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Tumor Necrosis Factor—Related Apoptosis-Inducing Ligand Receptor Deficiency Promotes the Ductular Reaction, Macrophage Accumulation, and Hepatic Fibrosis in the *Abcb4*^{-/-} Mouse

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The tumor necrosis factor—related apoptosis-inducing ligand (TRAIL; TNFSF10) receptor (TR) is a pro-apoptotic receptor whose contribution to chronic cholestatic liver disease is unclear. Herein, we examined TRAIL receptor signaling in a mouse model of cholestatic liver injury. TRAIL receptor-deficient (*Tnfr1*^{-/-} or *Tr*^{-/-}) mice were crossbred with ATP binding cassette subfamily B member 4—deficient (*Abcb4*^{-/-}, alias *Mdr2*^{-/-}) mice. Male and female wild-type, *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} mice were assessed for liver injury, fibrosis, and ductular reactive (DR) cells. Macrophage subsets were examined by high-dimensional mass cytometry (time-of-flight mass cytometry). *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice had elevated liver weights and serum alanine transferase values. However, fibrosis was primarily periductular in *Mdr2*^{-/-} mice, compared with extensive bridging fibrosis in *Tr*^{-/-}*Mdr2*^{-/-} mice. DR cell population was greatly expanded in the *Tr*^{-/-}*Mdr2*^{-/-} versus *Mdr2*^{-/-} mice. The expanded DR cell population in *Tr*^{-/-}*Mdr2*^{-/-} mice was due to decreased cell loss by apoptosis and not enhanced proliferation. As assessed by time-of-flight mass cytometry, total macrophages were more abundant in *Tr*^{-/-}*Mdr2*^{-/-} versus *Mdr2*^{-/-} mice, suggesting the DR cell population promotes macrophage-associated hepatic inflammation. Inhibition of monocyte-derived recruited macrophages using the CCR2/CCR5 antagonist cenicriviroc in the *Mdr2*^{-/-} mice resulted in further expansion of the DR cell population. In conclusion, genetic deletion of TRAIL receptor increased the DR cell population, macrophage accumulation, and hepatic fibrosis in the *Mdr2*^{-/-} model of cholestasis. (*Am J Pathol* 2020, 190: 1284–1297; <https://doi.org/10.1016/j.ajpath.2020.02.013>)

The bile ducts are lined by cholangiocytes, which modify bile and provide a barrier between biliary constituents in the ductular lumen and the surrounding tissue. Unfortunately, the cholangiocytes are affected by inflammation and fibrosis in a variety of human diseases termed the cholangiopathies.¹ These cholangiopathies are associated with impaired bile formation, causing cholestasis. Over time, the cholestatic liver injury can result in advanced hepatic fibrosis, cirrhosis, and end-stage liver disease; hence, these diseases are associated with considerable morbidity and mortality. The inflammatory and fibrotic processes mediating the cholangiopathies are incompletely understood, which has limited the development of rational therapies.

A histopathologic feature of the cholangiopathies is the development of a ductular reactive (DR) cell population; epithelial cells characterized by a biliary phenotype, organized into irregular shaped structures, often without a lumen, initially localized at the peripheral region of the portal space, from which they often extend

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into the hepatic parenchyma.^{2,3} The DR cells exist in a niche intimately associated with myofibroblasts, and accordingly, the magnitude of the DR cell population correlates with the severity of fibrosis in cholestatic liver diseases.³ DR cells have also been associated with macrophage-mediated inflammation.^{2,4–6} These observations implicate DR cells as mediators of liver injury in cholestasis. However, few studies have examined the mechanisms regulating the extent of the DR cell population during cholestasis.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), also known as TNF superfamily member 10 (TNFSF10), a death ligand, induces apoptosis in cells expressing its cognate receptors. Two pro-apoptotic TRAIL receptors (TRs) are expressed in humans, TRAIL-receptor 1 or death receptor 4 and TRAIL-receptor 2/death receptor 5. Mice have a single TR.⁷ Although TRs are ubiquitously expressed, TRAIL is mainly expressed on cells of the immune system, including macrophages.⁷ Interestingly, TR-mediated signaling has been shown to play a role in inducing acute cholangiocyte injury. Treatment of genetically susceptible mice (C57Bl/6) with agonistic anti-TR antibody induces acute cholestasis with fibrosis.⁸ However, the role of TR signaling in chronic cholestasis remains unclear, especially its role in DR cell biology.

As macrophages are a source of TRAIL, participate in cholestatic liver injury,⁹ and are associated with DR cells, their role in TR signaling in chronic cholestasis is likely important. The macrophages associated with DR cell population likely consist of diverse subsets. Macrophages exist as multiple subsets and are characterized using a variety of criteria. For example, liver macrophages are also frequently classified as resident macrophages (Kupffer cells) or recruited macrophages (ie, circulating bone marrow–derived monocytes differentiating into macrophages). Functionally, macrophages also exist as a continuum, with tissue damaging or proinflammatory at one end of the spectrum (M1-like) and restorative macrophages involved in tissue repair and healing at the other end (M2-like). However, the restorative macrophages have been implicated in tissue fibrosis as part of an unrelenting wound healing response.¹⁰ Restorative macrophages often express the scavenger receptors MER proto-oncogene tyrosine kinase (MERTK), CD206, and galectin-3 (alias LGALS3 or Mac-2). The MERTK-expressing macrophages are a cellular source of TRAIL.¹¹ Although recruited macrophages as opposed to resident macrophages participate in the inflammatory periductal milieu in *Abcb4*^{−/−} mice (a model of chronic biliary tract injury mimicking human cholestatic liver injury; alias *Mdr2*^{−/−}),⁹ the nature of the macrophage subsets associated with the cholangiocytes and the DR cell population in cholestasis has not been extensively examined. Herein, we report that *Mdr2*^{−/−} mice genetically lacking the murine TRAIL receptor (TR), the cognate receptor for TRAIL, are characterized by a substantially increased DR population, macrophage accumulation, and fibrosis.

