Indoleamine 2,3-Dioxygenase 1 (Ido1) Is Involved in the Control of Mouse Caput Epididymis Immune Environment

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Abstract
The epididymis maintains a state of immune tolerance towards spermatozoa while also protecting them and itself against infection and acute inflammation. The immunosuppressive enzyme indoleamine 2,3-dioxygenase 1 (Ido1) participates in this delicate local equilibrium. Using the mouse Ido1−/− model, we show here that the absence of Ido1 expression leads in the epididymis but not in serum to (1) an increase in the inflammatory state as evidenced by changes in the content of cytokines and chemokines, (2) the engagement of a Th1-driven inflammatory response as evidenced by changes in the Th17/Treg as well as Th1/Th2 equilibria, as well as (3) differences in the content of lipid intermediates classically involved in inflammation. Despite this more pronounced inflammatory state, Ido1−/− animals succeed in preserving the local epididymal immune situation due to the activation of compensatory mechanisms that are discussed.

Introduction
Tryptophan (TRP) metabolism in mammals via indoleamine 2,3-dioxygenase (IDO) has received considerable attention in recent years because of its role in modulating both innate and adaptive immune responses (reviewed in: [1,2]). First described as an effecter of innate immunity, IDO and its associated kynurenine (KYN) metabolites are now mostly known for modulating the adaptive immune response, in particular T-cell mediated responses [1–3]. In most tissues, high KYN production directly reflects the anti-inflammatory and immunosuppressive enzyme activity of IDO and thus, the inflammatory and tolerogenic states of tissues. In the mouse epididymis, IDO and the KYN pathway are constitutively activated [4–6]. In previous work we reported that epididymal IDO1 expression in the mouse is restricted to the caput region from segments 2 to 5 with a peak of expression in segments 3 to 4 [7]. Both principal and apical cells were shown to be reactive towards an anti-IDO1 antibody and the enzyme was shown to be cytosolic and absent from the epididymal luminal compartment [5,7]. IDO1 is not the sole TRP-recycling enzyme found expressed in the mouse epididymis epithelium since TDO (tryptophane 2,3 dioxygenase) and INDOL (or IODO2, an homologous enzyme coming from a gene duplication event in mammals) were also shown to be expressed however at much lower levels [7]. We also demonstrated that IDO1 is the major contributor to KYN formation in the caput epididymis [7] since none of the other TRP-recycling enzymes compensated for the lack of IDO1 either at the transcription level or by their increased activities in a mouse model invalidated for IDO1, leading to a drastic drop in KYN content in IDO1-deficient epididymis extracts [7]. Kynurenine levels in mouse epididymal extracts were shown to be very significant and, amongst the various downstream products resulting from IDO1 activity we reported that L-kynurenine (KYNU), kynurenic acid (KYNA) and 3 hydroxykynurenine (3OHK) were by far the most abundant species following the metabolic activities in a mouse model invalidated for IDO1, leading to a drastic drop in KYN content in IDO1-deficient epididymis extracts [7]. Kynurenine levels in mouse epididymal extracts were shown to be very significant and, amongst the various downstream products resulting from IDO1 activity we reported that L-kynurenine (KYNU), kynurenic acid (KYNA) and 3 hydroxykynurenine (3OHK) were by far the most abundant species following the metabolic activities in a mouse model invalidated for IDO1, leading to a drastic drop in KYN content in IDO1-deficient epididymis extracts [7]. 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engaged in both anti-inflammatory and immunosuppressive actions needed to preserve spermatozoa, which are allogenic strangers to the male immune system, from auto-immune responses while maintaining an efficient anti-infection surveillance of the immune-privileged epididymal duct.

In the present report, we have analyzed in more depth the immune/inflammatory status of the caput epididymidis in both WT and IDO1-deficient mice in order to gain further insights into the role of IDO1 in controlling the balance between immunological responsiveness and tolerance.

