Testosterone Increases Susceptibility to Amebic Liver Abscess in Mice and Mediates Inhibition of IFNγ Secretion in Natural Killer T Cells

Hannelore Lotter1*, Elena Helk1, Hannah Bernin1, Thomas Jacobs1, Cornelia Prehn2, Jerzy Adamski2, Nestor González-Roldán3, Otto Holst4, Egbert Tannich1

1 Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, 2 Helmholtz Center Munich, Institute of Experimental Genetics, Genome Analysis Center, Neuherberg, Germany, 3 Division of Immunobiology, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany, 4 Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany

Abstract

Amebic liver abscess (ALA), a parasitic disease due to infection with the protozoan Entamoeba histolytica, occurs age and gender dependent with strong preferences for adult males. Using a mouse model for ALA with a similar male bias for the disease, we have investigated the role of female and male sexual hormones and provide evidence for a strong contribution of testosterone. Removal of testosterone by orchietomy significantly reduced sizes of abscesses in male mice, while substitution of testosterone increased development of ALA in female mice. Activation of natural killer T (NKT) cells, which are known to be important for the control of ALA, is influenced by testosterone. Specifically activated NKT cells isolated from female mice produce more IFNγ compared to NKT cells derived from male mice. High level production of IFNγ in female derived NKT cells was inhibited by testosterone substitution, while the IFNγ production in male derived NKT cells was increased by orchietomy. Gender dependent differences were not a result of differences in the total number of NKT cells, but a result of a higher activation potential for the CD4+ NKT cell subpopulation in female mice. Taken together, we conclude that the hormone status of the host, in particular the testosterone level, determines susceptibility to ALA at least in a mouse model of the disease.

Introduction

Susceptibility to and outcome of infectious diseases may be greatly influenced by the gender. There are various examples, in particular for parasitic diseases, in which male individuals are more frequently infected with the respective pathogen, suffer from higher parasite burden or develop more severe clinical courses in comparison to females [1,2]. An extraordinary example for a male bias towards a parasitic disease is amebic liver abscess (ALA). The disease is endemic in most tropical and subtropical countries and is characterized by massive liver tissue destruction due to infection with the protozoan Entamoeba histolytica. This parasite primarily colonizes the human gut where it can reside and multiply for months or even years without inducing any clinical symptoms. However, in a small proportion of infected individuals the parasite invades the tissue and causes colitis or extraintestinal abscesses, most commonly in the liver. Interestingly, ALA is rare in children and women. The vast majority of more than 80% of all ALA cases occur in adult males [3,4,5]. This phenomenon is independent from ethnic or cultural background as it is found in all parts of the world in which ALA is endemic as well as in travelers from non-endemic countries acquiring the disease during their journey [6,7,8]. In one of the largest studies on the epidemiology of ALA in Central Vietnam, in which more than 2,000 ALA cases were analyzed, it was shown that the risk for ALA increases after puberty with peak incidence in adult males at the age between 30 and 50 years, suggesting that sexual hormones in particular testosterone might have an impact on ALA susceptibility [4,5,9].

The influence of steroid hormones on the outcome of parasitic diseases, either by direct interactions with the parasite or by altering immune functions is well documented [2]. In animal models for leishmaniasis or malaria, testosterone treatment decreases the resistance to parasite infections [10,11] either by suppressing functions of innate immune cells [12] or by promoting anti-inflammatory immune responses [13,14,15,16]. Interestingly, resistance to ALA mainly relies on a pro inflammatory type of immune response primarily based on the production of IFNγ. The importance of IFNγ in the early control of E. histolytica invasion has been documented from various in vitro studies as well as from animal models for experimental ALA [17,18,19,20].

