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Testosterone Suppresses Hepatic Inflammation by the Downregulation of IL-17, CXCL-9, and CXCL-10 in a Mouse Model of Experimental Acute Cholangitis

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Autoimmune liver diseases predominantly affect women. In this study, we aimed to elucidate how sex affects autoimmune hepatic inflammation. Acute experimental cholangitis was induced by adoptive transfer of OVA-specific CD8+ T cells into mice, which express the cognate Ag on cholangiocytes. In contrast to previous mouse models of cholangitis, this model displayed a strong sexual dimorphism: female mice developed marked cholangitis, whereas male mice were resistant to cholangitis induction. The recruitment of endogenous CD4+ T cells into female livers was strongly increased. These cells expressed higher amounts of the proinflammatory cytokine IL-17, which was at least in part responsible for the liver inflammation observed. The recruitment of endogenous CD4+ T cells was associated with increased expression of the chemokines CXCL-9 and CXCL-10 in female livers. The sex-specific factor responsible for the observed differences was found to be testosterone: male mice could be rendered susceptible to liver inflammation by castration, and testosterone treatment was sufficient to completely suppress liver inflammation in female mice. Accordingly, testosterone treatment of female mice significantly reduced the expression of IL-17A, CXCL-9, and CXCL-10 within the liver. Serum testosterone levels of untreated mice negatively correlated with the IL-17, CXCL-9, and CXCL-10 expression in the liver, further supporting a role for testosterone in hepatic immune homeostasis. In conclusion, testosterone was found to be the major determinant of the observed sexual dimorphism. Further study into the role of testosterone for liver inflammation could lead to novel treatment targets in human autoimmune liver diseases.

The online version of this article contains supplemental material.

Abbreviations used in this article: AIH, autoimmune hepatitis; AMA, anti-mitochondrial Ab; BEC, biliary epithelial cell; EAE, experimental autoimmune encephalomyelitis; mHAI, modified hepatitis activity index; NPC, liver nonparenchymal cell; PBC, primary biliary cirrhosis; PPRa, peroxisome proliferator activated receptor; PSC, primary sclerosing cholangitis; Treg, regulatory T cell.

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The online version of this article contains supplemental material.

Materials and Methods

Animals

K14-OVAp mice (C57BL/6 background) express the antigenic MHC class I restricted SIINFEKL peptide (residues 257–264 of OVA) under control of the human keratin 14 promoter and were provided by K. Hogquist (Minneapolis, MN). Although these mice were developed to study the thymic development of T cells, they were shown to express OVA on biliary epi-
thelial cells (BECs) and to develop liver inflammation when crossed to OT-1 transgenic mice (14, 15). K14-OVA+ mice were crossed to IL-17a−/− mice to investigate the role of endogenous IL-17 on liver inflammation. IL-17a−/− mice were described previously (16). CD8+ T cells from OT-1 mice (Jackson Laboratory, Bar Harbor, ME) express a transgenic receptor specific for the SIINFEKL peptide bound on H-2Kb. In our experiments, CD45.1 allelic-congenic OT-1 donor mice were used to distinguish transferred from recipient lymphocytes of CD45.2+ K14-OVA+ mice. All animals were age matched (8–10 wk). Animal care was in accordance with the governmental and institutional guidelines and all experiments were approved by the animal experimentation committee of the State of Hamburg (GI0103; GI2113).

Modulation of sex hormones via castration and pellet implantation

Castration of male mice and pellet implantations were conducted on 4-wk-old mice as described previously (17). Sixty-day release pellets of testosterone (12.5 mg/pellet) or β-estrogen (0.36 mg/pellet) were purchased from Innovative Research of America (Sarasota, FL) and implanted s.c. in the neck.

