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Unconventional RORγt+ T Cells Drive Hepatic Ischemia Reperfusion Injury

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An emerging body of evidence suggests a pivotal role of CD3+ T cells in mediating early ischemia reperfusion injury (IRI). However, the precise phenotype of T cells involved and the mechanisms underlying such T cell–mediated immune responses in IRI, as well as their clinical relevance, are poorly understood. In this study, we investigated early immunological events in a model of partial warm hepatic injury in genetically targeted mice to study the precise pathomechanistic role of RORγt+ T cells. We found that unconventional CD27−γ6TCR− and CD4−CD8− double-negative T cells are the major RORγt-expressing effector cells in hepatic IRI that play a mechanistic role by being the main source of IRI-mediating IL-17A. We further show that unconventional IRI-mediating T cells are contingent on RORγt, as highlighted by the fact that a genetic deficiency for RORγt, or its therapeutic antagonization via digoxin, is protective against hepatic IRI. Therefore, identification of CD27−γ6TCR− and CD4−CD8− double-negative T cells as the major source of IL-17A via RORγt in hepatic IRI opens new therapeutic options to improve liver transplantation outcomes. The Journal of Immunology, 2013, 191: 000–000.

In liver transplantation, organ exposure to ischemia and reperfusion during the transplantation process often leads to poor graft function; moreover, it may cause inflammatory activation in the intestine leading to multigain failure (1, 2). Unfortunately, a lack of transplantable human livers has led to the usage of “marginal” organs at high-risk for severe ischemia reperfusion injury (IRI) (3). IRI involves an early acute phase of Kupffer cell–mediated hepatic injury via reactive oxygen species, which is accompanied by activation of innate and adaptive immune responses that coordinate damaging neutrophil infiltration into the hepatic parenchyma by mechanisms including endothelial adhesion molecule expression and chemokine attraction (4–7). Perhaps counterintuitively, T cell activation (8, 9) appears to be pivotal in this early process. Although evidence to date suggests CD4+ T cells are involved in neutrophil recruitment, the operative cell type remains unclear. In this respect, STAT4 (11) signaling in infiltrating lymphocytes hints at Th1 polarization of IRI-mediating effector T cells (11); T cell Ig mucin (TIM)-1 (10) may play a role by inducing IFN-γ expression in Th1 cells to activate macrophages, or when expressed by Th2 cells, may activate macrophages via its TIM-4 ligand (12). Other T cell subpopulations may also be involved in mediating hepatic IRI, especially those producing IL-17A (13, 14). Although it is known that TGF-β and IL-6 induce the production of IL-17A by promoting Th17 cell differentiation via the hallmark transcription factor RORγt (15), the role of Th17 cells, per se, as a source of IL-17A in IRI remains known. Importantly, besides CD4+ Th17 cells, γδ T cells may be an early source of IL-17A in IRI, because they can rapidly release IL-17A without TCR ligation. Consistent with this capability, γδ T cells are effector cells in models of cerebral and cardiac IRI (16, 17). If the cellular sources for IL-17A are identified in IRI-related conditions, a novel therapeutic strategy could potentially be devised.

In this study, we investigated early immunological events in a murine model of partial warm IRI in mice and found a correlation of CD3, RORγt, and γδTCR positivity with development of IRI. With in-depth investigations of RORγt+ T cells using genetically modified mice, we discovered that unconventional IL-17A–producing, RORγt-dependent γδ and double-negative (DN) T cells are key effector cells in liver IRI. Furthermore, we ameliorated hepatic IRI by impeding RORγt activity with digoxin, a cardiac glycoside that antagonizes the receptor activity of RORγt (18).

Materials and Methods

Mice

Six- to eight-wk-old wild-type (wt) (C57BL/6) (B6) mice were used (Charles River Laboratories, Sulzfeld, Germany; γδTCR knockout (KO) mice, Rorc-reporter mice (RorcγδTCR), and Rorc KO mice (RorcγδTCR−/−) (B6 background) are JaxMice (The Jackson Laboratory, Bar Harbor, ME)). Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and regulations of Upper Palatinate, Germany.