Materials and Methods

Animals

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee of the Mayo Clinic (Rochester, MN). In-house colonies of Trail receptor–deficient (*Tnfrsf10*; alias *Tr*^{−/−})¹² and *Mdr2*–deficient (*Mdr2*^{−/−})¹³ mice on a C57Bl/6 background were used to generate all genotypes employed for these studies. Briefly, *Tr*^{−/−} and *Mdr2*^{−/−} were crossbred to generate the heterozygous *Tr*^{+/-}*Mdr2*^{+/-} animals that were further crossbred to generate *Tr*^{+/+}*Mdr*^{+/+} [wild type (WT)], *Tr*^{−/−}*Mdr*^{+/+} (*Tr*^{−/−}), *Tr*^{+/-}*Mdr2*^{−/−} (*Mdr2*^{−/−}), and *Tr*^{−/−}*Mdr2*^{−/−}. Mice were reared on a 12-hour light-dark cycle and had ad libitum access to food and water. At 45 ± 5 and 90 ± 5 days of age, male and female mice of the following genotypes (WT, *Tr*^{−/−}, *Mdr2*^{−/−}, and *Tr*^{−/−}*Mdr2*^{−/−}) were euthanized by exsanguination. Mice were weighed and anesthetized with ketamine (10 mg/kg, intraperitoneally) and xylazine (120 mg/kg, intraperitoneally). Blood was drawn from the inferior vena cava. The liver was isolated, rinsed in phosphate-buffered saline, and weighed. Subsamples of liver tissue were immediately preserved in 4% buffered formalin, embedded in OCT, or snap frozen. In a separate study, 45-day-old male and female (*Mdr2*^{−/−}) mice were treated with either the drug cenicriviroc, a CCR2/CCR5 inhibitor that prevents the tissue recruitment of macrophages, or placebo control for a period of 2 weeks. Cenicriviroc was administered subcutaneously daily at a dose of 15 mg/kg body weight.⁹

Serum Biochemistry Analysis

Blood samples were centrifuged at 6000 × g for 20 minutes, serum was separated, and 100 μL of serum was analyzed for alanine transferase, bile acids, bilirubin, and albumin in a Vetscan VS2 using the mammalian liver profile rotor (500-7128; Abaxis, Union City, CA).

Histology and Immunohistochemistry

Liver samples were preserved in 4% buffered formalin for 48 hours, paraffin embedded, and divided into section (5 μm thick). Hematoxylin-eosin staining was performed by the Biomaterials and Histomorphometry Core Facility of the Mayo Clinic. To assess fibrosis, deparaffinized and hydrated tissue sections were stained in 1% Sirius red (365548; Sigma, St. Louis, MO) dissolved in saturated picric acid (P6744; Sigma) for 60 minutes using 0.4% fast green (F7252; Sigma) as counterstain. Additional serial sections were used for immunostaining. Briefly, heat-induced epitope retrieval was performed with 10 mmol/L sodium citrate (pH 6.0) on deparaffinized and hydrated serial sections for 20 minutes. Tissue sections were blocked with Rodent Block M (RBM961; Biocare Medicals, Pacheco, CA) and were probed with antibodies against α-smooth muscle actin (ab21027; Abcam, Cambridge, MA) and F4/80 (ab6640; Abcam) at a

dilution of 1:100 and sex determining region Y-box 9 (SOX9) (AB5535; EMD Millipore, Burlington, MA) at a dilution of 1:500. Bound primary antibodies were detected with prediluted (K4010, K4065; Dako Cytomation, Glostrup, Denmark), species-specific, horseradish peroxidase–conjugated secondary antibodies using diaminobenzidine tetrahydrochloride as chromogen. Sections were counterstained with hematoxylin. Staining for all antibodies was optimized to ensure minimal background and verified for specificity using secondary antibodies alone. Bright-field digital images were acquired on a Zeiss microscope mounted with an Axiocam camera (Carl Zeiss, GmbH, Jena, Germany). Digital images of Sirius red staining for quantitative analysis were acquired under plane polarized light in an inverted microscope (Nikon Eclipse TE300; Nikon, Tokyo, Japan). Digital image analysis was performed using the software NIS Elements AR 4.60.00 (Nikon).

Immunofluorescence

OCT compound embedded frozen tissue was divided into sections (5 μm thick). Air-dried tissue sections were fixed in 4% formalin or acetone and immunostained with antibodies against proliferating cell nuclear antigen (sc-56; Santa Cruz Biotechnology, Dallas, TX) and macrophage inhibitory cytokine 1 (MIC1-1C3) (NBP1-18961; Novus Biologicals, Littleton, CO) or cytokeratin 19 (CK19) (ab52625; Abcam) at a dilution of 1:100. Fluorophore-tagged species-specific secondary antibodies (1:200; A21441, A21070; Thermo-Fisher, Waltham, MA; T6391; Life Technologies, Carlsbad, CA) were used to detect bound primary antibodies. Sections were stained with 300 nmol/L DAPI solution to visualize nuclei. Images were acquired on a confocal microscope (LSM 780; Zeiss, Jena, Germany). Digital image analysis for MIC1-1C3 was performed using the ImageJ software version 1.52i (NIH, Bethesda, MD; <http://imagej.nih.gov>), and results are presented as area percentage per field. For proliferation studies, proliferating cell nuclear antigen and CK19 double-positive cells were counted from a minimum of 15 fields per section; and data are presented as a percentage of the total number of CK19-positive cells.