Cell isolation and adoptive transfer experiments

For the induction of cholangitis, freshly isolated congenic OT-1 CD8+ T cells from female donors were injected i.p. (4 × 10^6 to 20 × 10^6 T cells) into K14-OVA+ male and female recipient mice as well as K14-OVA+PL-17/If knockout female mice. OT-1 CD8+ T cells were isolated with anti-CD8-FITC Ab (BioLegend, Fell, Germany) and anti-FITC immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

For the isolation of liver nonparenchymal cells (NPCs), mouse livers were perfused with PBS and dissected mechanically. Hepatocytes and debris were sedimented twice at 40 × g, and nonparenchymal cells were recovered by centrifugation over a 35% Optiprep (Sigma-Aldrich, Taufkirchen, Germany) gradient at 400 × g.

Cell restimulation and cytokine measurements

Spleen cells and liver NPCs (5 × 10^6 per well) were restimulated for 24 h with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Biosciences, Heidelberg, Germany). Abs. Supernatants were analyzed for IFN-γ and IL-17 levels by ELISA using DuoSet mouse ELISA kits (R&D Systems, Wiesbaden, Germany). The results shown are representative for at least three similar experiments.

TH17 differentiation assay

CD4+ T cells were isolated from spleens of C57Bl/6J WT mice using magnetic cell separation (Milteny, Bergisch Gladbach, Germany) according to manufacturer’s description. CD4+ T cells were cultured in the presence of irradiated APCs (4 × 10^5, 30 Gray, 11 min) for five days in ClinMACs medium supplemented with TGFβ1, IL-1β, IL-6, IL-23, anti-IFN-γ and anti-IL-4 (R&D Systems, Wiesbaden, Germany) (18). Ten percent testosterone-enriched serum (derived from mice supplemented with testosterone pellets) was added as control. FCS and serum derived from female mice were used as control.

Serum liver enzymes and anti-mitochondrial Ab assay

Serum liver enzymes were measured with a Hitachi Modular E170 analyzer (Boehringer Mannheim, Mannheim, Germany). Serum anti-mitochondrial Abs (AMAs) were measured using the QUANTA LiteM2 EP (MIT3) ELISA kit (INOVA Diagnostics, San Diego, CA).

Flow cytometry

Immunofluorescent surface staining of liver NPCs and spleen cells was performed with Abs to CD3, CD8, CD4, CD25, CD45.1, and CD45.2. For intracellular cytokine staining, cells were treated with Golgi Plug (1 μM; BD Biosciences, Heidelberg, Germany) and restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich, Taufkirchen, Germany) for 4 h. Fixed cells were then perforated in buffer containing Saponin/BSA (0.5%/2%; Sigma-Aldrich, Taufkirchen, Germany) and stained for IFN-γ and IL-17. All Abs for T cell staining were purchased from BioLegend. Intracellular Foxp3 staining was performed with Foxp3 staining buffer and anti-Foxp3 Ab (both from eBioscience, Frankfurt, Germany). Flow cytometry data were analyzed with FACS Diva software (BD Biosciences, Heidelberg, Germany).

Real-time PCR

Total RNA was isolated from total liver tissue using Nucleospin Kit (Macherey Nagel, Düren, Germany). cDNA was reverse-transcribed from total RNA (1st strand RNA cDNA Synthesis Kit; Roche, Mannheim, Germany) followed by RT-PCR using the Bio-Rad CFX96 real-time system (Bio-Rad, München, Germany) and absolute PCR SYBR mix (Thermo Fisher, Schwerte, Germany). Sequence-specific primers for CXCL9 (forward 5′-CTT GAG CCT AGT GAT GAT AAC 3′; reverse 5′-CCA GCT TGG TGA GGT CTA TC 3′), CXCL10 (forward 5′-CCA CGT GTG ATT GCT GC 3′; reverse 5′-AGT AGC AGC TGA TGT GAC C 3′) and CCL-20 (forward 5′-CGA CTG TTG CCT CTC GTA CA 3′; reverse 5′-AGG AGG TTC ACA GCA GCT TT 3′) were used. Relative mRNA levels were calculated after normalization to β-actin with male mice as calibrator (∼1) using the CFX96 Manager software. The expression of RORγ mRNA was measured using the Taqman Universal PCR master mix (Applied Biosystems, Darmstadt, Germany) and Taqman gene expression kits (Applied Biosystems) were used for amplification, namely RoR (RORY, Mm01261022_m1). Target gene expression was normalized to Hprt (Mm00449668_m1).

