice. To determine whether the targeted FL-Ron transgenic mice have ablation of SF-Ron expression, RT-PCR analysis was performed on RNA isolated from tissues that expressed SF-Ron constitutively. FL-Ron and SF-Ron were detected in the bone marrow of wild-type and heterozygous mice, whereas only FL-Ron is detected in FL/FL mice (Fig. 3, *A* and *B*). Moreover, FL/FL mice did not express SF-Ron in the spleen or testis (Fig. 3*B*), compared with wild-type mice. These data demonstrate that homozygous FL/FL mice do not produce SF-Ron transcript but retain FL-Ron expression.

To determine whether appropriately sized transcript is generated from the FL-Ron locus and to confirm the RT-PCR analyses shown earlier, Northern blot analysis was performed on testis RNA isolated from wild-type mice and FL/FL mice.

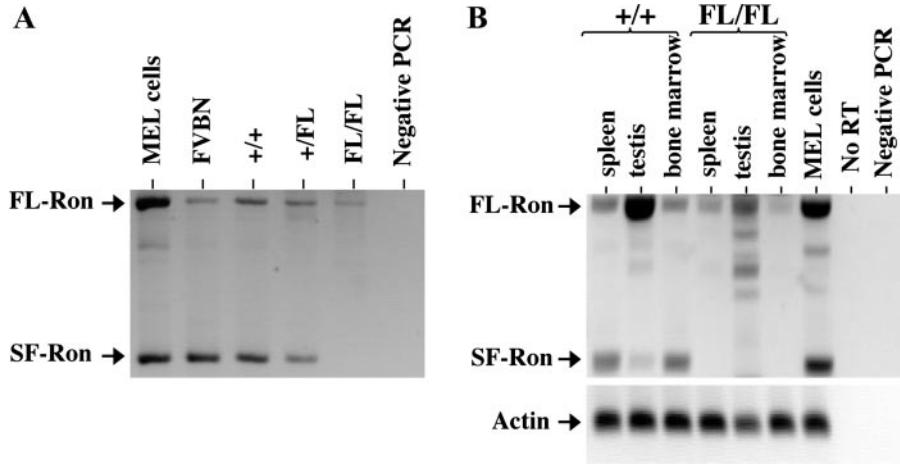

Fig. 3. Confirmation of elimination of the SF-Ron receptor transcript in FL-Ron-targeted mice. RT-PCR was performed using primers that allowed for simultaneous amplification of the FL-Ron and SF-Ron transcripts, as described in EXPERIMENTAL PROCEDURES. Migration of the FL-Ron and SF-Ron transcript PCR products are as indicated. RNA isolated from MEL cells served as a positive control. The negative PCR lacked cDNA template. No RT and Negative PCR control reactions lack reverse transcriptase or cDNA template, respectively. *A*: RT-PCR performed on bone marrow RNA specimens obtained from either wild-type FVBN mice (FVBN) or littermates from the FL-Ron gene-targeted colony that possessed either wild-type Ron receptor (+/+) or were hemizygous (+/FL) or homozygous (FL/FL) for the FL-Ron targeted allele. *B*, *top*: RT-PCR analysis of RNA isolated from spleen, bone marrow, and testes of wild-type (+/+) and homozygous FL-Ron (FL/FL) mice. *B*, *bottom*: RT-PCR analysis using primers specific for actin.

Slightly decreased levels of FL-Ron mRNA expression were observed in FL/FL mice compared with wild-type mice. However, as shown in Fig. 4, the expected 1.9-kb SF-Ron transcript was detected in testicular RNA from wild-type mice but not from FL/FL mice, further demonstrating that homozygous FL/FL mice have lost the ability to produce the SF-Ron transcript.

Friend erythroleukemia virus infection. Previous studies demonstrated that SF-Ron is required for susceptibility to Friend virus infection (21). However, these studies never precluded the involvement of FL-Ron in this process. To further define the impact of FL-Ron in Friend virus susceptibility, wild-type (+/+), heterozygous (+/FL), and homozygous FL/FL (FL/FL) mice were infected with FVP. As shown in Fig. 5, FL/FL mice had a significantly decreased spleen size compared with both wild-type mice and mice hemizygous for the FL mutation, confirming that SF-Ron is required for viral infection and that FL-Ron does not contribute to the susceptibility phenotype.