Mouse liver IRI model

Liver IRI was induced according to Abe et al. (10), where an atrumatic clip was placed across the portal vein, hepatic artery, and bile duct just above...
branching to the right lateral lobe. After 90 min of ischemia, the clamp was removed, and the liver was reperfused. Blood and tissues were collected from anesthetized animals at specified times (6 or 24 h) according to Kono et al. (14). Sham-operated animals underwent the same procedure without clamping. Serum alanine aminotransferase (ALT) was measured using a Dimension 1500 Vista Analyzer. The myeloperoxidase (MPO) Quantification Kit (Hycult Biotech, Uden, The Netherlands) was used to quantify MPO activity. Animals treated with neutralizing IL-17A Ab (MAB421; R&D Systems) or digoxin received daily i.p. injections starting on day 2 before IRI (0.1 mg IL-17A mAb/200 μl PBS or 40 μg digoxin/200 μl PBS); the last injection was given prior to abdominal closure after the ischemia reperfusion operation.

Cell isolation and adoptive transfer

Whole livers were dissociated using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany). Isolated liver-infiltrating mononuclear cells and splenocytes were activated with 50 ng/ml PMA and 550 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO). After 4 h, cells and supernatants were harvested for flow cytometry analyses and ELISAs, respectively. CD3+γδTCR+ T cells were isolated from B6 spleens using a presorting step with CD3 immunomagnetic beads (Miltenyi Biotec) and then sorted by FACS (FACSAria; BD Biosciences, Heidelberg, Germany) while gating on γδTCR+ cells. For reconstitution, 1.2 × 10^6 freshly isolated cells were injected i.v. into γδTCR KO mice 48 h before IRI.

Flow cytometry and cytokine measurement

Flow cytometry was performed on a FACSComp II (BD Biosciences). Cells were stained with the Abs listed in Table II. Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Cells from all displayed plots originate from positive gating on leukocytes and living cells while excluding doublets. Commercially available ELISA kits were used for determination of IL-17A (R&D Systems), IFN-γ and TNF-α (BD Biosciences) levels.

(Immunohistochemistry

Liver damage (percent necrosis) was determined morphometrically in paraffin sections (4 μm) using a Zeiss AxioVision Module where the percent necrosis was calculated from the total square micrometer of the tissue section; five sections per ischemic lobe tissue sample were measured (n = 8 animals/experimental point). Gr-1+ cells were immunohistochemically stained on acetone-fixed frozen sections. Dried sections were blocked with 10% goat serum (1 h). Slides were stained (30 min) with Abs against Gr-1 and CD3 (1/100; BD Biosciences). Secondary goat-anti-rat-Alexa 594 Ab (1/200) plus DAPI (1/10,000) were then applied. Gr-1+ and CD3+ cells were counted in necrotic areas (NA) per high-power field (HPF) (×200 magnification; five HPF per slide, eight animals per group). A blinded scientist received the slides randomly (without being aware of the experimental design) and performed all necrosis quantification measurements and cell counting procedures.
Real-time PCR
cDNA was isolated from mouse liver samples using the MACS ONEstep cDNA Kit (Miltenyi Biotec). For amplification of murine IL-17A, RNeasy Mini and Micro Kits (Qiagen, Hilden, Germany) were used to isolate mRNA. Reverse transcription was done with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Primer sets used (Qiagen) are listed in Table I.

Statistics
All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). All values are expressed as means and SDs. Data were analyzed with an unpaired two-tailed Student t test. Statistical comparison between multiple groups was analyzed using ANOVA, followed by a posttest using Bonferroni comparison of all pairs. Differences with p < 0.05 were considered significant.