Histology

H&E staining (Roht, Karlsruhe, Germany) was performed on formalin-fixed liver sections. SIINFEKL (eBioscience, Frankfurt, Germany) staining was performed on cryo-frozen tissue. All histologic scorings were performed by a pathologist in a blinded fashion. Scoring of liver inflammation was analyzed according to the modified hepatitis activity index (mHAI), which consists of the four subgroups A–D, with D representing the grade of portal inflammation (19). Scoring of skin and esophagus was analyzed according to an internal standard: 0 = no inflammation, 1 = minimal inflammation, 2 = mild inflammation, 3 = moderate inflammation, 4 = strong inflammation.

Statistics

Differences between two experimental groups were assessed for statistical significance with Student t test (p < 0.05 was considered significant). Differences between more than two groups were assessed using the one-way ANOVA Test and Tukey post test (p < 0.05 was considered significant). Correlation was calculated using the Pearson correlation coefficient (r), and p < 0.05 was considered significant. The p values are as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Results

Experimental cholangitis developed in female but not in male mice

There is a strong female preponderance in autoimmune liver diseases. We therefore sought to establish a model, in which the mechanisms behind this sexual dimorphism could be investigated. Experimental cholangitis was induced by the transfer of OVA-specific CD8+ T cells from OT-1 transgenic mice into male or female K14-OVA+ recipient mice. In this model, the adoptive transfer of 4 × 10^6 OVA-specific CD8+ T cells induced an increase in serum aminotransferase levels in female, but not in male recipient mice (Fig. 1A). Liver enzymes from female mice were significantly increased on day 8 and day 9 after OVA-specific CD8+ T cell transfer (Fig. 1B). Male mice were resistant to liver inflammation even after transfer of 10 × 10^6 OVA-specific CD8+ T cells (Fig. 1C). Histologically, cholangitis and lymphocytic portal tract inflammation was only observed in female recipient mice (Fig. 1D, 1E). Signs of autoimmune hepatitis such as interface hepatitis, lymphoplasmacytic infiltrate, rosetting, peripolesis or lobular inflammation were missing. In other models described, experimental cholangitis has been shown to be associated with the development of AMA, in humans a hallmark of PBC (2), but in mice a nonspecific sign of cholangitis (20, 21). Supporting the female predominance described in this model, we could detect AMA in 50% of K14-OVA+ female but not in male mice after the induction of cholangitis (female 4/7 [mean ± 58.10 U/ml]; male 0/7 [mean ± 22.45 U/ml]; cutoff, 30 U/ml). We did not find any differences in the hepatic expression levels of OVA between female and male mice that could account for the observed sexual dimorphism (Supplemental Fig. 1). As in this model, the expression of OVA is not restricted to BEC, we analyzed whether there is a sexual dimorphism also in the inflammatory activity in esophagus and skin of male and female recipient mice after OVA-specific CD8+ T cell transfer. In contrast to the prominent sex-dependent differences observed in the liver, there were no significant differences between male and female mice in skin and esophagus (Supplemental Fig. 2).
Hepatic recruitment and activation of transferred CD8+ T cells and the frequency of regulatory T cells were not different between female and male livers

As cholangitis was induced by the adoptive transfer of OT-1 CD8+ T cells, we analyzed whether the transferred OT-1 cells were differentially recruited to female or male livers. The differences in liver inflammation between male and female mice were not associated with altered frequencies of transferred OVA specific CD45.1+CD8+ T cells in the livers of recipient male or female mice (Fig. 2 A; 55% in males versus 59% in females of CD3+CD8+ T cells on day 8 after transfer). To exclude differences in the activation status of OVA-specific CD8+ T cells migrating into female and male livers, intraacellular IFN-γ levels were analyzed in CD45.1+ OT-1 T cells reisolated from livers on day 8 after transfer. The vast majority of
transferred CD8+ T cells expressed IFN-γ without differences between cells isolated from female or male livers (Fig. 2B).