TUNEL Staining

Apoptotic cells in frozen liver tissue sections were probed for DNA strand breaks with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with fluorescein using a commercially available kit following the manufacturer’s instructions (*In Situ* Cell Death Detection Kit; 11684795910; Roche, Indianapolis, IN). Sections were additionally immunostained with CK7 (1:100; sc-53263; Santa Cruz Biotechnology), as described above, to identify cholangiocytes/ductular reactive cells and counterstained with DAPI for visualization of nuclei. Digital images were acquired on a confocal microscope (Axiovert; Carl Zeiss Microimaging

GmbH, Jena, Germany) at a magnification of ×25. TUNEL-positive nuclei were individually verified for DAPI costaining. TUNEL and CK7 double-positive cells were quantified visually on a confocal microscope from 10 to 12 fields per section. Data are expressed as number of double-positive cells per field.

Hydroxyproline Content

Hepatic hydroxyproline content was determined from approximately 200 mg tissue, adopting previously published methods.¹⁴ Briefly, hepatic collagen was extracted from

Table 1 List of Markers and Antibody Clones Used for Mass Cytometry Analysis

Marker	Clone	Metal tag
CD45	30-F11	89 Y
LGALS3	202213	141 Pr
CD11C	N418	142 Nd
TcRb	H57-597	143 Nd
MHCI	28-14-8	144 Nd
TIM4	370901	149 Sm
MHCII	M5/114.15.2	150 Nd
CD3e	145-2C11	152 Sm
CD206	C06802	153 Eu
TER-119	TER-119	154 Sm
MERTK	108928	155 Gd
CCR2	475301	158 Gd
F4/80	BM8	159 Tb
CD64	290322	160 Gd
Ly6G	1A8	161 Dy
Cx3CR1	SA011F11	164 Dy
CD14	Sa14-2	165 Ho
CD19	6D5	166 Er
CD8a	53-6.7	168 Er
NK1.1	PK136	170 Er
CD11b	M1/70	172 Yb
CD115	AFS98	174 Yb
Ly6C	HK1.4	175 Lu
B220	RA3-6B2	176 Yb
TRAIL	N2B2	169Tm

B220, CD45 isoform expressed in B cells; CCR2, C-C motif chemokine receptor 2; CD11b, cluster of differentiation 11b, integrin alpha-M; CD11C, cluster of differentiation 11c, integrin alpha-X; CD115, cluster of differentiation 115, macrophage colony stimulating; CD14, cluster of differentiation 14, monocyte differentiation antigen; CD19, cluster of differentiation 19, B lymphocyte antigen CD19; CD206, cluster of differentiation 206, macrophage mannose receptor 1; CD3e, cluster of differentiation 3 e, T cell surface glycoprotein epsilon chain; CD45, cluster of differentiation 45, receptor-type tyrosine protein phosphatase C; CD64, cluster of differentiation 64, high affinity immunoglobulin gamma Fc receptor 1; CD8a, cluster of differentiation 8a, T cell surface glycoprotein CD8 alpha chain; Cx3CR1, CX3C chemokine receptor 1; F4/80, adhesion G protein-coupled receptor E1; LGALS3, galectin-3; Ly6C, lymphocyte antigen 6 complex c; Ly6G, lymphocyte antigen 6G; MERTK, tyrosine-protein kinase mer; MHCI, major histocompatibility complex I; MHCII, major histocompatibility complex II; mus, musculus; NK1.1, killer cell lectin-like receptor subfamily B, member 1c factor 1 receptor; TcRb, T-cell receptor beta chain; TER-119, erythroid specific antigen; TIM4, T-cell immunoglobulin and mucin domain-containing protein 4; TRAIL, TNF-related apoptosis inducing ligand.

Table 2 List of Primers Used in the Study (*Mus musculus*)

Gene	Reverse primer	Forward primer
18S	5'-CGCTCCACCAACTAAGAACG-3'	5'-TCAACACGGGAACCTCAC-3'
<i>Gapdh</i>	5'-CCTGTGCTGTAGCCGTATT-3'	5'-TTGTCTCCTGCGACTTCA-3'
<i>Hprt1</i>	5'-CCTGGTTTCATCATCGCTAATC-3'	5'-TCCTCCTCAGACCGCTTTT-3'
<i>TWEAK (Tnfsf12)</i>	5'-CCGAGACACCTGGCACAAA-3'	5'-TCAGGGCTGGGCTCTACTAC-3'
<i>Fn14 (Tnfrsf12a)</i>	5'-GCCAAAACCAGACCAGACT-3'	5'-GGACCTCGACAAGTGCATGG-3'
<i>Krt23</i>	5'-TCCAAGGTCTTTTCGGAGGCC-3'	5'-CGCCAGGATGGCAGTGGATGA-3'
<i>Trail (Tnfsf10)</i>	5'-GCAAGCAGGGTTGTTCAGA-3'	5'-ATGGTGATTGTCATAGTGCTCC-3'

tissue by homogenizing in 0.5 mol/L acetic acid containing 2 mg/mL pepsin (P7012; Sigma) at 50 mg/mL and incubating overnight at 4°C. Digested lysates were hydrolyzed with 4 volumes of 6N HCL. Hydroxyproline content in hydrolysates was determined using a standard biochemical protocol.¹⁵

