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Interleukin-27R (WSX-1/T-Cell Cytokine Receptor) Gene-Deficient Mice Display Enhanced Resistance to *Leishmania donovani* Infection but Develop Severe Liver Immunopathology

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The interleukin-27 (IL-27)/T-cell cytokine receptor (TCCR) pathway plays an important role in development of protective immunity against cutaneous leishmaniasis caused by *Leishmania major*. In this study, we analyzed the role of IL-27/TCCR pathway in the host defense against visceral leishmaniasis (VL) by monitoring the course of *L. donovani* infection in TCCR-deficient C57BL/6 (TCCR−/−) mice. TCCR−/− mice mounted a robust inflammatory response, produced high levels of pro-inflammatory cytokines, and developed severe liver pathology after *L. donovani* infection that eventually resolved. Interestingly, *L. donovani*-infected TCCR−/− mice controlled the parasite growth in their organs significantly faster than similarly infected TCCR+/+ mice. Adoptive cell transfer and cell depletion studies revealed that $CD4^+$ T cells were involved in mediating liver immunopathology and controlling *L. donovani* growth in TCCR−/− mice. These results indicate that the IL-27/TCCR pathway is not essential for the induction of protective Th1 response during VL but is involved in mediating susceptibility to *L. donovani*. Additionally, the data demonstrate that although the IL-27/TCCR interaction limits the severity of liver inflammation during VL by controlling $CD4^+$ T-cell activity, it is not required for the resolution of hepatic immunopathology. ***(Am J Pathol 2006, 168:158–169; DOI: 10.2353/ajpath.2006.050013)***

Visceral leishmaniasis (VL) is the most severe form of *Leishmania* infection caused by *Leishmania donovani* and *Leishmania chagasi*.[1] VL is characterized by hepatosplenomegaly, fever, abdominal pain, and weight loss associated with widespread dissemination of parasites throughout the reticuloendothelial system. Complications such as secondary infection, anemia, and malnutrition due to hepatosplenomegaly are primarily responsible for the mortality associated with this disease.

Interleukin (IL)-27 is a heterodimeric cytokine produced by endothelial cells, dendritic cells, and monocytes during inflammation.[2] It is composed of the interleukin-12 p40- and p35-related proteins EBI3 and p28, respectively.[3] IL-27 mediates its functions by signaling through the IL-27R, which is composed of the T-cell cytokine receptor (TCCR/WSX-1) and the signal transducer gp130.[4–6] Both immune and nonimmune cells express gp130, which is a shared receptor signaling chain used by several related cytokines.[7] However, the expression of WSX-1 is restricted to cells of the immune system such as monocytes, Langerhans cells, dendritic cells, NK cells, T cells, and B cells.[4–6,8]

The role of IL-27 in regulating the host immune response is complex because this cytokine has been shown to exert both pro- and anti-inflammatory activities.[2] For example, like IL-12, IL-27 induces interferon (IFN)-γ production from NK and $CD4^+$ T cells.[3] TCCR (WSX-1)−/− mice, which completely lack IL-27 signaling, fail to mount an efficient Th1-type response and thus show increased susceptibility to intracellular pathogens such

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as *Listeria monocytogenes* and *Leishmania major*.[6,9] Furthermore, IL-27 is also expressed at high levels in human Th1-associated granulomatous diseases such as tuberculosis, sarcoidosis, and Crohn's disease.[10] On the other hand, WSX-1−/− mice produce high levels of IFN-γ and develop significant immunopathology when infected with *Toxoplasma gondii* and *Trypanosoma cruzi*, indicating that IL-27 is also involved in antagonizing or modulating inflammatory responses in these diseases.[11,12]

The protective role of IL-27 in control of cutaneous *L. major* infection has been attributed to its ability to enhance IFN-γ production from both NK cells and $CD4^+$ T cells during the early phase of infection and subsequently to generate disease protective Th1 response.[6,9] However, a recent study has shown that early IL-4 production governs the requirement for IL-27 in the induction of Th1 responses in cutaneous leishmaniasis caused by *L. major*.[9] Interestingly, IL-27 is not required to maintain Th1 response during *L. major* infection because TCCR−/− mice develop large lesions during the early phase of infection but eventually resolve the infection and control parasite loads.[6,9]

To determine the role of the IL-27/TCCR (WSX-1) pathway in the outcome of VL, we examined the course of *L. donovani* infection in TCCR−/− C57BL/6 mice and compared it with that in the wild-type (TCCR+/+) C57BL/6 mice. TCCR−/− mice mounted a robust Th1 response after *L. donovani* infection and controlled parasite burdens significantly faster when compared with TCCR+/+ mice. However, rapid clearance of *L. donovani* in TCCR−/− mice was associated with the development of significant liver pathology accompanied by diffuse inflammation and aberrant granuloma formation. Depletion of $CD4^+$ T cells in TCCR−/− mice dramatically reduced liver pathology after *L. donovani* infection but rendered them more susceptible to the disease. Conversely, syngenic RAG2−/− recipient mice reconstituted with TCCR−/− T cells developed severe liver inflammation and rapidly controlled parasite growth in their organs. Finally, simultaneous neutralization of both tumor necrosis factor (TNF)-α and IFN-γ also reduced severity of hepatic pathology in TCCR−/− mice during VL but enhanced their susceptibility to infection. These findings demonstrate that the IL-27/TCCR pathway, in contrast to its protective role in *L. major* infection, is involved in the pathogenesis of *L. donovani* infection. The results also indicate that the IL-27/TCCR pathway suppresses the tissue inflammation associated with VL by regulating $CD4^+$ T-cell function.

Materials and Methods

Animals

TCCR (WSX-1) gene-deficient C57BL/6 mice were generated as described previously[8] and bred by mating homozygous parents. The deletion of TCCR in offspring was confirmed by genotypic analysis using PCR and by phenotypic analysis using flow cytometry. The wild-type C57BL/6 mice were purchased from Jackson Laboratories. The experiments were performed using 8- to 10-week-old sex-matched mice in a facility at The Ohio State University according to the guidelines for animal research as required by National Institutes of Health regulations. Under these guidelines, mice were bred and maintained in ventilated microisolator cages in a specific pathogen-free facility. Any mice showing signs of distress were immediately sacrificed. Animal care facilities at the Ohio State University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-International and follow the National Research Council's Guide for the Care and Use of Laboratory Animals.

Analysis of TCCR Deficiency in TCCR−/− Mice

Deletion of TCCR gene in the offspring was confirmed by genotypic analysis using PCR. Primer sequences used for amplifying the wild-type allele were sense, 5′GTTGAGATGCAGAACCTGGA-3′, and antisense, 5′-GCTGCTGATAAGTTCCCAAG-3′, and for mutated allele were sense, 5′-GCTTTCGTCTCCCGTGTGCT-3′, and antisense, 5′-TGAGCCCAGAAAGCGAAGGA-3′. The primers for detecting wild-type mice were designed to amplify a fragment in targeted region (exon 3 to exon 8) as described previously and hence were specific for the wild-type allele.[8] A primer set specific for *Neo* gene in the targeting vector was used for detecting mutated allele.[8]

Additionally, flow cytometric analysis using rat anti-mouse TCCR monoclonal antibody (mAb) was performed to demonstrate the absence of TCCR on T cells from TCCR−/− mice. Anti-mouse TCCR mAb was kindly provided by Dr. Fred de Sauvage (Genentech, San Francisco, CA).

Parasites and Infection Protocol

L. donovani (1 Sudan strain) was maintained by serial passage of amastigotes in Syrian golden hamster. Amastigotes were isolated from the spleen of an infected hamster, and experimental mice were infected with 1×10^7 *L. donovani* amastigotes by intravenous injection into the tail vein.