One of the main producer of IFNγ in early response to microbial infections are natural killer T (NKT) cells, which are immune cells, bridging the early innate and the adaptive immune response. NKT cells are activated by a range of microbial-derived lipids and glycolipids that are presented by the major histo-
patibility complex (MHC) class I-like molecule CD1d on antigen presenting cells (APCs) [21,22]. Upon activation, NKT cells exhibit regulatory functions by rapidly producing pro- and anti-inflammatory cytokines and mediate direct cytotoxicity against microbial pathogens [23,24,25,26]. Recently, we have shown that murine NKT cells could be activated to produce protective IFNγ by an E. histolytica lipopetidophosphoglycan (EhLPPG) present on the surface of ameba trophozoites [27]. In a C57BL/6 mouse model for ALA, knockout mice lacking functional NKT cells are impaired in their ability to control ALA [20], whereas activation of NKT cells by EhLPPG or by their most potent activator α-galactosylceramide (αGalCer) limit ALA development [27]. Interestingly, the C57BL/6 ALA mouse model revealed a strong sexual dimorphism in the susceptibility for ALA, mimicking at least in part the human situation. In this mouse model, female mice are able to clear parasites injected into the liver within 3 days, whereas in male mice viable ameba can be isolated up to 14 days. Accordingly, male mice develop larger abscesses and show a prolonged recovery from ALA [20].

In this communication, we report on the use of the C57BL/6 ALA mouse model to analyze the role of sexual hormones for ALA susceptibility and provide evidence that testosterone increases susceptibility for ALA by modulation the secretion of IFNγ by EhLPPG-activated NKT cells.

Materials and Methods

Ethic Statement

Mice were maintained in a specific pathogen-free microenvironment in the animal facility of the Bernhard-Nocht-Institute and received care in compliance with the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). All work was conducted with the approval of the Behörde für Gesundheit und Verbraucherschutz der Stadt Hamburg according to 48 TierSchG (German Protection of Animals Act; # 20/08; # 23/09; # 23/11).

Cultivation of Parasites and Preparation of EhLPPG

Trophozoites of the E. histolytica isolate HM1:IMSS were axenically grown in TY-S-33 medium (Diamond 1978). Parasites from the log growth stage were used for intrahepatic infection of C57BL/6 mice.

EhLPPG was purified from the membranes of E. histolytica trophozoites as described previously [27]. In brief, trophozoites of the late logarithmic phase of growth were washed, resuspended in pyrogen-free water and lysed by freeze and thawing. The homogenate was centrifuged at 430 g at 4°C for 10 min and subsequently the supernatant was recovered and the trophozoite membranes were enriched by ultracentrifugation at 150,000 g for 40 min. The obtained pellet was extracted with a mixture of chloroform/methanol/water 10:10:3 (by volume) and the insoluble material was recovered by centrifugation, dried, resuspended in distilled pyrogen free water and extracted three times with an equal volume of 90% phenol at 68°C for 30 min with constant stirring [28]. The water phase containing EhLPPG was recovered after centrifugation at 12,000 g for 30 min and dialysis against distilled water.

Ovariectomy, Orchietomy and Hormone Treatment

Female and male C57BL/6J mice at an age of 8 weeks were used for the experiments. The following groups of mice were assembled:

- Naive mice, ovariectomized (ovx) mice supplemented with testosterone, ovarioctomized and orchietomized mice treated with placebo, non-ovariectomized mice supplemented with testosterone and non-ovarioctomized mice treated with placebo.

Female mice were ovariectomized at an age of 8 weeks. The mice were prepared for surgery using an anesthetic protocol that enabled the abrogation of the narcosis immediately after finishing the surgical intervention. Initially, a solution consisting of 6.3 µl Domitor (Pfizer, Karlsruhe, Germany), 27 µl Ketanest (Inresa, Freiburg, Germany) and 66 µl phosphate buffered saline (PBS) was injected intraperitonially. The tolerance time using this method was approximately 60 min. The intra muscular injection of 5 µl Antisedan (Pfizer)/45 µl phosphate buffered saline/animal abrogated the narcosis within the next 10 min.