Intrahepatic Immune Cell Isolation

Intrahepatic leukocytes were isolated from whole livers of WT, *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-} *Mdr2*^{-/-} female mice. Briefly, liver tissue was isolated from euthanized mice, weighed, minced, and digested with a liver dissociation enzyme mix (130-105-807; Miltenyi Biotec, San Diego,

CA) in a gentleMACS Octo-tissue dissociator using the manufacturer's provided protocol. Dissociated cells were centrifuged at 300 × *g* for 5 minutes at 4°C. Red blood cells were lysed (155 mmol/L ammonium chloride, 12 mmol/L sodium bicarbonate, and 0.1 mmol/L EDTA) over ice for 5 minutes. The remaining cells were filtered through a 100-μm cell strainer. Kupffer cells and recruited monocyte and macrophage fractions were collected from the interphase of a 25% to 50% Percoll density gradient. The cell pellet containing the polymorphonuclear and lymphocyte fraction was also collected. Both fractions were combined, washed twice, and resuspended in buffer (phosphate-buffered saline with 10 mmol/L EDTA and 0.5% bovine serum albumin).

Figure 1 *Tr*^{-/-}*Mdr2*^{-/-} mice exhibit liver injury. Body weight (BW), liver weight (LW)/body weight ratio, and serum alanine transferase (ALT) levels of wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} male and female mice at 45 days (A) and at 90 days (B). *n* = 5 to 9 animals per group (A and B). **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001.

Figure 2 Liver injury is increased in *Tr*^{-/-}*Mdr2*^{-/-} mice. Representative images of hematoxylin-eosin staining of liver sections of wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Mdr2*^{-/-}*Tr*^{-/-} male and female mice at 45 days (A) and at 90 days (B). Scale bars = 100 μm (A and B).

Time-of-Flight Mass Cytometry

A minimum of three million freshly isolated intrahepatic leukocytes per animal were used for analysis. The mixed immune cell population was spun down at $300 \times g$ for 5 minutes, resuspended in ultrapure water, and stained with an antibody cocktail (Table 1) conjugated to lanthanide-based metal isotopes, according to manufacturer guidelines (Fluidigm, San Francisco, CA). Preconjugated antibodies were purchased directly from Fluidigm. Custom conjugations were performed by the Mayo Clinic Hybridoma Core (Rochester, MN). Mass cytometry was performed at the Mayo Clinic Immune Monitoring Core (Rochester, MN) on a Helios mass cytometry system (Fluidigm). EQ four element calibration beads were spiked into each sample to allow for signal normalization. Normalization was performed using time-of-flight mass cytometry software version 6.7.1014 (Fluidigm).

High-dimensional data analysis was performed after identifying cell singlets (191Ir⁺ and 193Ir⁺) and viable (195Pt⁻) events using the Cytobank software version 7.2 (Santa Clara, CA). All live singlet events were exported to new fcs files before analysis. Visualization for t-distributed stochastic neighbor embedding clustering analysis was performed on 10,000 equivalent events from each sample with selection of all parameters. Cellular phenotypes were assigned to the visualization for t-distributed stochastic neighbor embedding plot on the basis of distribution and expression characteristics of all markers after clustering. Phenographs were generated in the R language-based Cytofit software version 1.11.3.^{16,17}

Flow Cytometry

Intrahepatic macrophages collected from mice, as described above, were resuspended in serum-free RPMI 1640 medium

Figure 3 Lobular fibrosis is enhanced in $Tr^{-/-}Mdr2^{-/-}$ mice. **A:** Representative images of liver sections from male and female $Mdr2^{-/-}$ and $Tr^{-/-}Mdr2^{-/-}$ mice at 90 days, stained with Sirius red and imaged under plane polarized light for evaluating hepatic fibrosis. **B:** Representative images of liver sections from male and female $Mdr2^{-/-}$ and $Tr^{-/-}Mdr2^{-/-}$ mice at 45 and 90 days, stained with Sirius red for evaluating hepatic fibrosis. **C:** Quantification of collagen in wild-type (WT), $Tr^{-/-}$, $Mdr2^{-/-}$, and $Tr^{-/-}Mdr2^{-/-}$ male and female mice at 45 and 90 days, using plane polarized light on Sirius red-stained sections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$. Scale bars: 100 μ m (**A**); 50 μ m (**B**).

and Primocin (100 μ g/mL), seeded in 6-well plates, and incubated with protein transport inhibitors brefeldin A at 5 ng/mL (420601; BioLegend, San Diego, CA) and monensin at 2 nmol/L (420701; BioLegend). At 4 hours, cells were

detached using 2 mmol/L EDTA for 15 minutes. Cells were resuspended in PEB buffer (phosphate-buffered saline, 2 mmol/L EDTA, and 0.5% bovine serum albumin) and blocked with FcR blocking reagent (130-092-575; Miltenyi

Figure 4 Hydroxyproline content and α -smooth muscle actin (α -SMA) are increased in *Tr*^{-/-}*Mdr2*^{-/-} mice. **A:** Biochemical quantification of total hydroxyproline content in wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} male mice at 90 days. **B:** Representative images of liver sections stained for the marker of activated myofibroblasts, α -SMA, in WT, *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} male mice at 90 days. **C:** Quantification of α -SMA by digital imaging. **P* < 0.05, ***P* < 0.01, and *****P* < 0.001. Scale bars = 50 μ m (**B**).