Differences in regulatory T cell (Treg) numbers have been described to contribute to sexual differences observed in an experimental hepatitis model (22). We therefore investigated the CD4+ CD25+Foxp3+ Treg frequencies in the livers of male and female recipient mice on day 8 after cell transfer. There was no significant difference in hepatic or splenic Treg frequencies between female and male mice (Fig. 2C), suggesting that Tregs are not responsible for the observed sexual dimorphism.

**Increased recruitment of endogenous CD4+ T cells expressing IL-17 into the livers of female mice**

To elucidate the mechanisms behind the sex dimorphism of liver inflammation, the recruitment of endogenous lymphocytes of the recipient mice into the liver was investigated. The recruitment of endogenous CD45.2+CD4+ T cells was significantly increased in female as compared with male livers (28 ± 3 x 10^5 cells per 1 g female liver versus 9.4 ± 3 x 10^5 cells per 1 g male liver tissue; p < 0.0001; Fig. 3A), indicating a proinflammatory role for endogenous CD4+ T cells in our cholangitis model. To investigate the functional role of endogenous CD4+ T cells we performed transfer experiments of hepatic CD4+ T cells isolated from K14-OVAp mice after cholangitis induction into C57BL/6J wild-type animals. Twenty-four hours after CD4+ T cell transfer the largest fraction of K14-OVAp CD4+ T cells was detected in the liver (24%) or in liver-infiltrating lymph nodes (5%) of the wild-type recipient mice. We could hardly detect any K14-OVAp CD4+ T cells in the spleens of recipient mice (0.2%). As control, we analyzed the liver homing of activated CD4+ T cells derived from Smarta mice, which express a transgene TCR with specificity for LCMV (lymphocytic choriomeningitis virus) and which were activated in an Ag-specific manner. Smarta CD4+ T cells isolated from inflamed livers of K14-OVAp mice could cause liver inflammation in K14-OVAp recipient mice even in the absence of OT-1 cells (Fig. 3C). Thus, liver-derived CD4+ T cells from cholangitis mice are able to target the liver and cause hepatic inflammation even in the absence of Ag-specific OT-1 cells.

As we found sex-dependent differences in the recruitment of endogenous CD4+ T cells into inflamed livers, we next wanted to know whether IL-17 expression was also affected by sex. Of note, the cytokine IL-17 has been implicated in the pathogenesis of PBC, as well as PSC and experimental cholangitis models (23–26). To that end, spleen cells and liver NPCs were isolated from female and male mice on day 8 after transfer of OVA-specific CD8+ T cells. After restimulation of male or female spleen cells, IL-17 production was equally low; in contrast, lymphocytes isolated from female livers produced significantly more IL-17 as compared with cells isolated from male livers (220.8 pg/ml by female liver lymphocytes versus 57.5 pg/ml by male liver lymphocytes; p < 0.017). In accordance with the enhanced ex vivo IL-17 production by female liver-infiltrating lymphocytes, we detected an increased frequency of IL-17–expressing CD4+ T cells and higher amounts of RORc mRNA in female livers, as compared with male livers (Fig. 3D). The RORc gene encodes the lineage-specific master transcription factor that regulates the differentiation and function of Th17 cells.

**FIGURE 2.** The intrahepatic frequency of transferred CD8+ T cells and regulatory T cells is not different between male and female mice. (A) OT-1 CD8+CD45.1+ T cells (4 x 10^6) were injected into female and male K14-OVAp mice. On day 8 after injection, mice were sacrificed and the percentage of CD8+CD45.1+ T cells within the liver was determined with flow cytometry (mean ± SD; n = 4). (B) The activation status of transferred OT-1 CD8+CD45.1+ T cells in the liver of K14-OVAp female and K14-OVAp male mice on day 8 after transfer was determined by flow cytometry of intracellular IFN-γ expression (mean ± SD; n = 4). (C) On day 8 after OT-1 CD8+ T cell transfer, CD4+CD25+Foxp3+ Treg frequencies in spleen and liver were analyzed with flow cytometry (mean ± SD; n = 8).