Results
Unconventional T cells are the major IL-17–producing cells in early IRI in mice
We used a murine model of warm partial IRI to precisely study the role of RORγt+CD3+ T cells in IRI. Consistent with previous studies (12, 20) wt mice undergoing 90 min of warm partial IRI featured signs (increased ALT and hepatocellular necrosis) of hepatic injury 24 h after reperfusion (data not shown). Gr-1+ immunohistochemistry (IHC) and MPO results showed massive infiltration of neutrophils in the necrotic liver areas (data not shown). We also found significantly higher numbers of infiltrating CD3+ T cells (Fig. 1A) and high expression levels of RORγt in IRI versus control samples (Fig. 1B), indicating RORγt+CD3+ T cells may play a role in IRI. To address this, we isolated infiltrating leukocytes from IRI and sham liver samples and then measured expression of IL-17A upon PMA/ionomycin restimulation ex vivo; IL-17A is released by RORγt+CD3+ T cells and has been implicated in hepatic IRI by controlling neutrophil recruitment and Kupffer cell activation (14). Indeed, we found significantly more IL-17A produced by hepatic leukocytes with IRI (Fig. 1B, Table I), suggesting that infiltrating

Table I. Qiagen QuantiTect primers

<table>
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<th>Number</th>
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<tr>
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ROR γ T cells contribute to hepatic IRI via IL-17A production. These findings were supported by higher expression of IRI-related cytokines and chemokines downstream of T cell activation, such as TNF-α and CXCL-1 (data not shown).

Next, we determined the major cellular source of IL-17A by intracellularly staining isolated hepatic leukocytes from IRI and sham animals via flow cytometry (Table II). As expected, CD3⁺ NK1.1⁺ T cells—but not CD3⁺NK1.1⁺ NK T cells or CD3⁺NK1.1⁺ NK cells—were the main hepatic producers of IL-17A during IRI (Supplemental Fig.1). However, unexpectedly, the most prominent cellular source of IL-17A in liver of IRI animals was unconventional CD4⁺CD8⁺CD3⁺ T cells and not CD4⁺CD3⁺ T cells (Fig. 1C); previous speculation pointed toward CD4⁺CD3⁺ T cells as the main source of IL-17A during hepatic IRI (14).
Importantly, further subset phenotyping revealed that the IL-17A–producing unconventional CD4⁺CD8⁻CD3⁺ T cells in liver (Fig. 1C) consisted mainly of γδTCR⁺CD3⁺ T cells and a minor fraction of DN CD4⁺CD8⁺CD3⁺ T cells. Moreover, the IL-17A–producing γδTCR⁺CD3⁺ T cells belonged exclusively to the CD27⁻γδTCR⁺ T cell subset that is thought to be RORγt-dependent (21). Importantly, absolute numbers of CD3⁺ T cells as well as IL-17A–producing CD27⁻γδTCR⁺ T cells were statistically increased in IRI versus sham animals (Fig. 1D).

Absence of γδ T cells ameliorates early hepatic IRI

To directly address the distinct roles of γδ T cells versus DN T cells as major IL-17A–producing effector cells in IRI, we studied γδTCR KO mice. Importantly, γδTCR KO mice were well protected from partial warm IRI (versus wt controls), as shown by markedly better histological scores with less hepatocellular necrosis (Fig. 2A) and significantly lower levels of ALT (Fig. 2B) measured 6 and 24 h after reperfusion. This was accompanied by significantly less neutrophilic infiltration in IRI γδTCR KO animals, as shown by Gr-1-IHC and MPO measurements (Fig. 2B). Flow cytometry analyses confirmed that upon IRI, IL-17A–producing γδ T cells were absent in γδTCR KO mice and that the remaining cellular source of IL-17A in the liver (Fig. 2C, 2D) consisted mostly of DN T cells but not CD4⁺CD3⁺ T cells. Such an absence of IL-17A–producing CD27⁻γδ T cells was associated with significantly fewer absolute numbers of IL-17A–producing CD3⁺ T cells (Fig. 2E) in IRI-γδTCR KO versus wt animals and a strong trend toward less IL-17A production by restimulated bulk leukocytes from IRI liver samples of γδTCR KO mice (Fig. 2F). DN T cells apparently represent a remaining source of IL-17A in the absence of γδ T cells. Expression analyses support this conclusion, because reduced levels of IL-17A–related cytokines and chemokines (downstream of IL-17A) were measured by ELISA or real-time PCR (rtPCR), including TNF-α and CXCL-1 (Fig. 2F). Thus, a lack of γδ T cell–derived IL-17A leads to less neutrophilic infiltration and IRI amelioration.