After skin desinfection, a dorsal midline skin incision was made caudal to the border of the ribs. Lateral to the incision, the muscle layer of the posterior abdominal wall and the peritoneum were separated to enter the abdominal cavity. The periovarian fat tissue was grasped of to lift and exteriorize the ovary. The cranial fixation of the ovary and the arteria ovarica, as well as the fallopian tube were carefully heat coagulated and the ovary was removed. The blunt end of the uterine horn was slowly relocated in the abdominal cavern. When no further bleedings were

![Figure 1. Influence of gonadectomy on the size of ALA in the mouse model for the disease.](image-url)
observed, the peritoneum and the muscle layers were sutured and the skin was closed using skin clamps. The process was then repeated to remove the second ovary.

Surgical orchietomy was performed via a midline scrotal incision allowing bilateral access to the hemiscrotal contents. After exposing each testicle, a 3-0 vicryl suture was used to ligate the spermatic cord and then remove the testicle.

For hormone treatment, female mice were implanted subcutaneously with pellets releasing testosterone designed to yield blood levels of 6–9 ng/ml (12.5 mg/pellet/60 day release, Innovative Research of America, Sarasota, FL, USA). Control mice were implanted with Placebo pellets for testosterone (12.5 mg/pellet/60 day release, Innovative Research of America, Sarasota, FL, USA).

**Induction of Amebic Liver Abscess**

The mice were infected by direct hepatic inoculation of virulent trophozoites as previously reported [20]. Following anesthesia and disinfection of the surgery area, the skin, the muscle and the peritoneal layer were incized to enter the abdominal cavern. 1x10^5 axenically cultured trophozoites in a volume of 25 μl TY medium were injected in the visualized left liver lobe using a U-100 insulin syringe (BD, Heidelberg, Germany). Peritoneum and muscle layer were sutured and wound clips closed the skin. To maintain virulence, trophozoites were regularly passaged through the liver of male C57BL/6 mice. On day 7 post infection, the mice were sacrificed, the abscess size was calculated by measuring the size in mm and the introduction of score values (score 0: no abscess; score 1: abscess <1 mm; score 2: >1–<5 mm; score 3: >5 mm) and the abscess material was maintained in TY medium at 37°C to provide optimal conditions for the re-isolation of amebic trophozoites.

**Determination of Testosterone Concentrations in Serum Samples**

Since no steroid ELISA kits are available for mouse samples, a human ELISA kit has been adopted. Prior to the measurement, steroids were extracted from the matrix by liquid/liquid-extraction to avoid mouse plasma matrix effects. The plasma was extracted three times in each case with a tenfold excess of tetra-butylmethylether (TBME). After evaporation of the combined organic extracts the dried residues can be stored at −20°C. For the subsequent ELISA, the material is reconstituted in steroid free serum. The serum testosterone concentration was quantified using a competitive ELISA according to the manufacturer’s protocols (testosterone EIA-1559; DRG Instruments GmbH, Germany). The plates were read in a standard microplate reader at 450 nm (Tecan, Safire 2). The concentrations were calculated upon the calibration from the standard curve and reported in ng/ml. The sensitivity of the testosterone assay is 0.083 ng/ml.

**Isolation of Murine Liver Lymphocytes and FACS Sorting of NKT Cells**

Livers were perfused with ice cold PBS/20% FCS solution and subsequently filtered through a 40 μm mesh. Following centrifugation at 400 g, cell pellets were resuspended in RPMI medium, underlayered with a 30% Nycoprec solution (Nycoprep, Universal) and centrifuged at 900 g for 20 min. The liver lymphocytes were collected from the interface, treated with hypotonic ammonium chloride solution, washed and diluted in RPMI medium.

