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Cutting Edge: Chronic Inflammatory Liver Disease in Mice Expressing a CD28-Specific Ligand

Emily Corse,* Rachel A. Gottschalk,*†,1,2 Joon Seok Park,*†,1 Manuel A. Sepulveda,*3 P’ng Loke,‡,4 Timothy J. Sullivan,‡,5 Linda K. Johnson,‡,6 and James P. Allison*†

Inflammation of the normally tolerant liver microenvironment precedes the development of chronic liver disease. Study of the pathogenesis of autoimmune liver diseases, such as autoimmune hepatitis (AIH), has been hampered by a lack of autochthonous chronic animal models. Through our studies of T cell costimulation, we generated transgenic mice expressing a ligand specific for the CD28 receptor, which normally shares ligands with the related inhibitory receptor CTLA-4. The mice spontaneously develop chronic inflammatory liver disease with several pathologies found in AIH, including elevated serum aminotransferases in the context of normal alkaline phosphatase and bilirubin levels, lymphocytic inflammation, focal necrosis, oval cell hyperplasia, and fibrosis. The prevalence of IFN-γ-producing CD8+ T cells in the livers of transgenic mice suggests a role for autoimmune cytotoxicity in the chronic disease state. The CD28 ligand–specific transgenic mice will facilitate evaluation of CD8+ T cell function in liver disease pathologies found in AIH. The Journal of Immunology, 2013, 190: 526–530.

CD28 and CTLA-4 are related T cell transmembrane receptors that share the ligands B7-1 and B7-2 but have opposing effects upon T cell responses (1–3). Although stimulation of CD28 results in augmentation of TCR-signaling pathways and increased IL-2 production, CTLA-4 binding inhibits T cell responses, potentially by multiple mechanisms (4). Mechanistic insight into these opposing pathways of T cell regulation has been obscured by the inability to specifically ligate the CD28 and CTLA-4 receptors in cell culture systems. The development of membrane-bound single chain V region Ab reagents specific for CD28 and CTLA-4, known as single chain Fragment variable (scFv), represents a reductionist solution to this problem (5). Indeed, transgenic mice expressing anti–CTLA-4 scFv in B cells are resistant to autoimmune diabetes, underscoring the role of CTLA-4 in immune self-tolerance (6).

The liver is subject to a greater degree of immune tolerance than other organs, because of the unique APC environment (7) and its consequences for potentially reactive T cells (8). Disruption of this tolerogenic microenvironment occurs during chronic liver disease, which can result from persistent viral infection, drug toxicity, and autoimmune reactivity toward the liver. Although several mouse models of immune-mediated liver injury exist, models that reflect the chronic and complex pathologies of autoimmune liver diseases, such as autoimmune hepatitis (AIH), have been elusive (9, 10). Existing chronic mouse models of liver disease involve virus infection (11) and overexpression of inflammatory mediators (12). According to the National Institutes of Health, development of new models that reflect features of autoimmune liver diseases, such as AIH, including spontaneous development of chronic lymphocytic inflammation and fibrosis, is important for understanding the pathogenesis of this group of diseases (13). Activated CD4+ T cells have long been known to be in the liver and peripheral blood of AIH patients, and cytochrome P450 2D6 is an important autoantigen in the type 2 form of AIH (14). CD8+ T cells are also likely to be important in pathogenesis given the correlation between disease severity and IFN-γ secretion by cytochrome P450 2D6–reactive CD8+ T cells in AIH type 2 patients (15).
In the course of studies on T cell costimulation, we generated anti-CD28 scFv transgenic mice that allow selective ligation of the T cell transmembrane receptor CD28, which normally shares the ligands B7-1 and B7-2 with the T cell inhibitory receptor CTLA-4. The CD28 scFv mice, when maintained on a B7-1, B7-2 double-deficient background (16), spontaneously develop chronic inflammatory liver disease characterized by infiltration of IFN-γ-secreting CD8+ T cells, necrosis, and fibrosis. Engagement of CD28 in the absence of CTLA-4 may cause inflammatory liver disease by lowering the threshold for T cell reactivity in the normally tolerant liver microenvironment, a notion supported by the association between polymorphisms in the human CTLA-4 gene and susceptibility to the autoimmune liver diseases AIH and primary biliary cirrhosis (17). The CD28 scFv mice are ideal for the study of CD8+ T cell contributions to pathologies found in autoimmune liver diseases, such as AIH.