To further prove the significance of γδ T cells as IL-17A–producing effector cells in IRI, purified γδ T cells from wt mice were adoptively transferred to γδTCR KO recipients in the absence or presence of a neutralizing anti–IL-17A Ab; animals then underwent IRI. The reconstitution of γδTCR KO mice with γδ T cells aggravated IRI, as indicated by worsening of hepatocel-
lular damage (Fig. 3A). ALT, and neutrophilic infiltration (Gr-1-IHC; Fig. 3B). In contrast, adoptive transfer of γδ T cells in the presence of IL-17A Ab did not give rise to increased liver damage (Fig. 3). Flow cytometry analyses showed that upon reisolation from the adoptively transferred IRI γδ TCR KO animals, the IL-17A–producing γδ T cells had the CD27 - phenotype, supporting the hypothesis that CD27 - γδ T cells are the major IL-17A–producing effector cell in hepatic IRI (data not shown).

**IL-17A–producing γδ T cells and DN T cells mediating early IRI are RORγt dependent**

We tested whether the IL-17A–producing γδ T cells and DN T cells mediate early IRI contingent upon expression of the Th17 hallmark transcription factor RORγt. First, we used Rorc<sup>gfp/wt</sup> reporter mice in our IRI model; in this reporter mouse, RORγt is genetically linked to GFP coexpression (22). Importantly, Rorc<sup>gfp/wt</sup> mice showed similar levels of hepatic injury upon partial warm IR as wt mice, as demonstrated by hepatocellular necrosis (Fig. 4A) and ALT levels (Fig. 4B). Furthermore, the extent of neutrophilic infiltration, as shown by Gr-1-IHC (Fig. 4A) and MPO activities (Fig. 4C) in IRI livers were not appreciably different between Rorc<sup>gfp/wt</sup> and wt mice. Moreover, unconventional CD27 - γδ T cells and DN T cells were the main cellular sources of IL-17A in both Rorc<sup>gfp/wt</sup> mice and wt controls (data not shown). Exclusively IL-17A–producing CD27 - γδ T cells and DN T cells were GFP<sup>+</sup> in liver (Fig. 4D), indicating that RORγt is mainly expressed by unconventional IL-17A–producing T cells under IRI conditions in vivo.

To verify that RORγt expression is crucial for IL-17A–producing unconventional T cells in initiating hepatic IRI, we used Rorc<sup>gfp/gfp</sup> mice genetically lacking RORγt expression (22). Results confirmed that Rorc<sup>gfp/gfp</sup> mice are protected from IRI in comparison with wt and Rorc<sup>gfp/wt</sup> controls, as demonstrated by our usual panel of liver IRI tests (Fig. 5A, 5B). Moreover, lower secretion or expression levels (Fig. 5C) of IL-17A, TNF-α, and CXCL-1 were found in hepatic IRI samples from Rorc<sup>gfp/gfp</sup> mice. IL-17A–producing GFP<sup>+</sup>CD27 - γδ T cells and DN T cells were completely missing in hepatic (Fig. 5D, 5E) samples from IRI Rorc<sup>gfp/gfp</sup> mice, confirming that RORγt is necessary for unconventional T cells to mediate IL-17A–dependent hepatic IRI.