Gradient purified liver lymphocytes were further characterized by staining with APC-labelled anti-CD3, PE-labelled CD11b and FITC-labelled anti-GR1 in phosphate buffered saline containing 1% bovine serum albumin. The cells were subsequently subjected to flow cytometry on a FACS Calibur (BD Bioscience, Heidelberg, Germany) and the data were analyzed using the CellQuestPro software (BD Bioscience).

NKT-cells were stained for cell sorting using αGalCer (Alexis Biochemicals, Lausen, CH) loaded to PE labelled recombinant CD1d-tetramer (Proimmune, Oxford, UK) and FITC-labelled anti-CD4 (BD Bioscience, Heidelberg, Germany). Cell sorting was performed on a FACS Aria III (BD).

**In vitro Stimulation of Murine NKT Cells**

Generation of APCs. Bone marrow was harvested from femurs of male and female, 6- to 10-weeks-old C57BL/6 mice and cultured as described by Lutz et al. [29]. Cultures were supplemented with supernatants from Ag8653 myeloma cells transfected with the gene coding for murine GM-CSF [30]. The maturity of the bone marrow derived dendritic cells (BMDCs) was determined by FACS analysis using APC-labelled anti- CD11c, FITC-labelled anti - CD40-FITC and PE-labelled anti - CD86 and anti - CD80 (BD Bioscience). The ratio of immature to mature BMDCs was 80% vs 20%.

**In vitro stimulation of liver lymphocytes from testosterone treated female mice and orchietomized male mice.** In brief, gradient purified liver lymphocytes from hormone-modified mice were subjected to the PAN T cell isolation kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers instructions. Purified T cells (1x10^6) were added to gender matched APCs (5x10^4) previously pulsed with 4 μg/ml αGalCer and 8 μg/ml EhLPPG for 3 h. The cells were cultured in triplicates in 96-well round bottom plates with RPMI-20 medium supplemented with 200 munits L-glutamine, 50 μg gentamicin, 10% fetal cell serum, 1 mM sodium pyruvate and, 50 μM 2-mercaptoethanol. Cell supernatants were collected after 48 h of coincubation and assayed for IFNγ by enzyme-linked immunosorbent assay (ELISA) using antibody pairs purchased from R&D Systems (Abington, UK).

In vitro stimulation of purified murine NKT cells. Gradient purified liver lymphocytes from female and male C57BL/6 mice (10–12 weeks old) were stained with PE-labelled αGalCer -CD1d-tetramer, sorted by fluorescence activated cell sorting (BD-FACS-Aria) and added in a cell density of 5x10^4 to 1x10^5 gender matched APCs prepsulated for 3 h with either αGalCer (4 μg/ml) or purified amebic EhLPPG (8 μg/ml). The cell supernatant was assayed for IFNγ as described above.

In vitro stimulation assay using αNKT cell subpopulations. Gradient purified liver lymphocytes from female and male C57BL/6 mice (10–12 weeks old) were stained with PE-labelled-

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Figure 2. Influence of testosterone on the development of ALA. A) Time schedule of gonadectomy, testosterone substitution and intrahepatic infection with *E. histolytica* trophozoites. B) Serum testosterone levels in placebo treated naive female mice, testosterone substituted female mice and male mice measured by ELISA (ng/ml). C) Size of ALA in testosterone treated, ovariectomized (ovx) or naive female mice. Score values indicative for the size of ALA determined on day 7 post intrahepatic infection with 1x10^5 virulent *E. histolytica* trophozoites of naive female mice treated either with testosterone or placebo are shown (score 0: no abscess; score 1: abscess <1 mm; score 2: >1–<5 mm; score 3: >5 mm). D) Re-isolation rate of *E. histolytica* trophozoites from abscessed liver tissue of ovariectomized (ovx) and naive female mice treated either with testosterone or placebo. Results were obtained from at least 3 independent trials each comprising of 7 animals (statistics: Mann Whitney U test). doi:10.1371/journal.pone.0055694.g002
Testosterone Influences Amebic Liver Abscess