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FIGURE 3. Increased recruitment of endogenous CD4+ T cells into and increased expression of the chemokines CXCL-9 and CXCL-10 in the livers of female mice. (A) Total number of CD4+ T cells per gram of liver tissue in female and male recipient mice on day 8 after transfer of $4 \times 10^6$ OT-1 CD8+ T cells (mean ± SD; n = 5; one representative result from four independently performed experiments). (B) Activated Smarta CD4+ T liver cells and CD4+ T cells isolated from K14-OVAp mice livers after cholangitis induction were adoptively transferred into wild-type mice. The recruitment of transferred cells was analyzed 12 h after transfer (mean ± SD; n = 4). (C) ALT serum levels in female K14-OVAp mice after transfer of $2 \times 10^6$ CD4+ T cells isolated from K14-OVAp mice after cholangitis induction (mean ± SD; n = 3–4). (D) Liver NPCs and spleen cells were isolated from female and male mice on day 8 after transfer of $4 \times 10^6$ OT-1 CD8+ T cells and were restimulated for 24 h with anti-CD3 and anti-CD28. IL-17 levels were (Figure legend continues)
The role of endogenously produced IL-17 as a modulator of cholangitis in our model was confirmed by cell transfer experiments into K14-OVAp female recipient mice that lack endogenous IL-17A and F. OT-1 CD8 T cell transfer into female K14-OVAp IL-17a/f knockout mice led to significantly decreased ALT levels as compared with female K14-OVAp wild-type mice. Histologically, the severity of cholangitis in K14-OVAp IL-17a/f knockout mice was significantly decreased compared with female K14-OVAp wild-type mice (Fig. 3E).

Increased expression of the chemokines CXCL-9 and CXCL-10 in female livers

Because we observed sex-dependent differences in the recruitment of endogenous CD4+ T cells expressing IL-17 into the liver, we next investigated whether the expression of lymphotropic chemokines differed between female and male livers. The recruitment of effector T cell subsets into the inflamed liver is regulated by secretion of chemokine ligands (27, 28). The proinflammatory chemokines CXCL-9-11 are involved in the recruitment of CXCR3+, CD4+, and CD8+ T cells and are known to be expressed by hepatocytes, cholangiocytes, and stellate cells (29, 30). Furthermore, IL-17 has been demonstrated to increase the expression of chemokines, such as CXCL-9, CXCL-11, and CCL-20, by biliary epithelial cells (29–31). Therefore, it was possible that the increased IL-17 expression by female lymphocytes could feed chemokine secretion by liver cells, which in turn could lead to the recruitment of lymphocytes into portal tracts (31). In female livers, the expression of CXCL-9 and CXCL-10 was found to be significantly increased as compared with male livers. Differences in the hepatic expression of CCL-20 were less pronounced (Fig. 3F). These results suggested that increased expression of specific chemokines in female livers might stimulate the recruitment of endogenous proinflammatory lymphocytes to the site of biliary inflammation.