To test these findings therapeutically, we treated Rorc<sup>gfp/wt</sup> mice undergoing partial warm IRI with digoxin, which inhibits RORγt activity in CD4<sup>+</sup> Th17 cells (18). Rorc<sup>gfp/wt</sup> mice treated with digoxin showed an IRI-related phenotype similar to that of Rorc<sup>gfp/gfp</sup> mice, where the mice were at least partially protected from hepatic IRI (Fig. 6A). Although granulocyte infiltration was not consistently reduced in those mice, we found significantly lower levels of hepatocellular necrosis (Fig. 6B) and ALT (Fig. 6B), as well as evidence for reduced secretion or expression levels (Fig. 6C, 6D) of IL-17A, TNF-α, and CXCL-1. Digoxin-treated Rorc<sup>gfp/wt</sup> mice also had significantly lower expression levels of γδTCR and GFP (Fig. 6E), suggesting an antagonizing therapeutic effect of digoxin on IRI-mediating RORγt-expressing γδ T cells.

**Discussion**

Our detailed studies in a mouse model of warm IRI show that unconventional CD3<sup>+</sup> T cells, mainly consisting of γδ T cells and DN T cells, are the major RORγt-expressing effector cells mediating hepatic IRI via an IL-17A–dependent mechanism. These unconventional γδ and DN T cells involved in IL-17A–mediated IRI are clearly dependent on RORγt, because RORγt-targeted therapy using digoxin successfully diminishes hepatic IRI.

This hypothesis goes against a prevailing view that CD3<sup>+</sup> T cells of the CD4<sup>+</sup> effector phenotype are most critical in the early acute IRI-related injury (23) and that conventional CD4<sup>+</sup> Th17 cells are the major source of IL-17A in this situation (14). Nonetheless, our detailed mechanistic experiments using well-defined genetically modified mice with or without digoxin treatment upon hepatic IRI (original magnification, ×100; one representative image of eight is shown). IHC images show infiltrated Gr-1<sup>+</sup> cells (right, original magnification ×200; one representative image of six is shown). (B) IRI liver damage as measured by ALT levels and MPO activities (n = 8). NA were evaluated microscopically. Gr-1<sup>+</sup> cells were counted in NA (five HPFs per animal, five animals per group). (C) TNF-α and IL-17A protein levels in supernatants of restimulated liver-infiltrating leukocytes (ELISA, n = 8). (D) TNF-α, CXCL-1, γδ TCR (TCR), and GFP mRNA expression relative to GAPDH in IRI and sham livers (real-time PCR [qPCR], n = 8 independent experiments). n.d., Not detected; n.s., not significant; *p < 0.05, **p < 0.01.
modified mice show that only a minor fraction of conventional CD4-CD3- T cells produce IL-17A early after hepatic IRI. This is actually consistent with the Kono et al. (14) study that reported only 0.3 and 2.1% of CD4+ T cells in liver and spleen of IRI animals, respectively, producing IL-17A. An earlier report by Caldwell et al. (14) also did not definitively show that CD4+ Th17 cells were the IL-17A producers in this setting, because observations were based on work in CD4 KO mice or using IL-17A–neutralizing Abs. The contention that CD4+ Th17 cells are not the key source of IL-17A within 24 h of IRI is also consistent with the need for sustained Ag-specific TCR engagement for their differentiation into IL-17A–releasing effector cells (15). Our mouse experiments showed rather that γδ T cells and CD4-CD8- TCRγδ+ DN T cells mediate IL-17A–dependent immune responses in IRI. DN T cells are the major IL-17A–producing effector cells in systemic lupus erythematosus patients and in murine primary respiratory infections (24, 25). However, in our IRI model, DN T cells were a relatively minor source of IL-17A in IRI compared with γδ T cells. We confirmed this by showing γδTCR KO mice are protected from partial warm hepatic IRI but are again vulnerable after γδ T cell reconstitution. Induction of ischemia reperfusion–related tissue damage by adoptively transferred γδ T cells is dependent on the presence of IL-17, showing the significance of γδ T cells as IL-17A–producing effector cells. Furthermore, adoptively transferred CD4 cells did not lead to increased liver damage in this model (data not shown). Our data are consistent with γδTCR KO mice reportedly showing less neutrophil infiltration during hepatic IRI (26); although this same report showed no decrease in liver cell damage (ALT levels) or necrosis in γδTCR KO mice, the discrepancy could be explained by their analyses after a shorter reperfusion time (8 h) compared with ours (24 h: when neutrophilic effects on liver function predominate (23)). Interestingly, γδ T cells are also suggested as major IL-17A–producing effector cells in models of cerebral and cardiac IRI (16, 17), although their role has not been confirmed using γδTCR-deficient mice or γδ T cell–depleting Abs (16). It can be concluded that unconventional γδ and DN T cells found in livers but also spleens (data not shown) are emerging as critical regulators of early tissue injury. Although the warm ischemia model we used to study ischemia reperfusion is a standard well-characterized experimental system for this early basic research, we speculate that our findings could be even more pronounced in a mouse orthotopic liver transplantation model that includes a cold ischemia phase and features higher T cell infiltration levels post reperfusion; these studies are planned for future investigations.