Quantification of Parasite Loads, Histopathology, and Immunofluorescence Microscopy

Parasite loads in the liver and spleen of *L. donovani*-infected TCCR−/− and TCCR+/+ mice were measured on 15, 30, and 60 days after infection as described previously.[13] Briefly, livers and spleens were weighed and sectioned to prepare impression smears that were Giemsa stained to enumerate the amastigotes per 1000 nucleated cells. The parasite loads were expressed as Leishman-Donovan units (LDU):

$$\text{LDU} = \text{Number of amastigotes per 1000 nucleated cells} \times \text{Organ weight (g)}$$

At this time, tissue sections from livers and spleens of *L. donovani*-infected TCCR−/− and TCCR+/+ mice were excised and fixed in 10% buffered formalin for 48 hours. The tissues were processed and embedded in paraffin, and 5-μm sections were cut. The sections were stained by routine hematoxylin and eosin staining. The slides were coded and analyzed to assess the inflammation. Special staining was performed to analyze collagen deposition (Masson Trichrome) and reticulin fibers (reticulin).

For immunofluroscence microscopy, liver tissue was snap frozen in Tissue Tek OCT and cut into 5-μm sections using a cryostat (Leica Systems). Tissue sections were fixed in ice-cold acetone for 1 minute and blocked at room temperature for 30 minutes using protein-free block (DAKO). Subsequently, sections were incubated with rat anti-mouse CD11b (clone M1/70; BD Pharmingen, San Diego, CA) or rat anti-mouse CD4 (clone L3T4; BD Pharmingen, San Diego, CA) antibody at room temperature for 2 hours. Bound primary antibody was detected using biotin-labeled secondary Ab (BD Pharmingen, San Diego, CA) and streptavidin conjugated to appropriate fluorescent dye (e. g. AlexaFluor 594 (red) and AlexaFluor 488 (green); Molecular Probes).

T-Cell Proliferation Assay and Cytokine Analysis

The spleens were removed from *L. donovani*-infected TCCR+/+ and TCCR−/− mice on days 15, 30, and 60 after infection. Single cell suspensions were prepared by gentle teasing of the spleen, and live cells were enumerated by trypan blue exclusion after lysing the red blood cells using Boyle's solution. Cells (5×10^5) were added in quadruplicate to the wells of sterile 96-well flat-bottom tissue culture plates and stimulated with freeze-thaw Leishmania antigen (LdAg) (20 μg/ml). LdAg was prepared from stationary phase promastigotes of *L. donovani.* Promastigotes were washed three times in ice-cold phosphate-buffered saline and subjected to six cycles of freezing −70°C and thawing at 37°C. Freeze-thawed preparations were centrifuged to remove the particulate material, and protein concentration was determined using Bradford's assay as per manufacturer's instructions (Pierce Lab, Rockford, IL). The proliferation responses were measured by Alamar Blue assay as described previously.[14,15] Supernatants were collected after 72 hours of incubation at 37°C and analyzed for the production of IFN-γ, IL-12p70, IL-4, and IL-10 by enzyme-linked immunosorbent assay (ELISA). Reagents used for cytokine ELISA were all purchased from BD PharMingen, San Diego, CA.

Isolation of T Cells and Adoptive Cell Transfers

Spleens were removed aseptically from naïve TCCR+/+ and TCCR−/− donor mice, and single cell suspensions were prepared by gentle teasing in cold RPMI1640 supplemented with 10% fetal calf serum. Red blood cells were lysed using Boyle's solution, and cells were run through a nylon wool column twice to remove B cells. Depletion of B cells was confirmed by staining for B220+ cells using flow cytometry. For depletion of NK and natural killer T cells (NKT cells) B cell-depleted spleen cells were labeled using fluorescein isothiocyanate-conjugated anti-NK1.1 Ab (BD Pharmingen, San Diego, CA) and depleted by immunomagnetic separation using anti-fluorescein isothiocyanate Ab-coupled magnetic beads according to manufacturer's instruction (Miltyni Inc., Auburn, CA). Efficiency of depletion and purity of T-cell population was confirmed by flow cytometry. No NK1.1+ cells were detectable by flow cytometry after depletion, and 97% of the cells were T cells (data not shown). Subsequently, cells were washed twice and resuspended in sterile saline. Cells were enumerated by trypan blue exclusion, and 10^7 cells were injected intravenously via tail vein injections into recipient RAG2 knockout mice. The mice rested for 3 weeks after reconstitution before infection with parasites.

$CD4^+$ and $CD8^+$ T-Cell Depletions

TCCR−/− mice were administered 400 μg of anti-CD4 (clone GK1.5) or anti-CD8 (clone H35) or control IgG intraperitoneally 2 days before *L. donovani* infection followed by weekly single injection of the same dose thereafter. Efficiency of $CD4^+$- and $CD8^+$-cell depletion was nearly 90 to 92%, as determined by flow cytometry at the time of sacrifice (data not shown).

In Vivo *Neutralization of IFN-γ and TNF-α*

TCCR−/− mice were administered intraperitoneally with 400 μg of anti-IFN-γ mAb (clone XMG.6) or 250 μg of affinity-purified anti-TNF-α rabbit polyclonal Ab (Calbiochem, EMD Biosciences Inc., La Jolla, CA) or a combination of both 24 hours before *L. donovani* infection and weekly thereafter. A group of TCCR−/− mice received control antibodies in the same doses.

Statistical Analysis

Student's unpaired *t*-test was used to determine statistical significance of differences in the values. A value of $P < 0.05$ was considered significant. The statistical significance of antibody titers was determined by nonparametric tests using the Mann-Whitney *U*-Wilcoxon rank test.

Results

Analysis of TCCR Gene Deletion in TCCR−/− Mice

As anticipated, PCR analysis of tail DNA revealed the presence of the TCCR gene in TCCR+/+ but not TCCR−/− mice (Figure 1A). Furthermore, flow cytometric analysis of T cells from TCCR−/− mice confirmed the absence of TCCR on these cells (Figure 1, B and C).