A

unstained

anti-CD3 single stained

double stained

CD3

CD3

CD3

13.9 %

B

CD1d:GalCer+ or CD3+

female

male

6-8 weeks

9-11 weeks

16-18 weeks

C

IFNγ (pg/ml)

female

male

αGalCer

medium

mAPC / mNK7

mAPC / fNK7

fAPC / fNK7

fAPC / mNK7

ns

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***

***

ns

IFNγ (pg/ml)

αGalCer

medium

mAPC / mNK7

mAPC / fNK7

fAPC / fNK7

fAPC / mNK7

ns

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ns

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αGalCer -CD1d tetramer (Proimmune) and FITC-labelled anti-CD4. Tetramer positive, CD4⁺ or tetramer positive, CD4⁻ NKT cells were sorted by fluorescence activated cell sorting (BD-FACS-Aria) and added at a cell density of 3×10⁴ to 1×10⁴ gender matched APCs previously pulsed with αGalCer or EhLPPG as described above. The amount of IFNγ was measured by ELISA in the cell supernatant after 48 h of co-cultivation.

### Statistical Analysis

The Mann Whitney U test was applied to compare the liver abscess sizes between male and female mice over the time period monitored and the Paired t-test was used to determine the difference between abscesses that were culture positive for *E. histolytica*. The student`s t test was applied for the statistical analysis of the IFNγ values obtained by the NKT cell activation assay.

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**Figure 3. Characterization of NKT cells frequencies and NKT cell specific IFNγ production in female and male mice.**

A) NKT cell frequencies in the liver of female and male mice at different ages are shown. NKT cell numbers were determined as percentage of αGalCer -CD1d tetramer positive cells to CD3⁺ T cells. B) IFNγ production of NKT cells sorted (αGalCer -CD1d tetramer) from the liver of female or male mice after 48 h of co-incubation with αGalCer (4 μg/ml) or EhLPPG (8 μg/ml) pre-pulsed APCs. Medium control includes NKT cells co-cultured with naive APCs. IFNγ production was quantified by ELISA (5 independent experiments were performed, a summary of data is expressed as mean +/- SD, statistics: student t test). C) IFNγ production of male or female NKT cells upon stimulation with αGalCer pre-pulsed male or female APCs.

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**Figure 4. Characterization of the IFNγ producing gender specific murine NKT cell subpopulation.** NKT cell subsets (PE- αGalCer -CD1d tetramer/CD4⁺) from female and male mice. IFNγ was determined following 48 h of co-cultivation with pre-pulsed APCs as described above (2 independent experiments were performed, a summary of data is expressed as mean +/- SD, statistics: student t test).

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Results

Gonadectomy Decreases Susceptibility for ALA in Male Mice

The C57BL/6 mouse model for ALA is characterized by the development of significant larger abscesses in male mice within 7 days following intrahepatic ameba challenge, whereas female mice control the infection and develop only small lesions. To investigate whether sexual hormones might have an influence on ALA development, gonadectomy was performed in groups of 8 weeks old male and female mice. Seven weeks following castration, animals were challenged by intrahepatic application of cultured E. histolytica trophozoites. Development of ALA was assessed on day 7 after infection (Fig. 1). The results clearly indicate significant reductions in the sizes of ALA (p<0.009) and parasite survival rates (p<0.02) in orchiectomized male mice compared to respective male controls. The sizes of ALA in orchiectomized mice were similar to those found in female mice. In contrast, ovariectomy did not influence sizes of ALA or parasite survival rates in female mice suggesting that testosterone rather than estrogens influences susceptibility to ALA.