In vitro response of peritoneal macrophages to HGFL. Peritoneal macrophages show dramatic morphological changes in response to HGFL in vitro (10, 18, 24, 27, 28, 31, 32). To determine whether SF-Ron contributes to this HGFL/Ron-mediated morphological response, peritoneal macrophages isolated from wild-type mice and mice heterozygous (+/FL) or homozygous (FL/FL) for the FL-Ron allele were analyzed for HGFL-induced morphological shape changes indicative of activation. Macrophages from wild-type mice had similar baseline levels of activated macrophages and in response to HGFL underwent morphological shape changes to a degree consistent with published analyses (data not shown) (2, 18, 27, 28). Interestingly, both +/FL and homozygous FL/FL macrophages responded to HGFL to the same degree as macrophages isolated from wild-type mice. This suggests that SF-Ron signaling is not essential for this component of HGFL-induced macrophages activation.

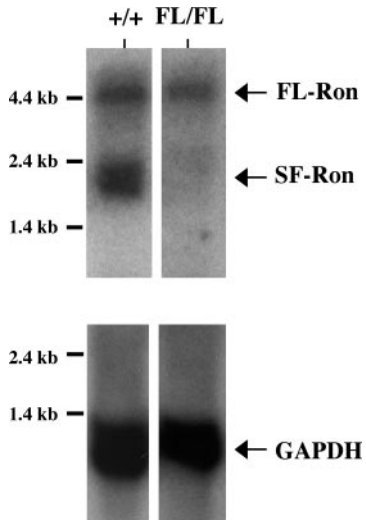

Fig. 4. Analysis of Ron receptor mRNA transcript expression by Northern hybridization. Northern blot analysis was performed on total RNA isolated from testes from wild-type (+/+) or homozygous FL-Ron (FL/FL) mice. The blot was probed with a mouse Ron cDNA fragment (*top*). The FL-Ron and SF-Ron mRNA transcripts are 4.8 and 1.9 kb, respectively, as shown at *right*. Mobility of RNA molecular weight markers is shown at *left*. The membrane was stripped and reprobed with a GAPDH cDNA probe (*bottom*).

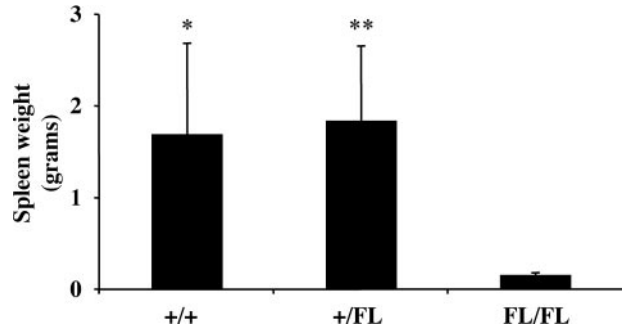

Fig. 5. SF-Ron is required for Friend virus disease susceptibility. Fourteen days following infection with the FVP strain of Friend virus, spleens isolated from mice that were wild-type (+/+), hemizygous (+/FL), or homozygous for the FL-Ron allele (FL/FL) were weighed. Values represent the average spleen weight from 3 to 6 mice per group, with standard error values indicated. Statistical comparisons were made to the FL/FL genotype (*$P = 0.02$; **$P < 0.01$).