Testosterone suppresses cholangitis and T cell recruitment into the liver

Sexual dimorphisms are to a large extent dependent on sex hormones. We therefore investigated whether the sexual differences observed in our cholangitis model depended on the activities of estrogen or testosterone. To that end, the susceptibility for cholangitis was analyzed in castrated male mice without estrogen supplementation. We observed that castration alone increased cholangitis severity to measured in the supernatants by ELISA (mean ± SD; n = 4; four independently performed experiments). Percentage of IL-17–producing CD4+ T cells were detected by flow cytometric analysis (mean ± SD; n = 4). Relative mRNA expression of RORc in liver tissue from male and female recipient mice on day 8 after cell transfer of 4 × 10⁶ OT-1 CD8+ T cells (mean ± SD; n = 4; four independently performed experiments). (E) ALT serum levels and grading of inflammatory activity in K14-OVAp × IL17A+F knockout and K14-OVAp female recipient mice after transfer of OT-1 CD8+ T cells on day 8 after transfer (mean ± SD; n = 8–12; performed twice independently). (F) Relative mRNA expression of chemokine ligands in liver tissue from female and male mice on day 8 after transfer of 4 × 10⁶ OT-1 CD8+ T cells (mean ± SD; n = 5; four independently performed experiments). *p < 0.05, **p < 0.01, ***p < 0.001.
was performed twice independently. Pretreatment of castrated male mice with estrogen could not further enhance the effect observed with castration alone (data not shown). These observations suggested that the higher androgen levels in male mice protect against disease development. To investigate the potential suppressive effect of androgens on portal inflammation, female mice were pretreated with testosterone pellets before the transfer of OT-1 T cells. Testosterone significantly suppressed inflammation in female mice, marked by decreased histologic scores (Fig. 4B, 4C) and decreased numbers of CD4+ liver-infiltrating T cells (Fig. 4D), as compared with female mice without testosterone treatment. Indeed, the portal inflammation of testosterone treated females was as low as that of male recipient mice.

**Testosterone suppresses the hepatic expression of CXCL-9 and CXCL-10 and the expression of IL-17 in liver infiltrating lymphocytes**

Because we hypothesized that chemokine expression is key to the sexual dimorphism of this model, we next investigated whether testosterone pretreatment could modulate the hepatic expression of the proinflammatory chemokine ligands, which we have found to be increased in female cholangitis. Indeed, testosterone pretreatment of female mice significantly decreased the hepatic mRNA expression of CXCL-9 and CXCL-10 (Fig. 5A, 5B) to mRNA levels similar to those observed in male livers.

Accordingly, a significant decrease in IL-17 levels and RORc mRNA expression (Fig. 5C, 5D) was observed in female mice after testosterone treatment. Our results suggested that hepatic expression levels of CXCL-9 and CXCL-10 and the production of IL-17 by liver lymphocytes were controlled by testosterone. To support this notion further, we analyzed whether serum testosterone levels in untreated wild-type animals correlated with IL-17 production of liver lymphocytes and hepatic chemokine expression. Indeed, liver lymphocytes isolated from untreated female mice produced significantly more IL-17 in response to anti-CD3/anti-CD28 stimulation than lymphocytes from untreated male mice did (Fig. 6A). Moreover, a significant negative correlation was observed between serum testosterone levels and the expression of IL-17 by liver-derived lymphocytes ($r = -0.6697; p = 0.0003$), as well as the relative hepatic expression of CXCL-9 and CXCL-10 (Fig. 6B–D). To investigate whether testosterone could act on CD4+ T cells directly, we isolated CD4+ T cells from male and female mice and analyzed them for androgen receptor expression. We found that male and female CD4+ T cells similarly express the testosterone receptor (Supplemental Fig. 3). Furthermore, we performed in vitro TH17 differentiation assays in the presence or absence of testosterone. We found that the addition of testosterone enriched serum, which was derived from mice supplemented with testosterone pellets, decreased TH17 differentiation compared with estrogen-enriched serum and FCS serum control (Fig. 6E). Taken together, these results identified testosterone as a novel and important modulator of autoimmune liver inflammation in experimental cholangitis.

**Discussion**

Sexual differences in autoimmune diseases are poorly understood. We describe an Ag-driven inducible model of cholangitis that features a similar female predominance as observed in human autoimmune liver diseases (2).

Because of the lack of a cholangiocyte-specific promoter, we chose a previously developed model that relies on the expression of an OVA peptide driven by the human K14 promoter, to target the immune response against cholangiocytes. The keratin-14 promoter is expressed in several tissues, including liver, skin, and thymus (14). However, we could detect only mild inflammation in extrahepatic organs (e.g., skin and esophagus) without differences between male and female mice after transfer of Ag-specific CD8+ T cells. Thus, the sexual dimorphism described in this study was specific to the liver.