Substitution of Testosterone Increases Susceptibility for ALA in Female Mice

To further determine the importance of testosterone for ALA development, testosterone substituted female mice were investigated. Substitution was performed by subcutaneous implantation of small pellets releasing either testosterone or placebo. Pellets were implanted into 10 weeks old C57BL/6 female mice or into female mice following ovariectomy (Fig 2A). Seven weeks later, prior to intrahepatic ameba challenge, serum testosterone concentrations were determined indicating similar levels between respective female controls. The sizes of ALA in orchiectomized female mice were similar to those found in female mice. In contrast, ovariectomy did not influence sizes of ALA or parasite survival rates in female mice suggesting that testosterone rather than estrogens influences susceptibility to ALA.
male and testosterone-substituted female mice (Fig. 2B). Intrahepatic infection of the various groups of testosterone and placebo substituted animals revealed significant increases of abscess sizes in testosterone-substituted animals compared to sham-substituted controls \( (p<0.05) \), regardless whether mice were ovariectomized or not (Fig. 2C). Moreover, 90% of abscesses of testosterone treated mice contained viable \( E. histolytica \) trophozoites, compared to only 30% of ovariectomized and placebo treated female mice and 10% of uncastrated and placebo treated female mice \( (p<0.02, p<0.0002) \) (Fig. 2D). In contrast, parallel experiments of estradiol substitution in naive or ovariectomized male mice had no effect on abscess sizes and parasite survival rates compared to respective placebo controls (data not shown).

**Gender Differences in IFN\( \gamma \) Production of \( \alpha \)GalCer or EhLPPG Activated Murine NKT Cells**

We have recently shown that NKT cells are critically important for the control of ALA and that an \( E. histolytica \) lipoproteinphosphoglycan (EhLPPG), similar to \( \alpha \)GalCer, specifically activates liver or spleen murine NKT cells to secrete significant amounts of protective IFN\( \gamma \) [27]. To investigate whether NKT cells are responsible for gender-related differences, the frequencies of \( \alpha \)GalCer -CD1d tetramer/CD3+ positive cells in the liver of female and male mice at different ages were determined (Fig. 3A). The results indicated lower numbers of NKT cells in young mice (6–8 weeks) compared to older animals (9–11 or 16–18 weeks) but there were no statistically significant differences between the genders. The activation of isolated NKT cells by \( \alpha \)GalCer or EhLPPG pre-pulsed APCs generated from untreated animals induced significant higher amounts of IFN\( \gamma \) in female compared to male derived cells (\( \alpha \)GalCer, \( p<0.003; \) EhLPPG, \( p<0.0001 \)) (Fig. 3B). To further determine whether this gender specific IFN\( \gamma \) secretion is indeed due to NKT cells and not a result of a gender specific presentation by the corresponding APCs, activation of male or female NKT cells by \( \alpha \)GalCer or EhLPPG-pulsed APCs (Fig. 3C). The results clearly indicate that higher secretion of IFN\( \gamma \) in female mice is primarily a function of NKT cells, as it was independent from the gender of the APCs.

**Mouse CD4+ NKT Cells Rather than CD4+ NKT Cells are Responsible for Gender Specific Differences in IFN\( \gamma \) Production upon Activation**

To further determine the NKT cell subpopulation that might be responsible for the observed gender specific differences, isolated NKT cells were further subdivided by the presence or absence of the surface marker CD4. NKT cell stimulation assays with \( \alpha \)GalCer or EhLPPG indicate that both CD4+ and CD4− NKT cells secrete IFN\( \gamma \). However, whereas we found no gender specific differences in the number of CD4− NKT cells (data not shown), CD4− NKT cells from female mice produced significant higher amounts of IFN\( \gamma \) (Fig. 4).