Influence of SF-Ron on IFN-γ production ex vivo. Given the prominent expression of SF-Ron in the spleen, we posited that SF-Ron alters the biological activity of splenocytes. To test this idea, splenocytes isolated from FL/FL mice were compared with those isolated from wild-type controls. Following stimulation with ConA, an antigen-presenting cell (APC)-dependent activator of T cells, splenocytes from FL/FL mice had a significantly greater production of IFN-γ compared with control splenocytes (Fig. 6*A*). Similar results were obtained when splenocytes were stimulated with a second APC-independent activator of T cells (anti-CD3/TCR monoclonal antibody; Fig. 6*B*). The impact of SF-Ron on splenocyte cytokine production appeared to be relatively specific to IFN-γ, because no alterations were observed in IL-4, IL-2, or IL-12 production or in the production of nitric oxide, following either ConA or endotoxin stimulation (Table 2 and data not shown). Moreover, these differences in IFN-γ production were not due to altered proportions of resident macrophages or of naive and nonnaive

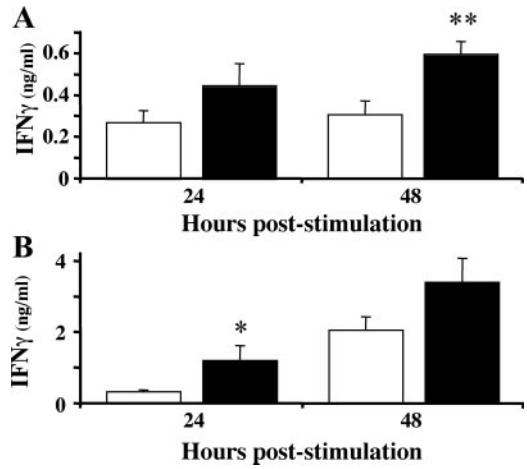

Fig. 6. SF-Ron is required for normal IFN-γ production in stimulated splenocytes. Splenocytes from wild-type (+/+, open bars) and FL-Ron (FL/FL, solid bars) mice were isolated and cultured ex vivo in the presence of concanavalin A (*A*) or plate-bound anti-CD3/TCR monoclonal antibody (*B*) as described in EXPERIMENTAL PROCEDURES. Results shown represent means ± SE, with $N = 8$ mice per experimental determination. Statistical comparisons were between genotypes (*$P < 0.05$; **$P < 0.01$).

Table 2. *FL-Ron spleenocytes produce similar levels of IL-2, -4, and -12 with ConA stimulation*

	24-h Stimulation			48-h Stimulation		
	+/+	FL/FL	*P* value	+/+	FL/FL	*P* value
IL-2	545.7±149.2	498.8±49.3	0.341	335.2±137.8	267.7±125.5	0.320
IL-4	24.5±14.9	29.8±19.9	0.379	14.4±14.9	20.5±23.6	0.385
IL-12	85.2±56.6	58.8±42.1	0.211	93.3±7.6	94.8±13.0	0.453

Levels of cytokines in the culture media ($n = 4$ wells per determination) are expressed as means ± SE, in pg/ml. Spleenocytes were isolated from FL-Ron mice and stimulated with conconavalin A (ConA) as described in EXPERIMENTAL PROCEDURES and Fig. 6.

T cells in the spleens of FL/FL mice compared with wild-type mice (Table 3).

Influence of SF-Ron on IFN-γ production in vivo. To establish the extent to which SF-Ron impacts IFN-γ-dependent immune-mediated disease processes in vivo, we employed an experimental model of immune-mediated acute liver failure triggered by exposure to ConA (15, 23). In this model, IFN-γ is produced mostly from natural killer T cells, and to a lesser degree from T cells and Kupffer cells (15, 23).

Six and 15 h postinjection of Con A, serum IFN-γ levels were significantly elevated in FL/FL mice compared with controls (Fig. 7*A*), and this was associated with a significant increase in serum alanine aminotransferase levels (Fig. 7*B*), a marker of liver injury, as well. In a separate experiment using a higher dose of Con A, FL/FL mice had a significantly worse survival outcome compared with control mice, with 100% of FL/FL mice succumbing under 10 h postexposure, compared with complete survival at the end of the 48-h experiment in the control group (Fig. 7, *C* and *D*).

Histological analysis of liver tissue following a 15 h exposure to ConA showed a corresponding increase in necroinflammatory liver injury in FL/FL mice compared with control mice (Figs. 8, *A* and *B*). In addition, pretreatment of mice with anti-IFN-γ antibody before administration of ConA blocked the development of histological liver injury in both control and FL/FL mice (Fig. 8, *C* and *D*), consistent with a prominent role of IFN-γ in mediating Con A-induced liver injury. Thus elimination of SF-Ron enhances IFN-γ production in response to ConA in an in vivo animal model of immune-mediated acute liver injury, and this correlates with a worse liver injury both biochemically and histologically.