Biotech). Macrophages were stained with fluorochrome-conjugated surface markers against CD45 (130-102-469; Miltenyi Biotec), CD11b (130-097-336; Miltenyi Biotec), and intracellular antigen TRAIL/CD253 (130-102-562; Miltenyi Biotec). Flow cytometry was performed on a MACSQUANT X (Miltenyi Biotec) using appropriate fluorescence minus one controls. The Viability fixable dye (Miltenyi Biotec) was used to discriminate between live and dead cells. Data were analyzed on FlowJo software version 10.6.1 (FlowJo, LLC, BD Life Sciences, Ashland, OR).

Quantitative Real-Time PCR

Total RNA was isolated from 50 to 100 mg of frozen liver tissue using standard TRIZOL protocol. Total RNA (1000 ng) was transcribed to cDNA using iScript cDNA synthesis kit (BioRad, Carlsbad, CA). Quantitative real-time PCR was performed on a Roche LC480 using SYBR Green technology. Details of primer pairs are provided in Table 2. Fold change in expression of genes of interest was calculated using the $\Delta\Delta$ Ct method, normalizing to the geometric mean of two housekeeping genes (18S and glyceraldehyde-3-phosphate dehydrogenase).

Statistical Analysis

Data are expressed as means $\pm$ SEM, representing mouse numbers within an experiment. Statistical significance between multiple genotypes was determined by two-tailed analysis of variance, whereas statistical difference between two groups was defined by paired *t*-test. *P* < 0.05 was considered statistically significant.

Results

Tissue Injury and Hepatic Fibrosis Are Pronounced in *Tr*^{-/-}*Mdr2*^{-/-} Mice Compared with *Mdr2*^{-/-} Mice

There was no significant difference in body weights of male and female mice among the different genotypes at 45 days

(Figure 1A). However, the *Tr*^{-/-}*Mdr2*^{-/-} male mice and all of the female *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice were significantly smaller than their WT counterparts by the later time point of 90 days (Figure 1B). The liver/body weight ratio was significantly higher in *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice for males and females at both time points. In male mice, serum alanine transferase levels were significantly elevated in the *Tr*^{-/-}*Mdr2*^{-/-} mice at 45 days and in both *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice by 90 days. In female mice, serum alanine transferase levels were comparatively higher and significantly elevated in *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} over that of the WT and *Tr*^{-/-} mice (Figure 1). Increased bile acids were also observed in both male and female *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice for both age groups (Supplemental Figure S1). Interestingly, serum albumin levels were significantly decreased in male and female *Tr*^{-/-}*Mdr2*^{-/-} and in *Mdr2*^{-/-} in the later age groups (Supplemental Figure S1), suggesting increased hepatic dysfunction.

Histologic evaluation of hematoxylin-eosin stained liver sections displayed the characteristic cholestatic pattern of injury in the male *Mdr2*^{-/-} mice with expanded portal tracts due to portal and periportal inflammation and increased number of bile ductules (Figure 2). In female *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice, inflammation extended into the hepatic lobule with injury more pronounced in the *Tr*^{-/-}*Mdr2*^{-/-} mice. No injury was observed in the *Tr*^{-/-} and the WT mice (Figure 2). Hepatic fibrosis was evaluated by Sirius red staining and indicated accumulation of collagen around the bile ducts under both plane polarized light (Figure 3A) and bright field (Figure 3B). Fibrosis was limited to a concentric periductal pattern in male *Mdr2*^{-/-} mice (Figure 3, A and B), whereas collagen extended into the hepatic lobule in female *Mdr2*^{-/-} mice. In both male and female *Tr*^{-/-}*Mdr2*^{-/-}, collagen deposition extended well into the parenchyma, often bridging from portal vein to portal vein, mimicking the stage 3/4 pattern of biliary fibrosis observed in humans (Figure 3, A and B). Image analysis of Sirius red-stained sections under plane polarized light confirmed the increased collagen deposition in *Mdr2*^{-/-} and

Figure 5 Ductular reactive cell population is increased in $Tr^{-/-}Mdr2^{-/-}$ mice. **A:** Representative images of liver sections from $Mdr2^{-/-}$ and $Tr^{-/-}Mdr2^{-/-}$ male and female mice at 45 and 90 days, stained for sex determining region Y-box transcription factor 9 (SOX9). **B:** Quantification of SOX9 by digital imaging. **C:** Representative images from liver sections of wild-type (WT), $Tr^{-/-}$, $Mdr2^{-/-}$, and $Tr^{-/-}Mdr2^{-/-}$ male and female mice, stained for macrophage inhibitory cytokine 1 (MIC1-1C3), and their quantification by digital imaging. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$. Scale bars = 50 μ m (**A** and **C**).

$Tr^{-/-}Mdr2^{-/-}$ mice (Figure 3C). $Tr^{-/-}$ and WT mice exhibited minimal collagen deposition. Total hydroxyproline content was likewise increased in the $Mdr2^{-/-}$ and $Tr^{-/-}Mdr2^{-/-}$ mice (Figure 4A). Immunohistochemical analysis of the activated myofibroblast marker, α -smooth muscle actin, indicated a significantly increased expression

in the $Tr^{-/-}Mdr2^{-/-}$ mice (Figure 4, B and C). Collectively, these observations are consistent with advanced liver injury and fibrosis in both male and female $Mdr2^{-/-}$ mice. However, the $Tr^{-/-}Mdr2^{-/-}$ mice displayed a more extensive pattern of parenchymal fibrosis, a pattern of fibrosis potentially implicating the ductular reaction as a mediator.