**Materials and Methods**

**Mice**

The anti-CD28 scFv ligand was generated by fusing 37N.51 variable regions to B7-2 in place of its membrane distal Ig domain (5) and subcloned into the pD06c vector containing the invariant chain promoter, a gift of D. Mathis, Department of Microbiology and Immunology, Harvard Medical School, Boston, MA (18). Linearized plasmid was injected into C57BL/6 embryos by the Memorial Sloan-Kettering Cancer Center (MSKCC) Mouse Genetics Facility. Founder mice were identified by PCR. B7-1, B7-2 double knockout (DKO) mice were purchased from The Jackson Laboratory and bred to anti-CD28 scFv mice. Male anti-CD28 scFv mice were analyzed. OTII RAG2−/− mice (19) were purchased from The Jackson Laboratory and bred to B7-1, B7-2 DKO mice. All mice were maintained in microisolator cages and treated in accordance with National Institutes of Health and American Association of Laboratory Animal Care regulations. Experiments in this study were approved by the MSKCC Institutional Animal Care and Use Committee.

**In vitro T cell proliferation**

CD11c+ APCs were positively selected (Miltenyi Biotec), pulsed with OVA (323–339) peptide, and used to stimulate OT-II B7-1, B7-2 DKO T cells. Proliferation was monitored by the addition of [3H]thymidine. Cells were cultured at 37°C/5% CO2 in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin/streptomycin, and 2 mM 2-ME. Cells formed on a BD LSRII and data analyzed with FlowJo software (TreeStar). All other Abs were from BD Pharmingen, eBioscience, or BioLegend. Liver and spleen T cells were positively selected for CD90.2 (Miltenyi Biotec) prior to stimulation. 

**Histology, immunohistochemistry, and liver enzyme measurements**

Preparation of liver tissue, sectioning, H&E and Mason’s trichrome staining, and liver chemistry panels were done by the Laboratory of Comparative Pathology, MSKCC.

**Isolation, quantitation, and stimulation of leukocytes from liver**

Livers were digested with Liberase TL (1.2 mg) and DNase I (0.8 mg) and passed through a 100-μm filter. Leukocytes were isolated by centrifugation in 33% isotonic Percoll. For analysis of IFN-γ secretion, cells stimulated with PMA/ionomycin were stained for intracellular IFN-γ with BD Biosciences reagents.

**Statistical analysis**

Data were analyzed for significance by one-way ANOVA with Bonferroni correction using Prism 5 software (GraphPad).

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**Results and Discussion**

**Anti-CD28 scFv mice express a functional ligand for CD28 on APCs**

The anti-CD28 scFv mice express anti-CD28 Ab fragments fused to the CD28 ligand B7-2 (5) (Fig. 1A) in MHC II–expressing APCs (18). Expression was visualized with recombinant soluble CD28-Fc and flow cytometry (Fig. 1B). Like natural CD28 ligands, the anti-CD28 scFv fusion protein is most highly expressed on APCs bearing the dendritic cell marker CD11c+ (Fig. 1B). To specifically activate CD28 with scFv ligand, anti-CD28 scFv–transgenic mice were crossed to mice deficient in CD28 and CTLA-4 ligands B7-1 and B7-2 (16). These mice, referred to hereafter as “B7-1, B7-2 DKO,” serve as littermate controls lacking CD28-mediated costimulation.

To test the function of the anti-CD28 scFv ligand, CD11c+ leukocytes from transgenic mice were used as APCs to stimulate Ag-specific T cells (Fig. 1C). The anti-CD28 scFv APCs induce increased proliferation compared with B7-1, B7-2 DKO APCs, consistent with costimulatory function of CD28 (Fig. 1C). Thus, the anti-CD28 scFv fusion protein is a functional CD28 ligand that enhances T cell stimulation. **Anti-CD28 scFv mice have chronic inflammatory liver disease**

Upon observing enlarged livers from 3 mo of age (Fig. 1D, Supplemental Table I), we were prompted to evaluate liver chemistry in the anti-CD28 scFv mice. Elevated levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were observed in all mice examined between 4 and 8 mo of age (Fig. 1E), whereas alkaline phosphatase, γ-glutamyl transpeptidase, bilirubin, and other liver chemistry measurements were normal (data not shown). Elevated transaminases in the context of normal levels of alkaline phosphatase and bilirubin is often seen in AIH patients (20).

H&E staining of liver sections from anti-CD28 scFv mice showed periportal lymphocytic inflammation (Fig. 1F, right panel). Microscopic signs of liver damage, such as single cell necroses and oval cell hyperplasia, were often observed in the inflamed anti-CD28 scFv livers (Fig. 2A, right panel, arrow and arrowhead, respectively). In addition, Mason’s trichrome staining highlighted collagen deposition, which is indicative of fibrosis, or formation of scar tissue, extending from the portal tract in anti-CD28 mice (Fig. 2B, right panel). A summary of liver pathology in mice of various ages can be found in Supplemental Table I.