Materials and Methods

Animals

The present study was approved by the Regional Ethics Committee in Animal Experimentation (CEMEA-Auvergne; Authorization CE2-04) and adhered to the current legislation on animal experimentation in France. Wild type and IDO1<sup>−/−</sup> BALB/c male mice [8] aged 6 months were used throughout the study unless otherwise indicated. Mice were housed under controlled environmental conditions (22°C, 12-h dark period), fed a basal diet (Global-diet, 2016S, Harlan, Gannat, France) ad libitum, and given free access to water. Mice were killed by cervical dislocation under CO₂ anaesthesia. Proteins were extracted from liquid nitrogen-frozen epididymal tissues stored at −80°C until use. Briefly, tissues were homogenized in 20 mM HEPES, 0.42 M NaCl, 1.5 m MgCl₂ M, 0.2 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₃VO₄, 0.1 mM NaF and complete 1X (Roche Diagnostics, Meylan, France). Blood samples were kept on ice in heparin-coated tubes, centrifuged 15 min at 1500 g at 4°C and then the plasma was recovered and kept at −80°C until use.

Kynurenine, Tryptophan Determinations

TRP and KYN concentrations were measured by high performance liquid chromatography (HPLC) using 3-nitro-l-tyrosine as internal standard [9]. For separation, reversed-phase cartridges 35-mm LiChroCART RP18 column were used. TRP was detected by a fluorescence detector at an excitation wavelength of 295 nm and an emission wavelength of 365 nm. A Shimadzu (Marne la Vallée, France) SPD-6A UV-detector in wavelength of 285 nm and an emission wavelength of 365 nm. Blood samples were kept on ice in heparin-coated tubes, centrifuged 15 min at 1500 g at 4°C and then the plasma was recovered and kept at −80°C until use.

Fatty Acids and Sphingolipids Measurements

Total plasma lipids were extracted as described in [10]. The phospholipid fraction was separated by thin-layer chromatography on a silica gel plate (Merck, Darmstadt, Germany) and 1-stage mobile phase development, which consisted of the solvents hexane, ethyl ether and acetic acid in an 80:20:2 (v/v/v) ratio. The plates were dried and sprayed with dichlorofluorescein to visualize cholesterol esters, phospholipids, triacylglycerols, and free fatty acids bands under ultraviolet light. The phospholipid band was scraped off into a separate test tube, and the fatty acids were converted into methyl esters. Fatty acid methyl esters were prepared and analyzed as previously described [11]. Ceramide and sphingomyelin analyses were performed at the lipidomic platform (INSERM IFR150 Metatoul Platform, Toulouse, France) as follows: cells were homogenized in methanol/EGTA 5 mM (2:1 v/v) with a FAST-PREP apparatus (MP Biomedicals), and an aliquot was taken up for protein measurement. Total lipids were extracted in chloroform/methanol/3 mM EGTA (2:5:2.5:2:1, v/v) in the presence of the internal standard stigmastanol (18 mg) as described in [12]. Ceramide-NC15 (2 mg), was prepared according to [13]. The lipid phase was dried down under a nitrogen flux, and lipid extract was submitted to a mild-alkaline treatment in 1 ml methanolic NaOH 0.6 N, followed by silylation in BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) containing 1% TMSCl (Chlorotrimethylsilane)/acetonitrile (1:1, v/v) [13]. The mixture (5 ml) was directly analysed by gas-liquid chromatography on a 4890 Hewlett Packard (Palo Alto, CA, USA) system using a RESTEK RTX-50 fused silica capillary columns (30 m x0.32 mm, 0.1 mm film thickness) [13]. Oven temperature was programmed from 195°C to 310°C at a rate of 3.5°C per min (for 12 min), and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 310°C and 340°C, respectively. SM and ceramide were separated on the basis of the number of carbons and the unsaturation of the amide-linked chain and quantified according to the internal standard. C16, C18, C22 and C24 SM or ceramide refer to the sum of the molecular species having this number of carbons in the lateral chain, independent of the unsaturation. Results are given as nmol per mg protein.