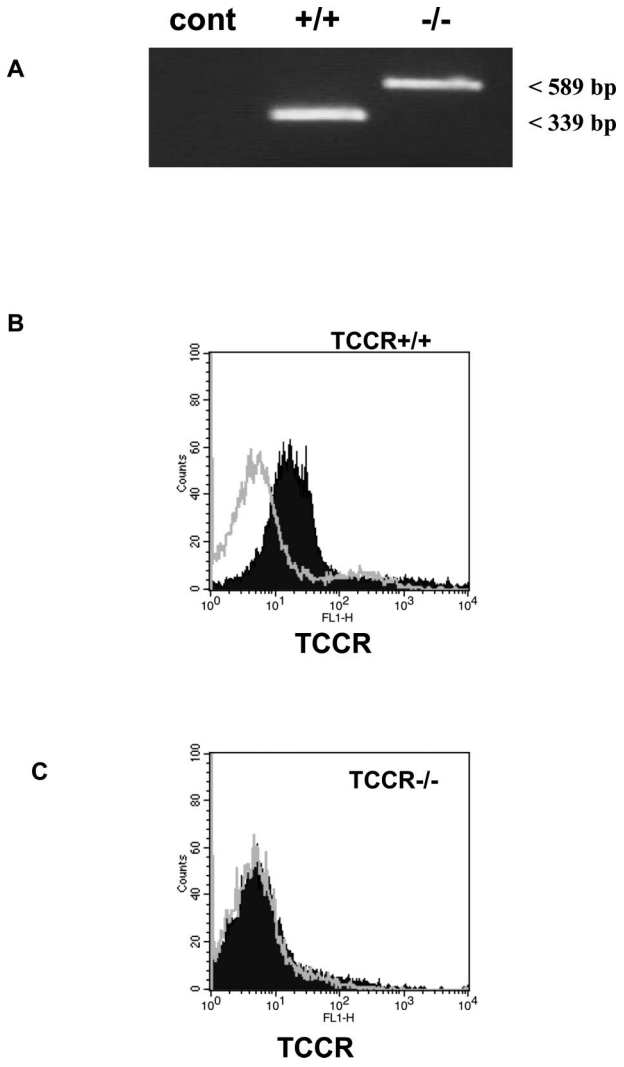

Figure 1. Analysis of TCCR gene deletion in TCCR−/− mice. **A:** Tail DNA isolated from TCCR+/+ and TCCR−/− mice were analyzed by PCR for the presence of TCCR gene. Cont, control with no DNA. Expression of TCCR on the T cells isolated from the spleens of TCCR+/+ (**B**) and TCCR−/− (**C**) mice was analyzed by flow cytometry. **Gray line** denotes the cells stained with an isotype control. **Solid black area** denotes the cells stained with anti-TCCR Ab.

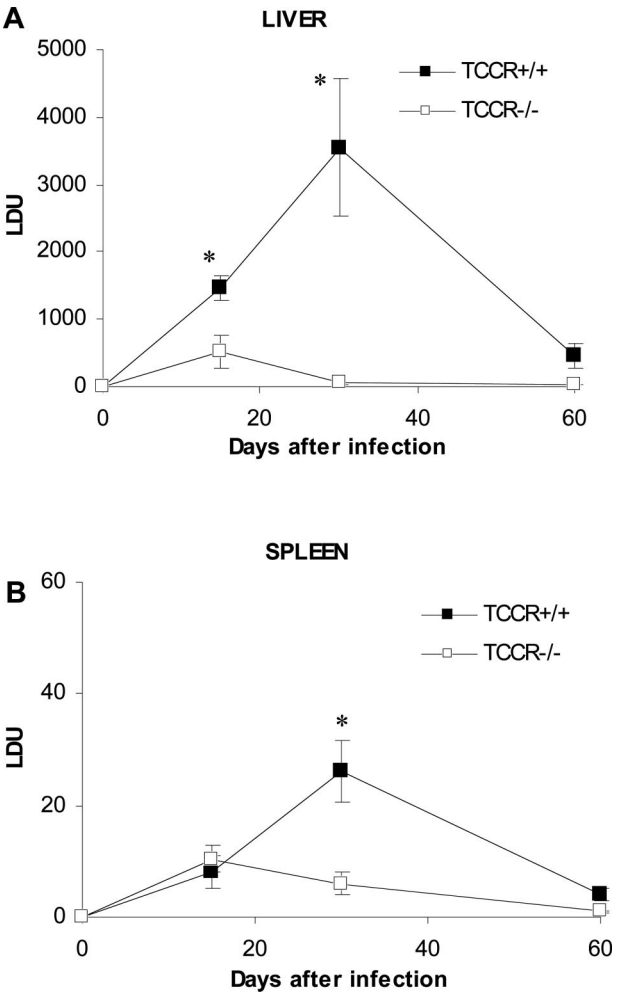

Figure 2. TCCR−/− mice are relatively resistant to *L. donovani* infection and rapidly control parasite burdens. Liver and spleen parasite loads (**A** and **B**) were determined 15, 30, and 60 days after intravenous inoculation with 1×10^7 *L. donovani*. Parasite burdens in liver (**A**) and spleen (**B**) are expressed as the mean LDU ± SEM. The data are the mean values from six to eight animals per group at each time point in two independent experiments with similar results. *Statistically significant differences between each group ($P < 0.05$).

TCCR−/− Mice Show Enhanced Resistance to L. donovani *Infection and Control Parasite Growth Significantly Faster Than the TCCR+/+ Mice*

On day 15 after intravenous inoculation with 1×10^7 *L. donovani* amastigotes, livers and spleens from TCCR−/− mice contained less parasites compared with those from TCCR+/+; however, the difference in parasite loads between the groups was only statistically significant for the liver (Figure 2). Liver and spleen parasite burdens increased in *L. donovani*-infected TCCR+/+ mice as the infection progressed and peaked at day 30 after infection (Figure 2). However, at 30 days, *L. donovani*-infected TCCR−/− mice showed a significant decrease in their liver parasite loads (Figure 2A). At this time, the spleens from TCCR−/− mice also contained fewer parasites compared with TCCR+/+ mice, but the difference was less profound when compared with the liver. Three replicate experiments showed that differences in spleen parasites loads were radically less profound compared with those in the liver (data not shown). By day 60 after infection, both TCCR+/+ and TCCR−/− mice efficiently controlled the growth of *L. donovani* in their liver and spleen, but parasite burdens in the liver were significantly lower in TCCR−/− mice (Figure 2A). These results demonstrate that the IL-27/TCCR pathway is not required for the development of host immunity to *L. donovani* but show that the IL-27/TCCR pathway delays the resolution of *L. donovani* infection and plays a role in pathogenesis of visceral leishmaniasis.

TCCR−/− Mice Develop Significantly More Severe Liver Pathology after L. donovani *Infection*

On gross examination, livers from *L. donovani*-infected TCCR−/− mice were larger and more fragile when compared with those from TCCR+/+ mice (data not shown). On days 15 and 30 after infection, a microscopic examination of livers from *L. donovani*-infected TCCR+/+ showed tissue inflammation associated with the formation of mature granulomas comprised of macrophages and T cells, with clusters of amastigotes within individual macrophages (Figure 3). On the other hand, *L. donovani*-infected TCCR−/− mice showed extensive destruction of the liver parenchyma associated with the presence of large granulomas and many foci of a diffuse inflammatory infiltrate comprised of macrophages, neutrophils, and lymphocytes (Figure 3). Livers from TCCR−/− mice also displayed foci of necrosis and an extensive perivascular inflammatory infiltrate composed of macrophages, lymphocytes, and few plasma cells in both portal and perisinusoidal spaces. Immunohistochemical analysis revealed that diffuse inflammatory foci in the livers of TCCR−/− mice predominantly contained CD11b+ macrophages and $CD4^+$ T cells (Figure 4). Both groups showed significant amounts of collagen in the liver granulomas on days 15 and 30 (Figure 5), but collagen deposition was more extensive in the livers from TCCR−/− mice (Figure 5, C and G). Similarly, livers from TCCR−/− mice had significantly more deposition of reticulin fibers at 15 and 30 days (Figure 5, D and H). Interestingly, *L. donovani*-infected TCCR−/− mice resolved liver inflammation more efficiently than wild-type mice and contained fewer granulomas in their livers on day 60 (Figure 3, I and K). These results demonstrate that the IL-27/TCCR pathway suppresses liver inflammation during *L. donovani* infection.