Figure 6 Gene expression of markers of ductular reaction is enhanced in *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice. Relative gene expression of keratin 23 (*Krt23*, also known as K23), TNF superfamily member 12 (*Tnfsf12*, also known as TWEAK), and TNF receptor superfamily member 12A (*Tnfrsf12a*, also known as Fn14) in male mice (A) and female mice (B) at 90 days. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, and *****P* < 0.001. WT, wild type.

The DR Cell Population Is Enhanced in *Tr*^{-/-}*Mdr2*^{-/-} Mice Compared with the *Mdr2*^{-/-} Mice

Chronic liver injury is associated with development of a DR cell population that promotes parenchymal hepatic fibrosis.³ Hence, the DR cell population was examined by immunostaining for established markers SOX9 and MIC1-1C3. Both markers were increased in the *Mdr2*^{-/-} mice compared with WT or *Tr*^{-/-} and were even further increased in male *Tr*^{-/-}*Mdr2*^{-/-} mice (Figure 5) and at 45 days in female *Tr*^{-/-}*Mdr2*^{-/-} mice (Figure 5). Gene expression of *Krt23*, a stress-inducible marker of DR cells,¹⁸ was also more abundant in the *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} male and female mice (Figure 6). Tumor necrosis factor–related weak inducer of apoptosis (TWEAK), also known as TNF superfamily member 12 (*Tnfsf12*), is recognized as a primary driver of ductular reaction in mice.⁵ Accordingly, gene expression of TWEAK and its receptor fibroblast growth factor-inducible 14 (Fn14), also known as TNF receptor superfamily member 12A (*Tnfrsf12a*), were elevated in *Tr*^{-/-}*Mdr2*^{-/-} and *Mdr2*^{-/-} male and female mice (Figure 6). As increased accumulation of DR cells may reflect enhanced proliferation, decreased cellular loss by apoptosis, or both processes, cell death was then evaluated by the TUNEL assay (Figure 7A) and cell proliferation by proliferating cell nuclear antigen immunohistochemistry (Figure 7B) in hepatic CK7/CK19-positive cells. TUNEL-positive cells were reduced in the *Tr*^{-/-}*Mdr2*^{-/-} versus the *Mdr2*^{-/-}, whereas cellular proliferation rates were similar in the *Tr*^{-/-}*Mdr2*^{-/-} and the *Mdr2*^{-/-} mice, albeit

elevated compared with WT mice (Figure 7). This observation suggests that TRAIL-TR signaling restrains the extent of the DR cell population in cholestasis, likely by inducing apoptosis of these cells.

Mass Cytometry Identifies Distinct Macrophage Subsets

The DR cells occur in close association with immune cells. Therefore, an unbiased approach was adopted to determine the composition of the immune cell population within the hepatic environment. High-dimensional mass cytometry based on 24 markers expressed by multiple immune cell types was performed on leukocyte populations isolated from livers of all genotypes. Visualization for t-distributed stochastic neighbor embedding plots from a representative animal from each genotype are presented in Figure 8A. Thirty-five distinct clusters were identified that could be categorized into macrophages, neutrophils, dendritic cells, natural killer cells, or B or T lymphocytes based on their unique marker profile (Supplemental Figure S2A). Multiple clusters were observed for each immune cell type and are individually enumerated in Supplemental Figure S2B. Furthermore, heat maps highlight the predominant clusters within individual animals (Supplemental Figure S2C) and the distinct immune profile of each cluster on the basis of label intensity (Supplemental Figure S2D).

The cumulative total of immune cell types varied across the four genotypes (Figure 8B). B cells predominated in the WT mice (36%) and *Tr*^{-/-} (31%) compared with *Mdr2*^{-/-} (25%) and *Tr*^{-/-}*Mdr2*^{-/-} (21%) mice. T cells were evenly

distributed among the four genotypes (15% to 20%), whereas the relative contribution of natural killer cells (2.9% to 4.5%) and dendritic cells (3.2% to 7.1%) to the total immune cell pool was modest. Of particular note, the total macrophage population comprising six distinct clusters (Supplemental Figure S2B) varied between the genotypes, being the least in WT (10%) and *Tr*^{-/-} (15%) mice, increased in the *Mdr2*^{-/-} (23%), and the greatest in the *Tr*^{-/-}*Mdr2*^{-/-} (28%) mice (Figure 8C). This was confirmed by immunohistochemistry for the pan-macrophage marker F4/80 (Figure 9A) that indicated peribiliary macrophages were significantly increased in the *Tr*^{-/-}*Mdr2*^{-/-} over that of the *Mdr2*^{-/-} mice in both males and females (Figure 9B). Furthermore, cluster analysis determined that the six macrophage subsets had unique profiles (Supplemental Figure S3). In particular, four clusters were prominent in the *Tr*^{-/-}*Mdr2*^{-/-} mice and, based on their profile, could be categorized as i) F4/80^{Hi} CD206^{Hi} CD11c^{Hi} LGALS3⁺ MERTK⁺; ii) F4/80^{Hi} CD11b^{Hi} CD64⁺ CX3CR1⁺ CD14⁺ CD11c⁺; iii) F4/80^{Hi} CD64^{Hi} CD11b⁺ CD206⁺ CD14⁺ MERTK⁺; and iv) F4/80⁺ CD206^{Hi} CD11c^{Hi} LGALS3⁺ MERTK⁺. Three of these clusters were characterized by the presence of MERTK, accounting for 15% in the *Tr*^{-/-}*Mdr2*^{-/-} compared with lower percentages in all other