Quantitative Reverse Transcriptase Polymerase Chain Reactions

Total RNAs were isolated with the NucleoSpin® RNA II kit (Macherey-Nagel, France) and were reverse transcribed by M-
MLV Reverse Transcriptase (Promega Corp., France) according to the manufacturer’s instructions. Quantitative real time PCR assays were performed using a RealPlex thermocycler (Eppendorf). Two μl of diluted cDNA template (1/20) were amplified using MESA GREEN qPCR MasterMix Plus (Eurogentec, France) according to the manufacturer’s instructions. Primer sequences are given in Table 1 and amplification efficiencies were provided. To ensure no genomic DNA contamination primers were designed in distinct exons separated by at least 500 bp-introns, so that the genomic DNA cannot be amplified. A standard curve of amplification efficiency for each set of primers was generated with a serial dilution of plasmids containing DNA of targeted genes. Melting curve analysis was carried out to confirm the specificity of primers. For quantification of transcripts, the relative expression between the tissues and genotypes.

Western Blots

Proteins (40 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Hybond ECL, GE Healthcare Biosciences, Piscataway, NJ). Blots were blocked with 10% low-fat dried milk/0.1% Tween 20/Tris Base Salt (TBS) and probed overnight at 4°C with anti-GAPDH (1/5000, Sigma-Aldrich) for loading controls and in parallel with various primary antibodies. For monitoring the activation of the STAT protein family, antibodies came from the STAT/Phospho-STAT antibody sampler kit (Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France). For monitoring the activation of the Smad intracellular effectors, rabbit monoclonal anti-mouse Smad1, rabbit monoclonal anti-mouse Smad3, rabbit polyclonal anti-mouse Smad5 as effectors, rabbit monoclonal anti-mouse Smad1, rabbit monoclonal anti-mouse Smad3 (Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France) were used. Each primary antibody was incubated with anti-rabbit horseradish peroxidase conjugate (1/5000, GE Healthcare, UK) that was detected using the ECL Western Blotting Detection kit on Hyperfilm™ (GE Healthcare). Densitometric analyses were carried out with “Quantity one” software (Bio-Rad, Marnes-la-Coquette, France).

Fluorescence-Activated Cell Sorting Analyses

Caput epididymides were dissected and processed for the preparation of stroma-vascular fractions (SVF) as described in [14]. Subpopulations of lymphocytes were identified by sorter (FACS; FacsCalibur, Becton Dickinson Biosciences, Roissy, France). Fluorescein (FITC)- or Phycoerythrin (PE) conjugated anti-cluster of differentiation 3 (CD3), CD4, CD8, CD19 antibodies were used (Miltenyi, France). Cells (around 2×10⁶/sample) were stained in PBS containing 5% mouse serum and re-suspended in PBS before counting on a Coulter counter (Beckman-Coulter, Roissy, France). Red cells were lysed in a buffer containing 155 mM ammonium chloride, 20 mM Tris, pH 7.6, for 5 min. Cells were then centrifuged (600g for 10 min) and resuspended in PBS before counting on a Coulter counter (Beckman-Coulter, Roissy, France). Fluorescein (FITC)- or Phycoerythrin (PE) conjugated anti-cluster of differentiation 3 (CD3), CD4, CD8, CD19 antibodies were used (Miltenyi, France). Cells (around 2×10⁶/sample) were stained in PBS containing 5% mouse serum and incubated with conjugated anti-mouse monoclonal antibodies for 20 min at room temperature in the dark. Cells were washed in PBS and then analyzed on a fluorescence-activated cell sorter (FACS; FacsCalibur, Becton Dickinson Biosciences, France). Subpopulations of lymphocytes were identified by

Table 1. Oligonucleotides used in the course of the study.

| Gene product | Primer sequences (5’ = >3’ ) | Insert length | Tm in °C | Efficiency |
|--------------|-------------------------------|--------------|----------|------------|
| Rorc NM,011281.2 | Fw AGCAGTGTAATGTTGGCCTAC | 179 bp | 59 °C | 0.9886 |
| | Rv GCACTTCCTGCATGTAAGCTG | | | |
| FoxP3 NM,001199348.1 | Fw CCCAGGAAGACAGCACACCTT | 90 bp | 59 °C | 0.9778 |
| | Rv TTTCAACAACCGACGACCTT | | | |
| Cyclophilin b NM,011149.2 | Fw GGAGATGCGACAGAGGAA | 76 bp | 55 to 63 °C | 0.9741 |
| | Rv GCCCGTAGTGCTTCAGCTT | | | |
| Gata-3 NM,008891.3 | Fw ACAGGAAGAGTTGGACCTACT | 242 bp | 58 °C | 0.9905 |
| | Rv GTGGTGGATGACGGCTTGG | | | |
| T-bet NM,019507.2 | Fw GTCCCCCATTCCTGGCTCCT | 211 bp | 60 °C | 0.9741 |
| | Rv CCTTTGTTGTTGAGCGCTT | | | |
| Cox1 NM,008969.3 | Fw CATGCGTCGCTGAAACTCCTA | 72 bp | 63 °C | 0.9923 |
| | Rv AAGGCCAGAGACGAGTGGCTT | | | |
| Cox2 NM,011198 | Fw CCAGCGCTTCACCACTCAGT | 52 bp | 63 °C | 0.9868 |
| | Rv ACCCAGGTCCTGGCTTATGA | | | |