TCCR−/− Mice Show Increased Levels of Pro-Inflammatory Cytokines in Their Sera during L. donovani *Infection*

The absence of IL-27/TCCR (WSX-1) pathway increases production of pro-inflammatory cytokines and induces systemic inflammation in TCCR−/− mice during certain infections.[11,12] Therefore, we measured levels of IL-12p70, TNF-α, and IFN-γ in sera of TCCR+/+ and TCCR−/− mice at different intervals after *L. donovani* infection. Because TCCR−/− mice efficiently resolve liver inflammation, we also determined the levels of anti-inflammatory cytokine IL-10 at these intervals. On day 15, *L. donovani*-infected TCCR−/− displayed significantly higher levels of IFN-γ and TNF-α in their serum compared with TCCR+/+ mice (Figure 6). Although both groups produced the same amounts of IL-12 on day 15 after infection, WSX-1−/− mice produced significantly more IL-12 on day 30. However, at this time point, no significant difference was noted in the serum levels of IFN-γ between the groups (Figure 6). By day 60, levels of IL-12, TNF-α, and IFN-γ dropped significantly in *L. donovani*-infected TCCR−/− mice. However, at the same time, sera from *L. donovani*-infected TCCR+/+ mice contained significantly more TNF-α. Throughout the course of infection, *L. donovani*-infected TCCR+/+ mice produced more IL-10 when compared with similarly infected TCCR−/− mice, but these differences were not statistically significant. None of these cytokines were detectable in sera from naïve TCCR+/+ or TCCR−/− mice. Together, these findings show that the IL-27/TCCR pathway controls the severity of VL-associated inflammation by negatively regulating production of pro-inflammatory cytokines.

TCCR−/− Mice Mount a Robust Th1-Like Response during L. donovani *Infection*

Because TCCR has been shown to suppress T-cell hyperactivity during infection with certain intracellular pathogens such as *T. gondii*, we compared LdAg-specific proliferation and cytokine production by spleen cells from *L. donovani*-infected TCCR+/+ and TCCR−/− mice. The spleen cells from *L. donovani*-infected TCCR+/+ and TCCR−/− mice proliferated efficiently in response to *in vitro* stimulation with LdAg on days 15 and 30 after infection, although the magnitude of proliferation was marginally higher in TCCR+/+ mice at day 30. Interestingly, on day 60 after infection, LdAg-stimulated spleen cells from TCCR−/− mice mounted a robust proliferation response (10- to 15-fold higher) compared with those from TCCR+/+ mice (data not shown). TCCR+/+ and TCCR−/− mice displayed significant levels of *Leishmania*-specific IgG1 and IgG2a antibodies during infection, and the differences between the groups were statistically not significant (data not shown).

Next, we analyzed levels of Th1-associated IL-12 and IFN-γ as well as Th2-associated IL-4 and IL-10 in the above supernatants by ELISA (Figure 7, A–C). Additionally, we also quantified levels of nitric oxide (NO) in these supernatants (Figure 7D).

The spleen cells from naïve TCCR+/+ and TCCR−/− mice displayed low but comparable basal levels of IL-12p70 (225 ± 82 and 343.6 ± 20 pg/ml in TCCR+/+ and TCCR−/− mice, respectively) and IFN-γ (116 ± 19 and 99 ± 30 pg/ml in TCCR+/+ and TCCR−/− mice, respectively). No IL-4 or IL-10 was detectable in culture supernatants from either group (data not shown).

LdAg-stimulated spleen cells from *L. donovani*-infected TCCR+/+ and TCCR−/− mice produced similar amounts of IFN-γ on day 15 after infection, but those from TCCR−/− mice produced significantly more on days 30 and 60 (Figure 7B). Similar differences were also observed in IL-12 production between the groups, although TCCR−/− mice displayed a significant drop in IL-12 production on day 60 (Figure 7A). TCCR−/− mice also displayed significantly higher levels of NO in their spleen cell supernatants on days 15 and 30 after infection but which dropped significantly by day 60 (Figure 7D). Al-

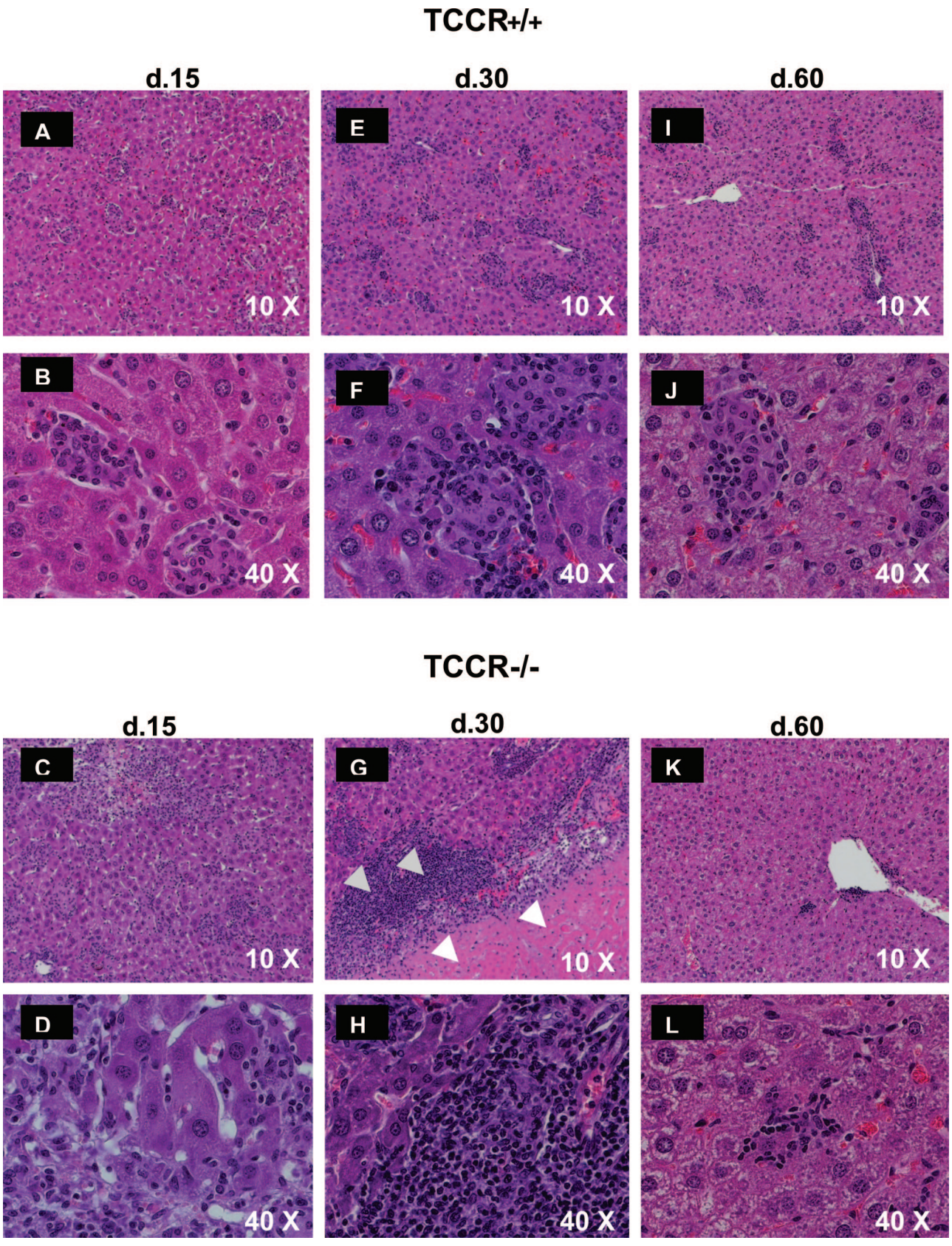

Figure 3. TCCR−/− mice develop severe liver inflammation after *L. donovani* infection. Histopathology of livers from both TCCR+/+ and TCCR−/− mice at 15 days (**A, B, C,** and **D**), 30 days (**E, F, G,** and **H**), and 60 days (**I, J, K,** and **L**) after *L. donovani* infection. On days 15 and 30 after infection, livers from TCCR+/+ mice displayed formation of well-organized granulomas with occasional clusters of amastigotes within individual macrophages (**A, B, E,** and **F**). By contrast, livers from TCCR−/− mice (**G** and **H**) displayed severe liver pathology with large granulomas associated with diffuse foci of inflammation (**gray arrows**) and necrosis (**white arrows**) compared with those from TCCR+/+ mice (**E** and **F**). By day 60, both TCCR+/+ (**I** and **J**) and TCCR−/− (**K** and **L**) mice resolved liver inflammation although some small granulomas were still visible in livers from TCCR+/+ mice (**I** and **J**).