**Testosterone Reduces IFN\( \gamma \) Production of Activated Murine NKT Cells**

To determine whether gender specific differences in IFN\( \gamma \) secretion of NKT cells following stimulation with EhLPPG is influenced by testosterone, purified liver lymphocytes from female and testosterone substituted female mice as well as from male and ovariectomized male mice were prepared. NKT cells present in these preparations were specifically activated upon co-culture with \( \alpha \)GalCer - or EhLPPG-pulsed APCs (Fig. 5). Experiments were performed with cells from naive (Fig. 5A u. B) as well as from ameba infected mice (Fig. 5C u. D). Analysis of IFN\( \gamma \) in supernatants of induced cells clearly indicated a testosterone dependent inhibitory effect on IFN\( \gamma \) secretion by NKT cells as \( \alpha \)GalCer - or EhLPPG-induced IFN\( \gamma \) production was significant lower in liver derived NKT cells from testosterone treated animals compared to respective female controls (\( \alpha \)GalCer, \( p_{\text{naive}}<0.05, p_{\text{treated}}<0.005 \), EhLPPG, \( p_{\text{naive}}<0.02, p_{\text{treated}}<0.02 \)) (Fig. 5A u. C). In addition, deprivation of testosterone in male mice by orchietomy significantly increased \( \alpha \)GalCer - or EhLPPG-stimulated IFN\( \gamma \) production (EhLPPG, \( p_{\text{naive}}<0.005, p_{\text{treated}}<0.05 \)) (Fig. 5B u. D).

**Discussion**

To further elucidate the mechanisms responsible for the higher susceptibility of males to develop ALA we have used a mouse model for the disease to investigate the role of sexual hormones and in particular the contribution of testosterone and of NKT cells. The mouse model used in our study is considered suitable for the analysis of ALA associated immune reactions and gender related functions as the histopathological cellular infiltrates of parasite infected livers and the strong gender bias towards males indicates clear similarities to findings in human ALA patients [20].

Because of the complex and strong host specificity of human parasites, rodent models that enable studies on the influence of hormones on infectious diseases are rare and often do not reflect the same gender bias that occurs in humans [31]. However, in rodent models for leishmaniasis, a parasitic disease with a male preference in humans [1,15], male mice bear higher parasite burden in the liver compared to female mice [15]. In addition, androgen deprivation by orchietomy decreases the number of leishmania amastigotes in the liver while testosterone treatment leads to the opposite effect [10]. Although the administration of testosterone substitution varies widely, comparable results are reported from other mouse models of infectious diseases such as malaria [11,32,33] [34], strongyloidiasis [35], schistosomiasis [36], trypanosomiasis [37] or tuberculosis [38].

There are numerous examples of infectious diseases with predispositions in males. In general, this phenomenon is attributed to a reduced ability of male individuals to generate an effective humoral or cellular immune response [1,2,39,40]. However, the extent of gender differences in most infectious diseases is usually low. In the case of ALA, gender differences are substantial with a male to female ratio of up to 7:1 in adult individuals depending on age [4]. Previous findings that the risk for ALA (i) is low and gender independent in children, (ii) increases after puberty in males, and (iii) shows peak incidence in middle-age men suggests that sexual hormones, in particular testosterone, might play a role in the susceptibility to ALA. The results presented here, strongly support this assumption. Gonadectomy selectively reduced the sizes of abscesses in male mice but did not alter susceptibility to ALA in female mice. On the other hand, substitution of testosterone in both ovariectomized female mice and untreated female mice significantly increased susceptibility to ALA as reflected by increases in abscess sizes and parasite survival rates in infected livers. In contrast, substitution of estradiol in male mice had no effect on ALA formation.

Previous studies have shown that NKT cells and IFN\( \gamma \) are critically important for the control of ALA in a mouse model for the disease and that NKT cells can be specifically activated not only by \( \alpha \)GalCer but similarly by EhLPPG to produce protective IFN\( \gamma \) [20,27]. Accordingly, to assess possible gender dependent immune functions underlying the testosterone dependent decrease in the control of ALA we have focused on the activation of murine.
NKT cells and their production of IFN\(\gamma\). Our results clearly indicate increased IFN\(\gamma\) production by \(\alpha\)GalCer or EhlPPG stimulated NKT cells from female mice compared to male mice, which is in line with earlier studies, indicating higher amounts of IFN\(\gamma\) in the serum of female mice after administration of \(\alpha\)GalCer [41]. Differences in the amount of IFN\(\gamma\) production between male and female mice are obviously not the result of differences in the numbers of NKT cells, as in our in vivo experiments identical numbers of isolated NKT cells were used. Furthermore the quantification of liver-derived NKT cells from mice at various ages did not reveal significant differences between the genders.