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were not elevated in caput epididymal extracts of \( \text{Ido1}^{+/+} \) animals, the KTR in the caput epididymis does not differ from that in plasma, suggesting that \( \text{Ido1} \) is the major provider of KYN in the former tissue. To monitor the inflammatory status of the caput epididymis, we measured the classical pro-inflammatory cytokines TNF-\( \alpha \) and INF-\( \gamma \) in both \( \text{wt} \) and \( \text{Ido1}^{-/-} \) caput epididymal extracts. As depicted in Figure 1B, the absence of \( \text{Ido1} \) these cytokines were significantly up-regulated in caput epididymal extracts, while plasma levels did not differ significantly between \( \text{wt} \) and \( \text{Ido1}^{-/-} \) animals. The up-regulation of these cytokines in the caput epididymitis of \( \text{Ido1}^{-/-} \) animals suggests an aggravated inflammatory status. This is supported by the fact that caput epididymal extracts of \( \text{Ido1}^{-/-} \) animals also contained higher levels of the soluble TNF receptors sTNF-R1 and sTNF-RII compared to \( \text{wt} \) extracts (Fig. 1C) which are often seen as classical counteractive phenomena to diminish TNF-\( \alpha \)-mediated pro-inflammatory signal. The increase in the inflammatory status of the \( \text{Ido1}^{-/-} \) caput epididymis was further confirmed by the observation, that several inflammatory chemokines including CCL3 (MIP-1\( \alpha \)), CCL5 (RANTES), CCL11 (Eotaxin-1), CCL24 (Eotaxin-2), CCL25 (TECK), CXCL9 (MIG), CXCL11 (I-TAC), and CX3CL1 (Neurotactin) were also up-regulated in \( \text{Ido1}^{-/-} \) caput epididymal extracts as shown in Figure 2A. The up-regulation of these chemokines was confined to the caput epididymis, as the corresponding plasma levels were not elevated (Fig. 2B). The specificity of the epididymal response is further attested by the fact that amongst the various chemokines surveyed not all of them were up-regulated. For instance, the concentrations of CCL1 (TCA-3), CCL2 (MCP1), CXCL5 (LIX) and CXCL13 (BCA-1) were not elevated in caput epididymal extracts of \( \text{Ido1}^{-/-} \) animals, and two of them, namely CCL2 (MCP1) and CXCL5 (LIX), were found to be even down-regulated in the corresponding plasma samples (Fig. 2A and 2B, right bars).

**Impact of \( \text{Ido1} \)-deficiency on T Cell-mediated Responses in the Caput Epididymis**

Both KYN production and tryptophan depletion resulting from \( \text{Ido1} \) activity are known to specifically control the activation and differentiation of T-cell subpopulations [20]. To verify whether \( \text{Ido1} \) deficiency affects T cell-mediated responses in the caput epididymis, we monitored the expression of various interleukins associated with T-cell differentiation and activation. It is obvious from Figure 4A that, with the exception of IL-4, all other T cell-related cytokines monitored including IL-18, IL-2, IL-3, IL-6, IL-9, IL-10, IL-12p70, IL-12p40p70, IL-13, and IL-17 were significantly up-regulated in \( \text{Ido1}^{-/-} \) caput epididymal extracts. In plasma, on the other hand, with the single exception of IL-4, no significantly increased lymphokine levels were observed (Fig. 4B). These data suggest that a T-cell response is engaged in the caput epididymis of \( \text{Ido1}^{-/-} \) animals, which is further supported by significantly increased concentrations of GM-CSF and G-CSF in the epididymis of \( \text{Ido1}^{-/-} \) animals, two colony-stimulating factors known to be produced by T cells, (Fig. 4C).