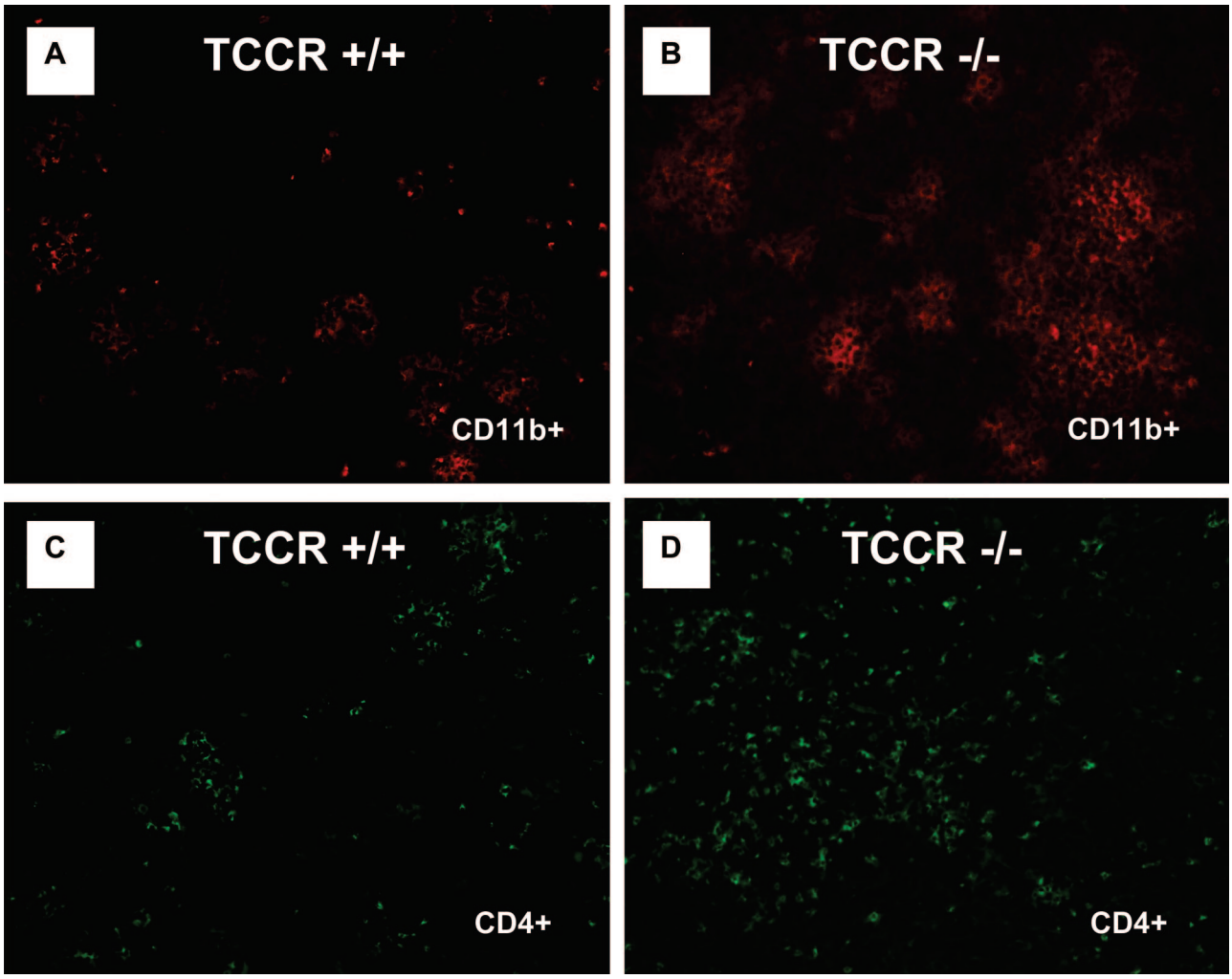

Figure 4. Immunohistochemical analysis of cell populations in livers from TCCR+/+ and TCCR−/− mice on day 30 after *L. donovani* infection. Cryostat cut sections from livers were stained using rat anti-mouse CD11b (**A** and **B**) or anti-mouse CD4 antibodies (**C** and **D**), and bound primary Ab was detected by appropriate secondary biotinylated Ab and streptavidin AlexaFluor 488 (green) or AlexaFluor 594 (red). Livers from TCCR+/+ mice showed well-organized granulomas composed of CD11b+ macrophages (**A**) and $CD4^+$ T cells (**C**), whereas those from TCCR−/− mice displayed diffuse parenchymal infiltration by these cell populations (**B** and **D**).

though no differences were noted in IL-10 production between TCCR+/+ and TCCR−/− mice on days 15 and 30, the latter produced significantly more IL-10 on day 60, coinciding with the resolution of liver inflammation (Figure 7C). No IL-4 was detectable in spleen cell supernatants from TCCR+/+ and TCCR−/− mice at all of the intervals examined (data not shown). These findings demonstrate that TCCR−/− mice mount a strong Th1-like response and produce high levels of IFN-γ after *L. donovani* infection.

Depletion of $CD4^+$ T Cells Significantly Reduces Hepatic Immunopathology but Renders Them More Susceptible to L. donovani

Because hyperactive T cells have been shown to mediate organ immunopathology in TCCR−/− mice during *T. gondii* and *T. cruzi* infections, we analyzed the effect of $CD4^+$ or $CD8^+$ cell depletion on liver inflammation and host resistance in TCCR−/− mice after *L. donovani* infection. TCCR−/− mice were administered anti-CD4, anti-CD8, or control antibodies during *L. donovani* infection to deplete specific cell subpopulations. Mice were sacrificed on day 21 after infection to assess liver pathology, organ parasite loads, and *in vitro* cytokine production by spleen cells.

Depletion of $CD4^+$ cells markedly reduced hepatic pathology in TCCR−/− mice but rendered them more susceptible to *L. donovani* infection (Figure 8, A, B, D, and E). Depletion of $CD8^+$ T cells also rendered TCCR−/− mice relatively more susceptible to *L. donovani* infection than controls but had minimal effect on the severity of liver inflammation (Figure 8, A, C, D, and E). Furthermore, spleen cell supernatants from these mice contained significantly less IFN-γ compared with those from TCCR−/− mice treated with control antibodies (data not shown). These findings show that although $CD4^+$ T cells mediate hepatic immunopathology in TCCR−/− mice during VL, they are also required for controlling parasite growth in the organs.