DISCUSSION

Several alternative forms of the Ron receptor having an altered cytoplasmic tyrosine kinase domain and increased receptor kinase activity have been identified in human cancers or cancer cell lines. The present research study explores the role of SF-Ron protein, a naturally occurring constitutively active form of the Ron receptor. The biological roles of these alternative forms of Ron have not been thoroughly studied, especially without the confounder of having a second Ron receptor form, namely the FL-Ron receptor, present concurrently during the experimental analyses. For example, the presence of FL-Ron could potentially impact the activity of SF-Ron, and vice versa, through either a dominant negative or dominant positive effect on basal or ligand-induced Ron receptor tyrosine kinase activity.

Table 3. *Distribution of splenocyte cell subpopulations in wild-type and FL/FL Ron mice*

Cell Type	+/+ Mice	FL/FL Mice	*P* Value
Resident macrophages	13.97±0.68%	15.53±0.71%	NS
Naive T cells	37.84±0.10%	35.04±1.35%	NS
Nonnaive T cells	13.60±0.30%	11.71±0.66%	0.039

Results are expressed as mean % ± SE ($N = 4$ for each genotype; significance at $P < 0.05$). The distribution of splenocyte cell subpopulations obtained from wild-type (+/+) and FL/FL mice was determined by flow cytometric analysis as described in EXPERIMENTAL PROCEDURES. NS, not significant.

We hypothesized that SF-Ron is involved in physiological roles in vivo distinct from those of the FL-Ron receptor. To explore the biological effects of the SF-Ron receptor isolated from the potential confounding influence of concomitant expression of FL-Ron, we generated mice that lack the capacity to express SF-Ron in any tissue in vivo, yet retain the ability to express FL-Ron. Indeed, homozygous FL-Ron mice thus generated do not express SF-Ron in any murine tissue that expresses SF-Ron in wild-type littermates (Fig. 1 and 3). Importantly, elimination of SF-Ron does not have a significant impact on the viability or overall development of mice.

Two viable experimental mouse models have been previously generated with the goal of targeted disruption of the Ron locus (5, 27). The first mouse model generated involved the targeted elimination of exon 1 of the murine Ron gene (5); however, these mice can theoretically still express SF-Ron generated from the alternative start site in exon 10. Although full characterization of SF-Ron expression in mouse organs was not reported, expression of SF-Ron in the bone marrow was reported to be eliminated (21). Indeed, these mice served in part as the experimental basis for the conclusion that expression of SF-Ron in adult bone marrow tissue confers susceptibility of mice to Friend erythroleukemia virus infection (21). In this report, we independently validate these prior findings that mice lacking SF-Ron expression in the spleen are resistant to Friend virus erythroleukemia infection (Fig. 5). Taken together, these collected data strongly suggest that SF-Ron is necessary for murine susceptibility to Friend erythroleukemia virus. Development of an experimental model to allow isolated expression of SF-Ron in the context of absent FL-Ron expression would establish that SF-Ron expression is sufficient for Friend virus susceptibility. The second mouse model of Ron receptor-deficient mice was generated in our laboratory and involves the deletion of the majority of the cytoplasmic tyrosine kinase domain of the receptor (27). Thus, by definition, these mice are incapable of producing either FL- or SF-Ron.