The anti-CD28 scFv mice also exhibit splenomegaly (Supplemental Fig. 1A, 1B). Although this could result from constitutive costimulation in the anti-CD28 scFv mice, splenomegaly is also often found in patients with chronic liver disease, including that caused by AIH (21, 22), due to shunting of blood from liver to spleen as a result of fibrosis and portal hypertension. Examination of other organs revealed occasional mild inflammation of the lung, kidney, pancreas, and skin (data not shown). Although present in only some anti-CD28 scFv mice, these phenotypes are reminiscent of additional autoimmune disorders in patients with AIH (21–23). We did not find evidence of circulating autoantibodies (data not shown), which is likely precluded by the defect in germinal center formation and diminished serum IgG levels in B7-1, B7-2 DKO mice (16). Although autoantibodies are commonly seen in autoimmune liver disease, evidence for their involvement in pathogenesis is lacking (21, 24). In summary,
anti-CD28 scFv mice spontaneously develop Ab-independent chronic liver disease that recapitulates several features of human AIH, including persistently elevated ALT and AST, periportal lymphocytic inflammation, and portal-based fibrosis.

**IFN-γ-secreting CD8⁺ T cells are prevalent in livers of anti-CD28 scFv mice**

We examined liver infiltrates for the presence of T cells using anti-CD3 immunohistochemistry (Fig. 2C), and this revealed substantial numbers of CD3⁺ lymphocytes in anti-CD28 scFv liver tissue (brown-labeled cells, Fig. 2C, right panel) compared with very few in B7-1, B7-2 DKO tissue (Fig. 2C, left panel). To further characterize liver infiltrates, we analyzed isolated leukocytes by flow cytometry. Significantly more CD8⁺ T cells exist in livers of anti-CD28 scFv mice (31% of total leukocytes) compared with control livers (11% of leukocytes) (Fig. 2D). This corresponded to a 5-fold average increase in the absolute number of CD8⁺ T cells in livers of 3–5-mo-old anti-CD28 scFv mice compared with controls (Fig. 2D). We did not observe increases in CD4⁺ or NK T cells (data not shown), making it likely that a significant portion of the CD3⁺ staining in Fig. 2C represents CD8⁺ T cells. Enrichment of CD8⁺ T cells in liver of CD28 scFv mice is in contrast to increases in CD4⁺ and CD8⁺ T cells and B cells in spleen (data not shown).

Trapping of T cells in liver was described as a mechanism of apoptotic removal (25); to directly examine the functional activity of CD8⁺ T cells in livers of anti-CD28 scFv mice, we analyzed IFN-γ production upon ex vivo restimulation. Compared with B7-1, B7-2 DKO controls, nearly twice the percentage of CD8⁺ T cells in the livers of anti-CD28 scFv mice secrete the inflammatory cytokine IFN-γ (Fig. 2E). Thus, in addition to increased numbers of CD8⁺ T cells in livers of anti-CD28 scFv mice, more of these cells produce IFN-γ, corresponding to a >8-fold increase in the number of IFN-γ-secreting CD8⁺ T cells, suggesting that liver disease in anti-CD28 scFv mice could be IFN-γ mediated. IFN-γ production by CD8⁺ T cells correlates with disease severity in AIH type 2 patients (15). Initial experiments show that CD8⁺ T cell depletion minimizes lymphocytic infiltration and other pathological features, such as fibrosis, in anti-CD28 scFv liver tissue (Supplemental Fig. 1C–F).

To examine the diversity and potential specificity of liver CD8⁺ T cells in anti-CD28 scFv mice, we stained T cells from liver and spleen of anti-CD28 scFv and B7-1, B7-2 DKO mice with Abs against specific TCR Vβ subunits (Supplemental Fig. 1G–I). The frequency of Vβ8.1/8.2⁺

T cells stimulated with OVA-pulsed CD11c⁺ APCs from anti-CD28 scFv mice (DKO αCD28 scFv) or B7-1, B7-2 DKO mice. Data in (B) and (G) are representative of three independent experiments. (D) Livers from 4-mo-old anti-CD28 scFv and control mice (left panel); scale bars, 1 cm. Liver weights from 3–5-mo-old anti-CD28 scFv, B7-1, B7-2 DKO, and B6 control mice (right panel). ***p = 0.0002. (E) Serum ALT (left panel) and AST (right panel) were measured in 4–8-mo-old mice. ALT, ***p = 0.0006; AST, ***p = 0.0004. Each symbol in (D) and (E) represents an individual mouse. (F) H&E staining of livers from 4-mo-old B7-1, B7-2 DKO mouse (left panel) and anti-CD28 scFv mouse (right panel). These photomicrographs are representative of the 11 mice evaluated per group. Scale bars, 100 μm.
In this article, we report a new spontaneous mouse model of chronic liver disease characterized by infiltration of IFN-γ-secreting CD8+ T cells and the development of fibrosis. Engagement of the T cell costimulatory receptor CD28 with scFv ligand in the absence of inhibition by the T cell inhibitory receptor CTLA-4 may lower the threshold for T cell-mediated immune attack in the normally tolerant liver microenvironment. Interestingly, polymorphisms in human CTLA-4 are associated with AIH and another autoimmune liver disease, primary biliary cirrhosis (17). Of the three types of autoimmune liver disease—AIH, primary biliary cirrhosis, and primary sclerosing cholangitis—the pathologies found in the anti-CD28 scFv mice most closely resemble those seen in AIH, including lymphocytic inflammation, development of portal-based fibrosis, elevated transaminases, and occasional mild inflammation of other organs.