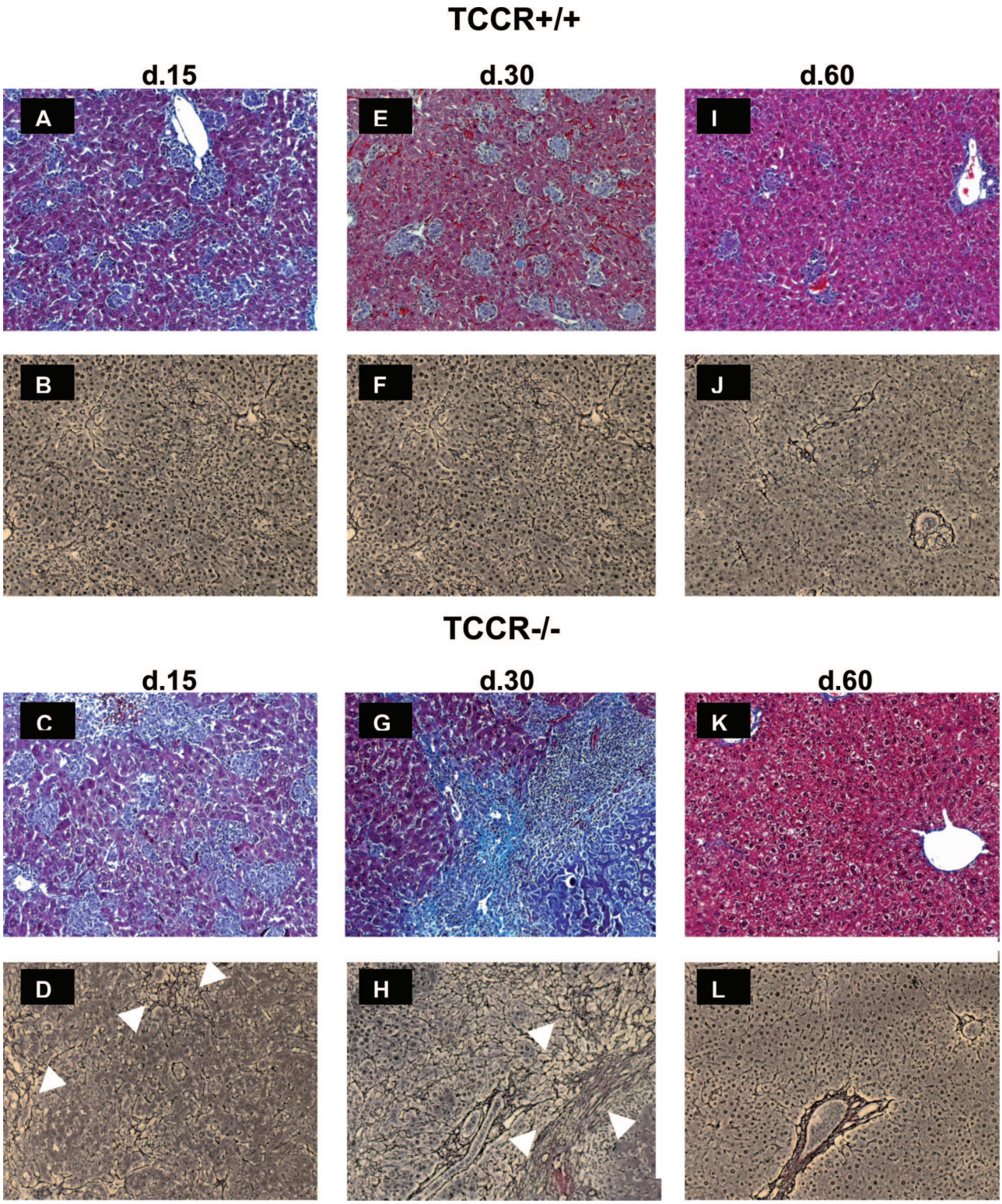

Figure 5. Analysis of collagen and reticulin fiber deposition in livers from TCCR+/+ and TCCR−/− mice on days 15 (**A, B, C,** and **D**), 30 (**E, F, G,** and **H**), and 60 (**I, J, K,** and **L**) after *L. donovani* infection. Livers from TCCR−/− mice showed significantly more deposition of collagen (**C** and **G**) and reticulin fiber (**D** and **H**) on days 15 and 30 after infection compared with those from the wild-type mice (**A** and **E** (collagen); **B** and **F** (reticulin fibers)). At day 60 after infection, livers from TCCR−/− mice displayed less collagen and reticulin fiber deposition compared with TCCR+/+ mice (**I, J, K,** and **L**). Note that collagen is stained blue and that reticulin fibers, which stain black, are denoted by **white arrows**. Magnification, ×10.

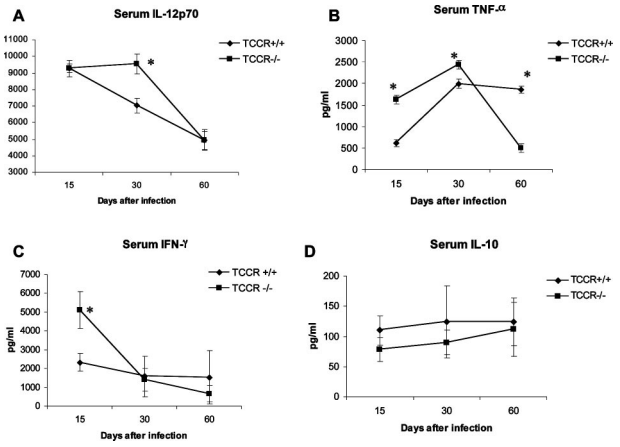

Figure 6. Kinetics of serum cytokine responses in TCCR+/+ and TCCR−/− mice after *L. donovani* infection. Mice were bled on days 15, 30, and 60 after infection and levels of IL-12p70 (**A**), TNF-α (**B**), IFN-γ (**C**), and IL-10 (**D**) were measured in serum by ELISA. Data shown are the mean ± SD of triplicate measurement from six to eight mice per group. The data are representative of two independent experiments. *Statistically significant differences between each group ($P < 0.05$).

RAG2−/− Mice Reconstituted with TCCR−/− T Cells Develop Severe Liver Pathology after L. donovani *Infection but Display Significantly Enhanced Resistance to Infection*

To determine whether TCCR−/− T cells alone are sufficient to mediate liver inflammation during VL, we compared the course of *L. donovani* infection in RAG2−/− mice that were reconstituted with B cell-depleted spleen cells (NKT+ T+) or B cell- and NK1.1+ cell-depleted spleen cells (NKT− T+) from TCCR+/+ or TCCR−/− mice. RAG2−/− mice reconstituted with B cell-depleted splenocytes from naïve TCCR−/− mice developed severe liver inflammation and displayed significantly lower hepatic parasite loads compared with RAG2−/− recipients reconstituted with TCCR+/+ T cells (Figure 9, A, C, E, and F). Similar results were also observed in RAG2−/− mice that received B cell- and NK1.1+ cell-depleted TCCR−/− spleen cells (Figure 9, B, D, E, and F). These results indicate that TCCR-deficient T cells are sufficient to induce liver inflammation during VL. Furthermore, they demonstrate that neither TCCR−/− NKT cells nor B cells mediate immunopathology.

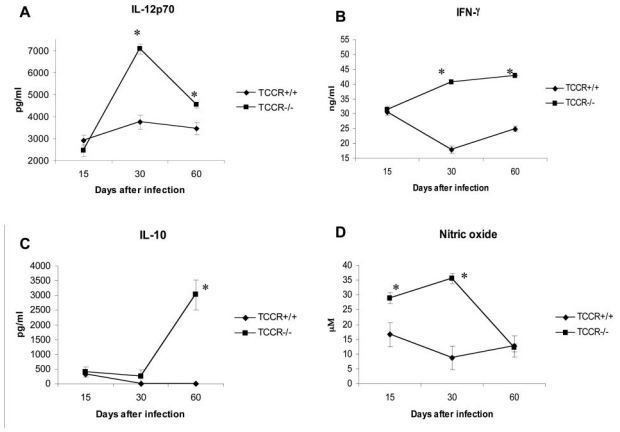

Figure 7. Kinetics of *in vitro* IL-12p70 (**A**), IFN-γ (**B**), IL-10 (**C**), and NO (**D**) production by spleen cells from *L. donovani*-infected TCCR+/+ and TCCR−/− mice. Spleen cells were stimulated with 20 μg/ml of LdAg, and levels of cytokines were measured by ELISA. NO production was measured using Griess's assay. Note that the levels of IFN-γ are shown in nanograms per milliliter. Data shown are the means ± SD of triplicate from four to five mice per group and are representative of two independent experiments. *Statistically significant differences between each group ($P < 0.05$).