genotypes (Supplemental Figure S3). MERTK⁺ macrophages were also characterized by high expression of CD206 and LGALS3. Hence, these results suggest that the increased lobular fibrosis in the *Tr*^{-/-}*Mdr2*^{-/-} mice may be attributed, in part, to the increased presence of alternatively activated CD206-positive macrophages, which are known to be profibrogenic.¹⁹ On the other hand, Ly6C⁺ infiltrating macrophages, comprising two clusters categorized as F4/80⁺ CD11b^{Hi} Ly6C^{Hi} CX3CR1⁺ CCR2⁺ CD64⁺ and F4/80⁺ CD11b^{Hi} Ly6C⁺, were greatest in the *Mdr2*^{-/-} mice (15%) compared with the *Tr*^{-/-}*Mdr2*^{-/-} (9.5%) or WT and *Tr*^{-/-} mice (4% to 5%) (Supplemental Figure S3). Macrophages are known to adopt microniche, context-dependent phenotypes. Intrahepatic macrophages are a source for multiple factors that may influence the microenvironment. These include TRAIL, which may promote the removal of unwanted cells by inducing apoptosis. Gene expression of TRAIL was increased in *Mdr2*^{-/-} and the *Tr*^{-/-}*Mdr2*^{-/-} mice compared with the WT and *Tr*^{-/-} mice (Figure 9C). In concordance with these results, flow cytometric analysis of freshly isolated intrahepatic macrophages indicated that TRAIL⁺ macrophages were up-regulated in both *Mdr2*^{-/-} and the *Tr*^{-/-}*Mdr2*^{-/-} mice compared with the WT and *Tr*^{-/-} mice (Figure 9D). Of note, mass cytometric analysis

Figure 7 Cholangiocyte-specific apoptosis is decreased in *Tr*^{-/-}*Mdr2*^{-/-}, whereas proliferation is similar to *Mdr2*^{-/-}, in 90-day-old male mice. **A:** Representative images of immunofluorescence staining of frozen liver sections for cytokeratin 7 (CK7)-positive cholangiocytes (magenta) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells (green; yellow arrows point to TUNEL⁺ nuclei, and white arrows point to CK7⁺TUNEL⁺ cells) with quantification of CK7⁺TUNEL⁺ cholangiocytes per field of view. **B:** Representative images of immunofluorescence staining of liver sections for CK19-positive cholangiocytes (red) and proliferating cell nuclear antigen (PCNA)-positive cells (green) in wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} animals with quantification of CK19⁺PCNA⁺ (as percentage of total cholangiocytes). *n* = 3 per group (**B**). ***P* < 0.01, ****P* < 0.005. Scale bars = 50 μm (**A** and **B**).

of isolated WT macrophages indicated that TRAIL⁺ macrophages are overwhelmingly MERTK⁺ (Figure 9E). Furthermore, *Mdr2*^{-/-} mice treated with cenicriviroc, which inhibits recruitment of monocyte-derived hepatic macrophages, demonstrated significantly increased SOX9-positive cells (Figure 9F), reinforcing the concept that macrophages may play a role in keeping the DR cell expansion in check.

Discussion

In this study, we report on the significance of TRAIL receptor-mediated cell death signaling in restraining the extent of the DR cell population in a mouse model of chronic cholestatic liver injury. Indeed, *Tr*^{-/-}*Mdr2*^{-/-} mice exhibited increased lobular hepatic fibrosis, expansion of the DR cell population, and increased macrophage accumulation. High-dimensional mass cytometry revealed that a subset of MERTK⁺CD206⁺LGALS3⁺ was enhanced in the *Tr*^{-/-}*Mdr2*^{-/-} mice. It was also demonstrated that MERTK⁺ macrophages express TRAIL. These results are discussed in detail in the following paragraphs.

The *Mdr2*^{-/-} mouse has long been used as a model for chronic biliary injury as this genetic effect occurs in

humans.^{20–23} The characteristic features of the *Mdr2*^{-/-} mice include onion skin periductular fibrosis and bile duct proliferation. However, the extent of injury and the degree and pattern of fibrosis are influenced by the mouse strain in which the *Mdr2* gene deletion is generated.^{24–27} In the present study, we used the *Mdr2*^{-/-} mouse on a C57BL/6 background,⁹ as it enabled crossbreeding with the *Tr*^{-/-} mice that were developed on this strain,¹² and hence permitted an unbiased comparison between all of the genotypes used in the study. The *Mdr2*^{-/-} mice on the C57BL/6 background with genetic deletion of TRAIL receptor (*Tr*^{-/-}*Mdr2*^{-/-}) displayed a pattern of fibrosis that was characterized by interportal fibrotic septa apparent as early as 45 days, whereas in the *Mdr2*^{-/-} mice, fibrosis was restricted to periportal areas with partial extension of septa to neighboring portal tracts. Consistently, total collagen content (by Sirius red quantification) and total hydroxyproline (by biochemical quantification) were greatest in the *Tr*^{-/-}*Mdr2*^{-/-}.