Our data demonstrate that IL-17A–producing γδ T cells belong exclusively to the CD27- subset, which is a critical subset in other models of IL-17A–mediated immune responses (21). Although some data suggest that IL-17A–producing CD27- γδ and DN T cells may be RORγt dependent (21), we could show this definitively using well-characterized reporter and knockout models for RORγt. More specifically, IL-17A–producing CD27- γδ and DN T cells were the only GFP-expressing cell subsets in Rorcγt+ Gfp reporter mice, whereas IL-17A–producing CD27- γδ and DN T cells were absent in Rorcγt+ Gfp KO mice; importantly, neither CD4+ Th17 cells nor neutrophils were induced to express RORγt and IL-17A in hepatic IRI. Notably, a recent study suggests that neutrophils are the major IL-17A–producing effector cells in renal IRI (27), meaning that there may be a temporal shift toward IL-17A secretion by neutrophils once the process is initiated by γδ and DN T cells; this would need to be confirmed. Finally, it remains to be addressed whether factors upstream of RORγt may drive CD27- γδ and DN T cells to upregulate RORγt and produce IL-17A in hepatic IRI. Most likely, IFN regulatory factor 3–dependent events control the IL-23/IL-17 axis in the liver, which is relevant to liver IRI (28). Our own data confirming expression of IL-1β and IL-23 in liver samples from IRI wt mice (data not shown) suggest these cytokines may play a role in driving T cells into IL-17A–releasing effector cells during IRI. This hypothesis is in line with previous cerebral IRI work (17). More detailed studies are clearly warranted.

We translated our findings in mice by antagonizing the receptor activity of RORγt with digoxin, a cardiac glycoside that impedes RORγt activity (18). Indeed, digoxin is not only partially effective therapeutically against IRI in our model, we now know that unconventional RORγt+ T cells are targeted by digoxin in addition to the RORγt+CD4+ Th17 cells in autoimmune models (18). Further testing and experimentation will clearly be necessary to confirm the effects of digoxin in settings of IRI. If confirmed, the question becomes whether a realistic therapy could be designed to target RORγt+ cells in the hepatic IRI. Although digoxin is toxic for human cells at high concentrations, nontoxic synthetic derivatives could become an option. Nevertheless, we anticipate that a better understanding of the mechanisms of early IRI will allow for even more specific targeting of responsible cell populations with less collateral side effects, which may benefit transplant recipients or patients with IRI to other organs or tissues.

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Disclosures

The authors have no financial conflicts of interest.

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