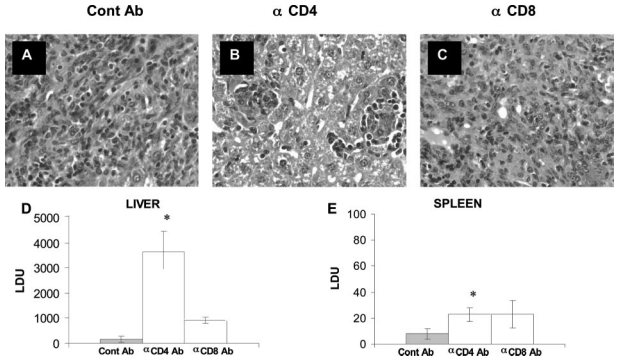

Figure 8. Effect of $CD4^+$ and $CD8^+$ cell depletion on liver pathology and host resistance in TCCR−/− mice during *L. donovani* infection. Histopathology of livers on day 21 after infection from TCCR−/− mice treated with control Ab (**A**), anti-CD4 (**B**), and anti-CD8 (**C**) antibodies. Parasite burdens in liver (**D**) and spleen (**E**) are expressed as the mean LDU ± SEM. The data are the mean values from three to four animals per group.*Statistically significant differences compared with mice treated with control Ab ($P < 0.05$).

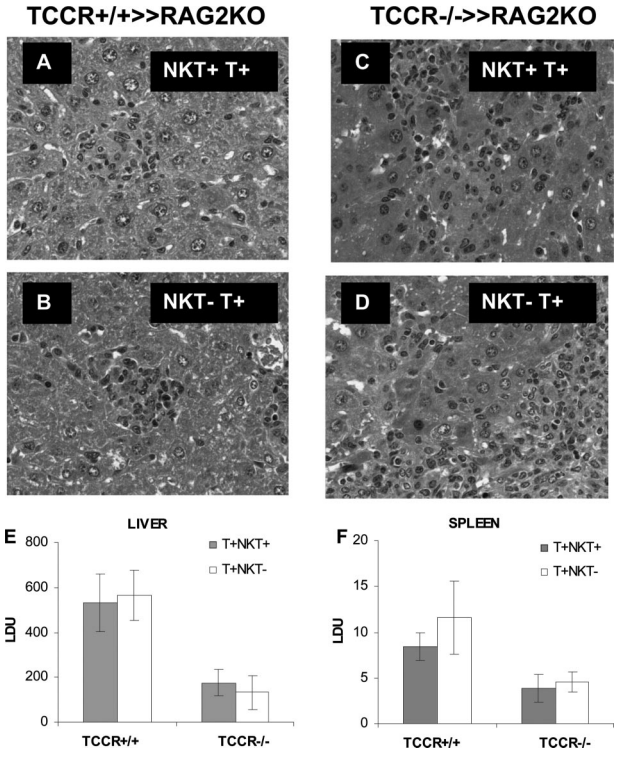

Figure 9. Course of *L. donovani* infection in RAG2−/− mice reconstituted with TCCR+/+ or TCCR−/− T cells. Histopathology of livers on day 30 after *L. donovani* infection from RAG2−/− mice reconstituted with (**A** and **B**) TCCR+/+ T cells or (**C** and **D**) TCCR−/− T cells. Parasite burdens in liver (**E**) and spleen (**F**) are expressed as the mean LDU ± SEM. The data are the mean values from three to four animals per group.*Statistically significant differences between each group ($P < 0.05$). Note: **B** and **D** are livers from RAG2−/− mice that only received T cells from TCCR+/+ and TCCR−/− mice, respectively.

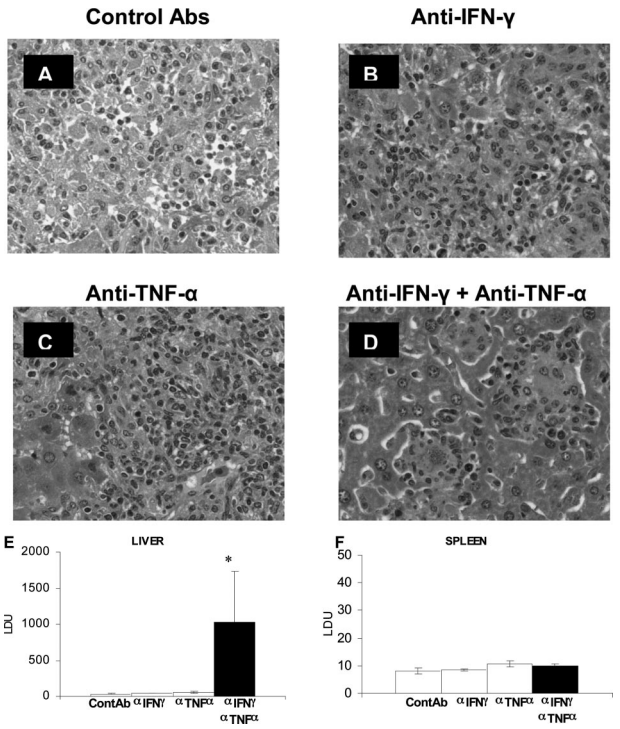

Figure 10. Effect of IFN-γ and/or TNF-α blockade on liver pathology and host resistance in TCCR−/− mice during *L. donovani* infection. Histopathology of livers on day 30 after infection from TCCR−/− mice treated with control antibodies (**A**), anti-IFN-γ (**B**), anti-TNF-α (**C**), and anti-IFN-γ/anti-TNF-α (**D**). Magnification, ×40. Parasite burdens in liver (**E**) and spleen (**F**) expressed as the mean LDU ± SEM. The data are the mean values from three to four animals per group.*Statistically significant differences between each group ($P < 0.05$).

In Vivo *Neutralization of Both IFN-γ and TNF-α Is Required to Reduce Hepatic Immunopathology in TCCR−/− Mice during VL*

Because *L. donovani*-infected TCCR−/− mice produced high levels of pro-inflammatory cytokines IFN-γ and TNF-α, we determined the effect of their blockade on liver pathology and host resistance in TCCR−/− mice during VL. Administration of anti-IFN-γ or anti-TNF-α antibodies alone failed to reduce liver inflammation and host resistance in TCCR−/− mice (Figure 10, A–C). On the other hand, TCCR−/− mice receiving both anti-IFN-γ and anti-TNF-α antibodies developed markedly reduced liver pathology but became more susceptible to infection. These results show that either IFN-γ or TNF-α alone is sufficient to mediate hepatic immunopathology and host resistance in TCCR−/− mice during VL.

Discussion

The results presented in this study clearly show that the IL-27/TCCR (WSX-1) pathway is not required for the induction of a protective Th1 response and IFN-γ production during *Leishmania donovani* infection. In fact, these results demonstrate that the lack of TCCR enhances resistance against *L. donovani*. The findings also suggest that TCCR plays a critical role in suppressing hepatic inflammation during the acute phase of VL, but it is not essential for the resolution of liver pathology.