One of the notable features of chronic cholestatic injury and fibrosis is its correlation with the presence and extent of the DR cell population.^{2,3} The origin and fate of these DR cells are incompletely understood but are believed to be

Figure 8 High-dimensional flow cytometry (time-of-flight mass cytometry) identifies unique clusters of macrophages in *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice. **A:** Representative visualization for t-distributed stochastic neighbor embedding plots of wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} mice showing distinct immune cell clusters. **B:** Composition of intrahepatic immune cells comprising macrophages, B cells, T cells, neutrophils, dendritic cells (DCs), and natural killer (NK) cells. **C:** Percentage of total macrophages in WT, *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} animals.

dependent on type and extent of liver injury and are considered to be clinically relevant because of their correlation with poor clinical prognosis.^{4,28} DR cells, as determined by immunostaining for at least two markers (SOX9, MIC1-IC3) and mRNA expression of the stress-inducible marker of DR, *KRT23*, were significantly increased in the *Mdr2*^{-/-} mice over the WT or *Tr*^{-/-} mice. DR cells were localized around the portal tract at the interphase with the hepatic parenchyma. More important, DR cells were significantly increased in *Tr*^{-/-}*Mdr2*^{-/-} mice compared with the *Mdr2*^{-/-} mice in both males and females. DR cells were present around periportal areas and additionally along the fibrotic septa extending well into the hepatic lobule. Interestingly, TWEAK, a mitogen for the progenitor cell proliferation,⁵ was equally up-regulated in the *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice. To ascertain whether the expansion of DR cells was the consequence of excess proliferation or deregulated elimination, the number of proliferating cell nuclear antigen–positive and TUNEL⁺ cholangiocytes was quantified. TUNEL⁺CK7⁺ cells were significantly decreased in *Tr*^{-/-}*Mdr2*^{-/-} mice despite similar levels of proliferation in *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} mice, suggesting that the expansion of DR cell population in the double knockout animals was because of their reduced elimination by TRAIL-mediated apoptosis.

The immune system plays an important role in the maintenance and restoration of tissue homeostasis in response to chronic liver injury. Indeed, a strong link exists between macrophage-mediated debris clearance and regeneration.²⁹ Hence, an analysis of the immune cell population

using high-dimensional mass cytometry was performed in conjunction with conventional methods. This allowed for a greater insight into distinct phenotypes in normal (WT and *Tr*^{-/-} mice) and injured (*Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-}) hepatic environments. Overall, the immune cell profile varied greatly between genotypes. In confirmation of our previous observations in acute and chronic cholestasis,³⁰ macrophages contributed to the largest pool of immune cells in the *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-}. High-dimensional mass cytometry enabled identification of multiple clusters of macrophages that were dissimilar between the genotypes. Notably, a cluster of infiltrating proinflammatory M1-like macrophages having a CCR2⁺Ly6C⁺ profile was up-regulated in the *Mdr2*^{-/-} mice, whereas a population of M2-like alternatively activated macrophages having a MERTK⁺CD206⁺LGALS3⁺ profile was up-regulated in the *Tr*^{-/-}*Mdr2*^{-/-} mice. These observations are in keeping with the large body of evidence that macrophages are heterogeneous, evolving to exhibit features that are shared by more than one macrophage population, and adapting to specific environmental cues.^{31,32} These observations suggest that the dissimilarities observed in the *Mdr2*^{-/-} and the *Tr*^{-/-}*Mdr2*^{-/-} mice reflect their distinctive hepatic microenvironments.

TRAIL is the only ligand known to activate the death receptor TR in mice,^{33,34} and macrophages are a source of TRAIL.³⁵ The most conspicuous feature of *Tr* deletion against the background of *Mdr2* deficiency-induced liver injury was the increased number of DR cells. The parenchymal fibrosis, the increase in DR cells, the reduced

Figure 9 Macrophages are enhanced in *Tr*^{-/-}*Mdr2*^{-/-} mice and are uniquely represented within genotypes. **A:** Representative images of liver sections of *Mdr2*^{-/-} and *Tr*^{-/-}*Mdr2*^{-/-} male mice at 90 days, immunostained for the macrophage marker F4/80. **B:** Quantification of peribiliary F4/80⁺ macrophages in male and female wild-type (WT), *Tr*^{-/-}, *Mdr2*^{-/-}, and *Tr*^{-/-}*Mdr2*^{-/-} mice at 90 days. **C:** Relative gene expression of TRAIL in whole liver of male mice at 90 days. **D:** Percentage of TRAIL⁺ hepatic macrophages, as measured by flow cytometry. **E:** Percentage of MER proto-oncogene tyrosine kinase (MERTK⁺) macrophages in TRAIL⁺ macrophages from WT mice, as determined by time-of-flight mass cytometry. **F:** Sex determining region Y-box transcription factor 9 (SOX9⁺) ductular reactive cells are enhanced in *Mdr2*^{-/-} mice treated with cenicriviroc, a CCR2/CCR5 inhibitor that prevents tissue recruitment of macrophages. **P* < 0.05, ****P* < 0.005, and *****P* < 0.001. Scale bars = 50 μm (A).

apoptosis of CK7⁺ cells, and the enhanced macrophage accumulation observed in the *Tr^{-/-}Mdr2^{-/-}* mice can all be attributed to the loss of the death receptor TR. These observations implicate a crucial role for this death receptor along with its ligand TRAIL in tissue homeostasis during cholestatic liver injury. The most parsimonious interpretation of these data is that MERTK⁺ macrophages expressing TRAIL limit the size of the DR cell population. The signals that stimulate the recruitment and differentiation of these macrophages to the DR niche and the role of the proapoptotic signaling in modulating these cell populations will need to be clarified. On the basis of this concept, we speculate that therapy purposefully inducing DR cell apoptosis may be salutary in cholestatic liver disease.

Author Contributions

A.K., T.K., A.I.A., and M.E.G. designed and performed experiments, acquired data, analyzed the data, and prepared the manuscript; N.B.O. and C.T. acquired and analyzed data; and A.K. and G.J.G. conceptualized the study, designed experiments, analyzed and interpreted data, and prepared the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.02.013>.

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