Interestingly, cytokine production of murine NKT cells can be modulated by testosterone. Liver lymphocytes isolated from testosterone substituted female mice produced significantly less IFN\(\gamma\) upon stimulation with \(\alpha\)GalCer or EhlPPG compared to female mice, while orchectomy increased NKT cell dependent IFN\(\gamma\) secretion in male mice. These results were independent whether cells were isolated from naive or from ameba infected mice. Testosterone, or steroid hormones in general, have been shown to activate lymphocyte immune functions either directly or via the modulation of macrophages [2,42,43,44,45]. In the mouse model, however, it is likely that testosterone affects NKT cells directly rather then via modulation of APC functions since the results were independent whether the APCs used for stimulation were prepared from gender matched or unmatched control mice. Our results indicate that the increased IFN\(\gamma\) production in response to stimulation with EhlPPG in female mice is not due to an expansion of NKT cells but primarily due to IFN\(\gamma\) secretion by CD4\(-\)NKT cells, which are also present in male mice but without producing significant amounts of IFN\(\gamma\). However, it remains to be determined whether NKT cells possess plasma membrane, intracellular or nuclear androgen receptors as conventional T lymphocytes do [46,47,48]. Moreover, studies are required to establish the importance of our findings in mice for the observed gender differences in human amoebiosis. It has to be considered that compared to humans, mice contain substantially more NKT cells, in particular within the liver [49] and that there are differences in NKT cell subsets between mice and men. Previous studies on the frequency of peripheral blood NKT cells in healthy blood donors [50,51,52] or cancer patients [53] indicated gender-related differences with higher NKT cell numbers in females. On the contrary, in one recent study this finding could not be confirmed [54]. Human NKT cell subsets consist of CD4\(^+\), CD4\(^-\)CD8\(^+\)(DN) and CD8\(^+\) NKT cells. In contrast, mice lack CD8 as a marker for NKT cells. Thus, murine NKT cells are subdivided by the presence or absence of the CD4 marker only [23,55]. In agreement with our observations in mice, the CD4\(^-\)CD8\(^-\)NKT cell subset in humans exhibits a Th1-biased cytokine profile (IFN\(\gamma\), MIP-1\(\alpha\) and TNF\(\alpha\)) [55,56,57] while the CD4\(^+\)CD8\(^-\) NKT cell subset in human is supposed to induce a Th2-type immune response similar to the CD4\(^+\)NKT cell subset in mice [55,58]. Although gender differences in the circulating human NKT cell subsets have not been observed, a comparison of the functional NKT cell profiles between males and females revealed higher frequencies of cells producing IFN\(\gamma\) and MIP1-\(\alpha\) in males but similar frequencies of cells in both genders producing IL-4 [54]. However, although gender distribution and functional activity of NKT cells might be similar among mice and human, differences in frequencies and organ distribution occur. In general, NKT cell numbers in humans are lower compared to mice. NKT cell frequencies among peripheral blood lymphocytes range between 0.01–1.0% in humans and between 0.2–1.0% in mice. The differences are more pronounced in the liver, where human NKT cells represent not more than 1% of the lymphocyte population while in mice 20–40% of liver lymphocytes are NKT cells [49,59].

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**Author Contributions**

Conceived and designed the experiments: HL ET TJ. Performed the experiments: HL EH HB NGR CP TJ. Analyzed the data: HL TJ EH. Contributed reagents/materials/analysis tools: JA OH. Wrote the paper: HL ET.

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