The results reported in this study suggest a novel biological role for SF-Ron, namely in regulating the mammalian immune response ex vivo and in vivo. Stimulation of splenocytes ex

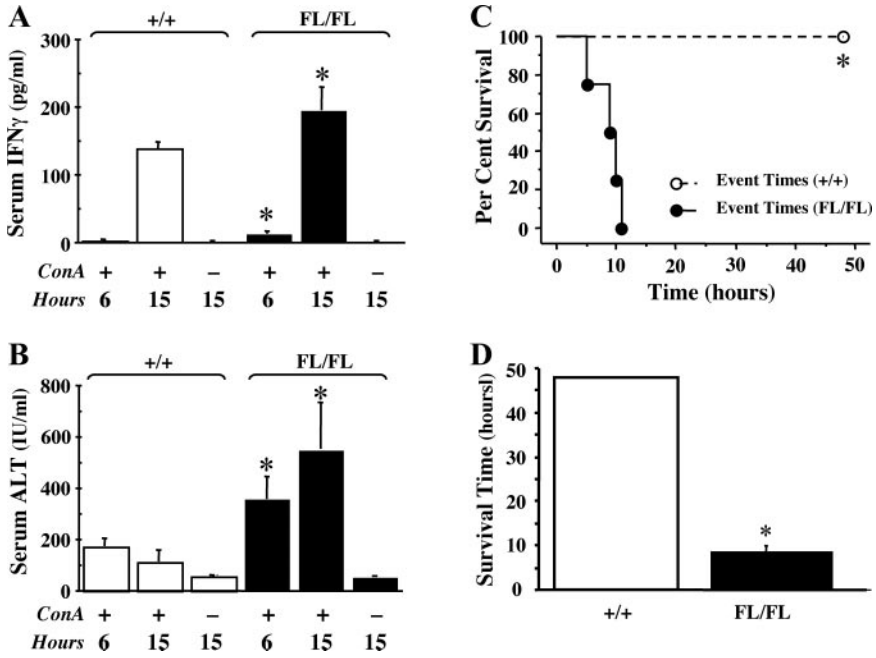

Fig. 7. Elimination of SF-Ron augments liver injury, IFN-γ production, and death in a murine model of T cell-mediated acute liver injury. *A* and *B*: blood was obtained from wild-type (+/+, open bars) and FL-Ron (FL/FL, solid bars) mice injected with either 20 mg/kg concanavalin A (ConA) or saline vehicle 6 or 15 h before death. Serum IFN-γ (*A*) and alanine aminotransferase (ALT; *B*) levels were analyzed as described in EXPERIMENTAL PROCEDURES. Results shown represent means ± SE, with $N = 8$ mice per ConA-treatment group ($N = 2$ for saline control groups). Statistical comparisons were between genotypes (*$P < 0.05$). *C*: cumulative survival plot for wild-type (○, $n = 6$) and FL/FL mice (●, $n = 4$) mice injected with 40 mg/kg ConA. Log-rank (Mantel-Cox) statistical comparisons were between genotypes (*$P = 0.01$). *D*: survival time for mice treated as in *C*; statistical comparisons were between genotypes (*$P < 0.0001$).

vivo either with ConA (an APC-dependent activator of T cells) or anti-CD3/TCR antibody (an APC-independent activator of T cells) leads to SF-Ron-dependent inhibition of IFN-γ production, suggesting that SF-Ron either directly or indirectly suppresses T cell effector function (Fig. 6). However, peritoneal macrophages isolated from mice that are either heterozygous or homozygous for the FL-Ron allele respond to HGFL in a morphological assay in a manner similar to macrophages isolated from wild-type mice (data not shown), suggesting that SF-Ron is not essential for HGFL-induced activation of this macrophage subpopulation.

The type of immune response generated in vivo is influenced by the profile of cytokines that are secreted by lymphocytes and other immune cells in response to antigen. Immune responses characterized by an increase in IFN-γ production are prototypically a type 1 immune response, whereas a type 2 immune response is associated with production of IL-4, IL-5, and IL-10 (17, 20). Type 1 responses are associated with macrophage activation driven in part by IFN-γ production and other aspects of cell-mediated immunity, whereas type 2 responses typically lead to inhibition of macrophage activation and antibody production. The in-