We aimed to investigate the mechanisms behind the observed sexual differences in liver inflammation. Differences in Treg numbers have recently been described to account for the sex bias observed in a murine model of AIH (22, 32). In our model, Treg numbers were similar between female and male livers. The different modes of disease induction (i.e., hepatitis versus cholangitis) may in part explain these findings. Our data suggest that Ag-specific

**FIGURE 5.** Testosterone suppresses the hepatic expression of CXCL-9 and CXCL-10 and the expression of IL-17 in liver infiltrating lymphocytes. Relative hepatic mRNA expression of (A) CXCL-9 and (B) CXCL-10 from male and female K14-OVAp recipient mice with and without prior testosterone treatment 8 d after transfer of $4 \times 10^6$ OT-1 CD8+ T cells (mean ± SD; $n = 4$). (C) IL-17 levels in supernatants of liver NPCs isolated from testosterone treated K14-OVAp female mice in comparison with controls on day 8 after cell transfer. Cells were restimulated with anti-CD3 and anti-CD28 (2 μg/ml; mean ± SD; $n = 4$). (D) Relative mRNA expression of RORc in liver tissue from testosterone-pretreated females compared with male and female recipient mice without hormone treatment on day 8 after cell transfer (mean ± SD; $n = 4$). Each experiment was performed twice independently. $^*p < 0.05$, $^*^*p < 0.01$.
CD8+ T cells initiate cholangiocyte activation and subsequent recruitment of endogenous CD4+ T cells, which exert their proinflammatory activities at least in part by the production of IL-17. This activity was suppressed by testosterone in a sex-dependent fashion. These findings are in accordance with earlier findings that TH17 cells may play a crucial role in human PBC, PSC, and other inflammatory liver diseases (26, 33), as well as in a murine mouse model of PBC (13). Recently, it has been shown that in the inflamed human liver, IL-17 stimulation of BEC induces the expression of the chemokine ligands CXCL-9-11 and CCL-20 (31). Indeed, we observed a significantly higher expression of the proinflammatory chemokines CXCL-9 and CXCL-10 in the livers of female recipient mice after OVA-specific CD8+ T cell transfer. Thus, enhanced CD4+ T cell recruitment into female livers with CD8+ T cell–induced cholangitis can be driven by a positive feedback loop involving the IL-17–induced upregulation of chemokines by BECs (31). In the original publication of the K14-OVAp model using OT-1/K14-OVAp double-transgenic mice (14), serum levels of TNF-α and IL-1β were found to be increased. In our transfer model, these cytokines were not increased in male mice after castration (data not shown), which rendered the mice susceptible to cholangitis induction; therefore, our subsequent studies focused on IL-17. However, an additional effect of these cytokines on liver inflammation cannot be excluded.