**IDO1 Deficiency in the Caput Epididymidis Alters the Th17/Treg/Th1/Th2 Equilibrium**

Depletion of tryptophan via \( \text{Ido1} \) activity as well as KYN actions have been shown to induce the differentiation of naïve T cells into the Treg lineage [21]. The latter is characterized by its immunosuppressive effect mediated by its ability to secrete immunomodulatory cytokines such as IL-10 and TGF\( \beta \) [22]. Both cytokines in return slow down the differentiation of the Th17/Th1/Th2 immunosubpopulations [23]. Thus, \( \text{Ido1} \) activity influences the Th17/Treg equilibrium in favor of Tregs, allowing immunosuppressive actions and the creation of a tolerogenic situation (Fig. 5A). To evaluate whether this Th17/Treg equilibrium has been modified in caput epididymal tissue from \( \text{Ido1}^{-/-} \) animals we used Western blot and real-time PCR to respectively monitor, the STAT3-dependent signal transduction pathways and the accumulation of key transcription factors that specifically govern Th17 and Treg differentiation from naïve T cells. Figures 5B and 5C show significant increases in the phosphorylation status of the STAT3 signal transducer intermediate and in the expression of the Th17-lineage specific transcription factor RORc1, respectively, in \( \text{Ido1}^{-/-} \) caput tissue.
extracts as compared to wt extracts. In the same samples we observed neither a change in the phosphorylation status of STAT5 nor in the expression of the FOXP3 transcription factor, both of which are specific for the Treg lineage differentiation program. These data suggest that in caput epididymis extracts of Id01^{2/2} animals the Th17/Treg equilibrium is altered in favor of the Th17 subpopulation.

As increased representation of the inflammatory Th17 subpopulation in Id01^{2/2} caput epididymis should affect the Th1/Th2 equilibrium [23] we show in Figures 6A-B that the Th1-lineage specific transcription factor T-bet was significantly up-regulated in...
caput epididymal extracts of Ido1/2 animals, while expression of the Th2-lineage specific transcription factor GATA-3 did not change. These data support the notion that the caput epididymis of Ido1/2 animals is engaged in a Th1 response. Consistent with this hypothesis, a significant increase in the leptin content of caput epididymal extracts from Ido1/2 animals was found, while plasma levels did not differ significantly from those of wt animals (Fig. 6C). Leptin is known to prime the generation of pro-inflammatory adaptative Th1 responses after T<sup>CD4+</sup>-cells have encountered antigens following their interaction with antigen presenting cells (APC) [24,25].

IDO1 Deficiency does not Alter the Overall Leucocyte Representation in Caput Epididymis

FACS analysis was used to look at the distribution of leucocytes in caput epididymal tissue samples dissociated by collagenase treatment in both wt and Ido1/2 animals. Figure 7 shows cytofluorometric analyses of gated lymphocyte subpopulations in wt and Ido1/2 caput epididymis samples (n = 5). Globally there is no dramatic change in the representation of the various leucocyte lineages that we have been able to monitor in the different issue extracts. In addition, the analysis revealed that B cells and CD3<sup>+</sup> cells were quite rare in the caput epididymal tissue, whatever the genetic background, and that most of the lymphoid cells were triple negative (CD3<sup>-/CD4<sup>-/CD8<sup>-</sup>) cells. Regarding the few CD3<sup>+</sup> cells, they were essentially found to be of the CD3<sup>+</sup> double negative type (devoid of CD4 and CD8), the so-called CD3<sup>+</sup>DN or TDN.