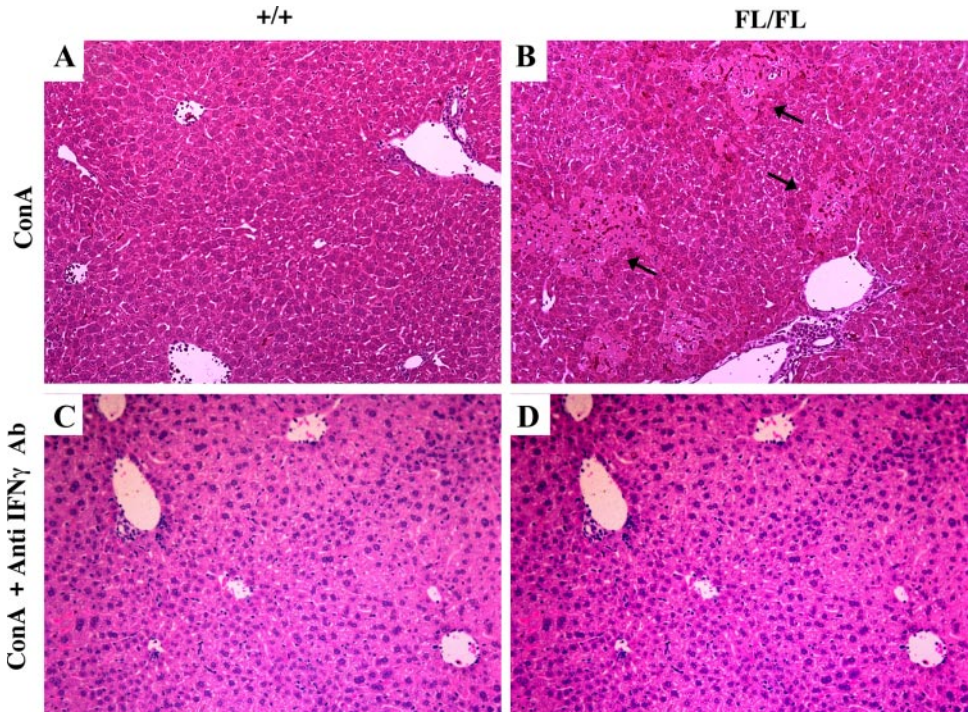

Fig. 8. Enhanced liver injury in FL-Ron mice is reversed by pretreatment with anti-IFN-γ antibody. Representative hematoxylin and eosin stained liver sections from wild-type (*A* and *C*) and FL-Ron (*B* and *D*) mice, following a 15-h exposure to intravenously dosed ConA. Mice *C* and *D* were pretreated with anti-IFN-γ antibody before exposure to ConA. The livers of FL-Ron mice showed marked hemorrhagic hepatocellular damage (black arrows; *B*), whereas the livers of wild-type mice, and both strains of mice pretreated with anti-IFN-γ antibody, were normal appearing.

crease in IFN-γ production observed in this study following stimulation of splenocytes from wild-type and FL-Ron mice with ConA suggests that FL-Ron mice are preferentially generating a type 1 immune response relative to a type 2 response, and as a corollary, that SF-Ron is critical in maintaining the proper immunological balance between a type 1 and type 2 immune response.

In addition to the ex vivo experiments demonstrating an involvement of SF-Ron in immune regulation, we show that SF-Ron impacts disease progression in vivo in a commonly used experimental murine model of T-cell mediated acute liver injury. In this model, stimulation with ConA leads to infiltration of mononuclear cells, activation of T cells and natural killer T cells, and the release of proinflammatory cytokines, resulting in severe liver destruction (14, 26). Of these proinflammatory cytokines, INF-γ is especially critical in driving the pathological process, because liver injury is significantly reduced in INF-γ-deficient mice or in mice pretreated with INF-γ neutralizing antibody (16, 25). Our results show that FL-Ron mice lacking SF-Ron, compared with wild-type control mice, have a more severe liver injury in this model of immune-mediated liver disease, as manifest by increased serum levels of IFN-γ and alanine aminotransferase, worsened liver histology, and poorer overall survival (Figs. 7 and 8). Moreover, the heightened liver injury observed in Con A-treated FL/FL mice is reversed if mice are pretreated with antibody directed against IFN-γ, suggesting a significant mechanistic role for IFN-γ in the enhanced FL/FL liver injury phenotype. Thus, in the normal setting, this suggests that SF-Ron dampens the immune response and that SF-Ron is critically important for generation of a normal immune response in vivo.

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