In castration and hormone supplementation experiments, we could clearly show that the sexual differences observed in our cholangitis model depended on the activities of testosterone. It has previously been described that female sex hormones, such as estrogen, drive autoimmune diseases rather than androgens being suppressive (34). In the model described in this study, testosterone supplementation clearly induced resistance to disease in female mice, and castration alone increased susceptibility of male mice to levels observed in female mice (Fig. 4A, 4B). In support of our findings, a protective effect of testosterone has been previously described in EAE, which is also driven by IL-17. In that model, castration of male mice worsened EAE (35), whereas ovariectomy did not have a major effect on disease (36, 37). In human multiple sclerosis, it has been shown that 24% of affected men present with reduced serum testosterone levels (38). In addition, it was demonstrated in a recent publication that testosterone activity was essential for the protection from islet inflammation in the nonobese diabetic mouse model (39). To our knowledge, testosterone levels in male patients with autoimmune liver diseases have not been studied. Whereas protection from EAE may be mediated by the cytokines IL-10 and IL-4 (36, 37), there were no differences in the expression of these cytokines in our model (data not shown). In our study, the protective effects of testosterone seemed to be mediated by downregulation of the inflammatory TH17 response; females rendered resistant by testosterone supplementation exhibited decreased IL-17 production of liver-infiltrating lymphocytes (Fig. 5B). Androgen receptors have been identified in CD4+ T cells initiate cholangiocyte activation and subsequent recruitment of endogenous CD4+ T cells, which exert their proinflammatory activities at least in part by the production of IL-17. This activity was suppressed by testosterone in a sex-dependent fashion. These findings are in accordance with earlier findings that TH17 cells may play a crucial role in human PBC, PSC, and other inflammatory liver diseases (26, 33), as well as in a murine mouse model of PBC (13). Recently, it has been shown that in the inflamed human liver, IL-17 stimulation of BEC induces the expression of the chemokine ligands CXCL-9-11 and CCL-20 (31). Indeed, we observed a significantly higher expression of the proinflammatory chemokines CXCL-9 and CXCL-10 in the livers of female recipient mice after OVA-specific CD8+ T cell transfer. Thus, enhanced CD4+ T cell recruitment into female livers with CD8+ T cell–induced cholangitis can be driven by a positive feedback loop involving the IL-17–induced upregulation of chemokines by BECs (31). In the original publication of the K14-OVAp model using OT-1/K14-OVAp double-transgenic mice (14), serum levels of TNF-α and IL-1β were found to be increased. In our transfer model, these cytokines were not increased in male mice after castration (data not shown), which rendered the mice susceptible to cholangitis induction; therefore, our subsequent studies focused on IL-17. However, an additional effect of these cytokines on liver inflammation cannot be excluded.

In castration and hormone supplementation experiments, we could clearly show that the sexual differences observed in our
T cells (40), and androgens have been shown to suppress IL-17 expression in T cells, as demonstrated by an increased expression of IL-17 in male T cells after castration (40). Accordingly, we found that testosterone acts directly on CD4+ T cells by suppressing TH17 differentiation in vitro (Fig. 6). However, the IL-17 response of T cells could also depend on their anatomical location, as female CD4+ T cells isolated from lymph nodes expressed more IL-17 than male cells did after in vitro restimulation, whereas female CD4+ T cells isolated from spleen expressed less IL-17 than male cells did (17). The role of androgens in regulating the IL-17 response in the liver has not been evaluated.

Of interest, the nuclear receptor peroxisome proliferator activated receptor (PPARα) has been identified as an androgen-sensitive suppressor of NF-κB activation (17). PPARα expression was higher in male than in female T cells, and lack of PPARα involvement in sexual differences in human autoimmune liver diseases was suggested. PPARα has been shown to play a role in the suppression of immune cholangitis that, in contrast to previously described cholangitis models, showed a female preponderance similar to human autoimmune liver diseases. We could identify an important immuno-suppressive effect of testosterone on Ag-induced cholangitis.

In conclusion, we describe an inducible mouse model of autoimmune cholangitis that, in contrast to previously described cholangitis models, showed a female preponderance similar to human autoimmune liver diseases. We could identify an important immunosuppressive effect of testosterone on Ag-induced cholangitis. These findings should stimulate further research into the mechanisms involved in sexual differences in human autoimmune liver diseases and could lead to novel treatment options for liver inflammation.

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Disclosures

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References

Supplemental Figure 1. Expression of the SIINFEKL peptide by cholangiocytes

A: RT-PCR analyses of the expression of the transgene in liver tissue of male and female mice as well as hormone pretreated animals. B: Immunohistochemical staining specific for the SIINFEKL peptide (purple) of liver sections demonstrated an equal expression in cholangiocytes (* bile duct) of male and female mice with or without hormone pretreatment (DAPI nuclei stain blue). Representative sections are given.
Supplemental Figure 2. The grade of inflammation in skin and esophagus in male and female K14-OVAp mice.

Histological grading of inflammatory activity in organs expressing the K14-promoter was performed. No differences in inflammation of esophagus (left) or skin (right) could be observed between male and female recipient mice on day 8 after OT-1 CD8 T cell transfer.
Supplemental Figure 3. Androgen receptor expression on CD4+ T cells

CD4+ T cells isolated from male and female mice similarly express the testosterone receptor.