IL-27/TCCR (WSX-1) signaling plays a critical role in mediating host resistance against several intracellular bacteria and parasites.[8,11,12] The importance of IL-27/TCCR (WSX-1) signaling in antibacterial immunity was first demonstrated in a study that found TCCR−/− mice to be more susceptible to *Listeria monocytogenes* when compared with the wild-type littermates and to display significantly higher bacterial loads in their livers.[8] WSX-1 (TCCR)−/− mice were also highly susceptible to protozoan parasites, like *T. gondii* and *T. cruzi*, and rapidly succumbed to disease after infection with these pathogens.[11,12] Whereas increased mortality in *T. gondii*-infected WSX-1 (TCCR)−/− mice was due to immune-mediated immunopathology,[11] enhanced susceptibility of these mice to *T. cruzi* was associated with the development of prolonged parasitemia.[12] In contrast, in the present study, TCCR−/− mice were highly resistant to *L. donovani* infection and rapidly controlled parasite growth in their organs. These observations indicate that IL-27/TCCR signaling is not required for the development of protective immunity against *L. donovani* but may be involved in mediating susceptibility to this pathogen. These findings substantially differ from those reported in the experimental model of cutaneous leishmaniasis, showing that IL-27/TCCR signaling plays a critical role in mediating host resistance to *L. major*.[9,16] Specifically, *L. major*-infected WSX-1 (TCCR)−/− C57BL/6 mice failed to produce optimal amounts of IFN-γ during the early phase of infection and later developed large lesions containing large numbers of parasites.[9] This is perhaps not surprising because *L. major* and *L. donovani* cause different diseases in man and trigger complex immune responses that determine the outcome of disease in the host.[1] For example, cutaneous *L. major* infection in man usually manifests as a self-resolving skin lesion, whereas *L. donovani* infection is associated with systemic spread of parasites to organs such as the liver, spleen, and bone marrow.[1] Furthermore, resolution of cutaneous *L. major* infection is associated with induction of a predominant Th1 response and IFN-γ production.[1] On the other hand, patients infected with *L. donovani* mount a mixed Th1/Th2 response.[17–20] Finally, cytokines such as IL-4 have been shown to play opposite roles in determining the outcome of murine *L. major* and *L. donovani* infection. Although IL-4 is a susceptibility factor in murine *L. major* infection,[1] it is required for successful vaccination and chemotherapy against *L. donovani* infection.[21,22]

Although the protective role of TCCR (WSX-1) signaling in control of *L. monocytogenes* and *L. major* infections has been attributed to its ability to promote Th1 development by inducing T-bet expression via a STAT1-dependent pathway,[9,16,23] WSX-1 signaling also prevents immune-mediated inflammation by inhibiting T-cell hyperactivity and suppressing production of pro-inflammatory cytokines during *T. gondii* and *T. cruzi* infections.[11,12] In the present study, *L. donovani*-infected TCCR−/− mice produced significantly more IL-12, IFN-γ, and TNF-α during the early phase of infection and developed marked hepatic immunopathology when compared with the wild-

type mice. Depletion of $CD4^+$ T cells significantly reduced liver pathology in TCCR−/− mice but rendered them susceptible to *L. donovani*. On the other hand, depletion of $CD8^+$ T cells had no effect on liver inflammation, although $CD8^+$ T cell-depleted mice displayed enhanced susceptibility to infection. $CD4^+$ and $CD8^+$ T-cell depletion also increased spleen parasite burdens in TCCR−/− mice, but these increases were less dramatic compared with those observed in liver. Furthermore, RAG2−/− mice reconstituted with B cell-depleted (NKT+ and T+) or B cell- and NK1.1+ cell-depleted (NKT− and T+) spleen cells from TCCR−/− mice also developed severe hepatic inflammation after *L. donovani* challenge and displayed enhanced resistance to infection, indicating that TCCR-deficient NKT cells are not involved in mediating immunopathological damage in the liver during VL. Interestingly, despite severe liver injury, TCCR−/− mice did not succumb to *L. donovani* infection but showed a marked drop in serum TNF-α and IFN-γ and successfully controlled spleen and liver inflammation by day 60. On the other hand, by this time, the TCCR+/+ mice displayed higher levels of TNF-α and showed more granulomas and foci of residual inflammation in their livers compared with TCCR−/− mice. Although LdAg-stimulated spleen cells from TCCR−/− mice produced significantly more IFN-γ compared with those from TCCR+/+ mice on day 30 and thereafter, both showed markedly reduced but comparable levels of IFN-γ in their sera at these time points. This suggests that high levels of IFN-γ produced by spleen cells alone may not be sufficient to induce immunopathology in liver. In fact, neutralization of both IFN-γ and TNF-α in TCCR−/− mice reduced hepatic pathology but increased their susceptibility to *L. donovani.* However, blockade of IFN-γ or TNF-α alone was not sufficient to block liver inflammation or compromise host resistance in TCCR−/− mice. This is most likely due to the fact that either cytokine compensates for the blockade of the other and mediates hepatic pathology. Surprisingly, blockade of both IFN-γ and TNF-α had no significant effect on spleen parasite burdens, suggesting that the IFN-γ/TNF-α mechanism may be involved in controlling parasite growth in the spleen of TCCR−/− mice during VL. Alternatively, it is also possible that the amount of Ab administered was insufficient to optimally neutralize high amounts of IFN-γ and TNF-α produced in the spleen of these mice. On day 60 after infection, spleen cells from TCCR−/− mice also produced significantly more IL-10, but both groups displayed low levels of IL-10 in their sera, suggesting that this cytokine may be involved in controlling splenic but not hepatic inflammation in TCCR−/− mice after *L. donovani* infection. This is perhaps not surprising because previous studies have shown that cytokines and chemokines play organ-specific roles in controlling parasite loads and pathology in liver and spleen during *L. donovani* infection.[24,25] Nevertheless, taken together, these results demonstrate that the IL-27/TCCR pathway plays a critical role in limiting the severity of injury to target organs during the acute phase of *L. donovani* infection by inhibiting production of the pro-inflammatory cytokines, but it is not essential for the resolution of VL-associated liver and spleen inflammation. Furthermore, they demonstrate that $CD4^+$ T cells play a critical role in mediating immunopathological liver damage in TCCR−/− mice during *L. donovani* infection.

Susceptibility to *L. major* is associated with the development of a Th2-like response, with production of cytokines such as IL-4 and IL-10.[26–29] In contrast, we and others have shown that IL-4 is required for vaccine-induced protection against *L. donovani* as well as successful resolution of *L. donovani* infection after chemotherapy.[13,21,22] TCCR (WSX-1)−/− mice infected with *L. major* or *T. cruzi* mount a Th2 response and display a significant increase in production of IL-4 and IL-13. However, no IL-4 was detectable in serum samples or culture supernatants from *L. donovani*-infected TCCR+/+ and TCCR−/− mice, suggesting that IL-4 is not involved in enhancing resistance of TCCR−/− mice to *L. donovani*.

In conclusion, our results demonstrate that C57BL/6 mice lacking TCCR rapidly control parasite growth in their organs and show increased resistance to *L. donovani*. TCCR−/− mice mount a strong Th1 response, produce high levels of pro-inflammatory cytokines, and develop severe hepatic inflammation after *L. donovani* infection that eventually resolves. $CD4^+$ T cells are primarily responsible for inducing hepatic immunopathology in TCCR−/− mice during *L. donovani* infection, but they are also involved in controlling parasite growth. These results indicate that although the TCCR pathway is not essential for the development of protective Th1 response during VL, it may mediate susceptibility to *L. donovani*. The unexpected and novel findings herein demonstrate that TCCR signaling also limits severity of immune-mediated liver damage by suppressing production of pro-inflammatory cytokines, but it is not required for resolution of hepatic and splenic inflammation.

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