Although early studies of AIH emphasized the involvement of CD4+ T cells, it is now appreciated that CD8+ T cell reactivity is relevant (14), and the anti-CD28 scFv mice will facilitate study of CD8+ T cell contributions to AIH-like pathologies. Transgenic mice expressing liver-specific IFN-γ were shown to have inflammatory liver disease, although these mice did not develop fibrosis (26). Deletion of the immunomodulatory cytokine TGF-β also results in inflammatory liver disease and death of BALB/c mice at ~2 wk of age (27). Although genetic manipulation of IFN-γ and other cytokines demonstrates their ability to mediate liver disease (9), the anti-CD28 scFv mice are unique as the result of the spontaneous CD8+ T cell response initiated in the absence of virus infection, immunization, or other manipulation; this makes them an advantageous experimental model.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


### Supplemental Table 1. Summary of gross liver pathology in αCD28 scFv transgenic mice

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<tr>
<th>Age at Analysis</th>
<th>Genotype</th>
<th>Liver weight (g)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>Hepatitis</th>
<th>Fibrosis (trichrome)</th>
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<tr>
<td></td>
<td>DKO αCD28 scFv</td>
<td>1.7+/−0.34</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4 months</td>
<td>B7-1, B7-2 DKO</td>
<td>1.5+/−0.16</td>
<td>28+/−4.9</td>
<td>74+/−37</td>
<td>0/12</td>
<td>0/3</td>
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<tr>
<td></td>
<td>DKO αCD28 scFv</td>
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<td>315+/−184</td>
<td>275+/−120</td>
<td>11/12</td>
<td>3/3</td>
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<td>5 months</td>
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<td>2.0+/−0.43</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DKO αCD28 scFv</td>
<td>2.1+/−0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6 months</td>
<td>B7-1, B7-2 DKO</td>
<td>1.8+/−0.15</td>
<td>31</td>
<td>39</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>DKO αCD28 scFv</td>
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<td>142</td>
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<tr>
<td>8 months</td>
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<td>-</td>
<td>39+/−13</td>
<td>77+/−24</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>DKO αCD28 scFv</td>
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<td>292+/−62.2</td>
<td>262+/−91.2</td>
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Supplemental Figure 1. T cell responses in liver and spleen of αCD28 scFv mice. A) Photographs of spleens from 4 month old αCD28 scFv mice (DKO αCD28 scFv) and littermate control mice (B7-1, B7-2 DKO). B) weights of spleens from 3-5 month old DKO αCD28 scFv, B7-1, B7-2 DKO, and B6 control mice. Scale bars=1 cm. ***P<0.0001. C-F) αCD28 scFv mice were injected with 100 μg anti-CD8 antibody (clone 2.43; D and F) or isotype control antibody (C and E) once per week from 6 weeks of age. Mice were sacrificed at 4 months of age and livers examined by H&E staining. Images are representative of two independent mice per group. 40X images in E and F (scale bars=0.05 mm) are increased magnifications of the 10X images in C and D (scale bars=0.2 mm), respectively. Arrows indicate perportal infiltration of lymphocytes in isotype control-treated samples (C and E), which are largely absent in samples from mice depleted of CD8+ T cells (D and F). The asterisk indicates fibrosis (E), absent in CD8 depletions (F). Single and double arrowheads indicate oval cell hyperplasia and ductular proliferation, respectively (E), largely absent from CD8 depletions (F). G) TCR Vβ1/8.2 frequency in liver T cells of αCD28 scFv and B7-1, B7-2 DKO mice. Numbers on histograms indicate percent of CD8+ T cells. CD44 histograms are gated on indicated populations. H) TCR Vβ1/8.2 frequency in liver and spleen of αCD28 scFv and B7-1, B7-2 DKO mice. Error bars represent mean +/- s.d. (n=3 mice). **P=0.0018, ns=not significant (P=0.2839). I) TCR Vβ frequency in liver and spleen of αCD28 scFv and B7-1, B7-2 DKO mice. Error bars represent mean +/- s.d. (n=3 mice).