Discussion

The present data indicate that IDO1 and its downstream metabolites, the KYNs, participate in the establishment of an immunological equilibrium within the caput epididymis of mice. This is supported by our observations that in the absence of IDO1 expression (i.e., in the Ido1<sup>−/−</sup> mouse model [8]) an increase in the concentrations of classical inflammatory cytokines (IFN-γ, TNF-α, IL-6, IL-1β) as well as in various chemokines associated with T cell-driven inflammatory situations was seen, but solely in caput epididymis extracts and not in plasma. A change in the
Figure 3. Other inflammatory markers of the IDO1<sup>−/−</sup> caput epididymidis. A: Quantitative RT-PCR estimations of COX1 and COX2 mRNA accumulation in caput epididymidis samples from wt (black bars) and IDO1<sup>−/−</sup> (grey bars) male mice. For quantification of transcripts, the relative method was used to calculate mRNA levels relative to Cyclophilin B standard. WT levels were set as 1 in the Y-axis. Mean +/- SEM; n = 12. *P<0.05; **P<0.01. B: Schematic representation of the omega 6 (ω6) and omega 3-derived (ω3) fatty acid (FA) intermediates (DGLA, Dihomo-gamma-linolenic acid; AA, Arachidonic acid; EPA, Eicosapentaenoic acid and DHA, Docosahexaenoic acid) precursors of the eicosanoids derivatives including prostaglandins, leukotrienes and thromboxanes. Bracketed numbers given above each FA species indicate difference recorded in the representation of these FA in caput epididymidis extracts of IDO1<sup>−/−</sup> animals versus wt (n = 6). C: Histograms illustrate the recorded differences in sphingolipid intermediates (sphingomyelin and ceramides) concentration in caput epididymidis extracts of wt (black bars) and IDO1<sup>−/−</sup> (grey bars) from 6 month-old animals. Cholesterol level was taken as a reference to show that the transgenic animals do not present a general disruption in their epididymal lipidic profile.

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inflammatory status of the caput epididymidis in Ido1−/− animals was also indicated by the up-regulation of several T cell-generated lymphokines and the over-expression of the inflammation-induced cyclooxygenase, COX2. As expected, COX2 induction in the epididymis of Ido1−/− animals was associated with increased consumption of ω3- and ω6-PUFA, which serve as precursors for...
both pro-inflammatory and anti-inflammatory effectors including prostaglandins, leukotrienes and thromboxanes [16]. In addition, we show here an increase in the sphingolipid/ceramide pathway in caput epididymidal extracts of \textit{Ido1} \textsuperscript{+/−} animals (Fig. 3C) that may, in part, explain the activation of COX2, because sphingolipids and particularly ceramides have been shown to actively participate in TNF-α-mediated inflammatory responses (for a recent review see: [19]). It is worth noting that the epididymis is rather unusual in this regard as it is characterized by its constitutive expression of COX2 [26,27]. In any other tissue, COX2 expression is solely induced by inflammatory stimuli. The constitutive expression of both COX2 and IDO1 reinforces the idea that the caput epididymidis is characterized by the maintenance of a permanent immune-tolerant state. We show here that both COX1 and COX2 expression levels were upregulated in \textit{Ido1} \textsuperscript{+/−} caput extracts compared to \textit{wt}. Although it is commonly believed that the constitutive isoform COX1 has little or no involvement in regulating immune responses [28], there are recent reports

**Figure 5. Th17/Treg equilibrium in the caput epididymidis. A:** Differentiation of the Th17 and Treg lineages from naïve T cells are mutually exclusive via their respective cytokines. STAT3/RORc drive the differentiation of Treg cells, while STAT5/FOXP3 drive the differentiation of Treg. In inflammatory or/and tolerogenic situations, IDO1 activity and the resulting KYNs promotes the differentiation of Treg cells and their immunosuppressive actions. **B:** Representative western blots showing the levels of STAT3 and STAT5 proteins and their phosphorylated counterparts phosphoSTAT3 and phosphoSTAT5 upon activation in caput epididymidis extracts from \textit{wt} and \textit{Ido1} \textsuperscript{+−} mice at 6 months of age. Bar graphs display means ± SEM using GAPDH as an internal standard for quantification. (n = 7 for Stat3, and n = 3 for Stat5; **p < 0.01). **C:** Quantitative RT-PCR estimations of RORc and FOXP3 mRNA accumulations in caput epididymidis samples from \textit{wt} (black bars) and \textit{Ido1} \textsuperscript{−−} (grey bars) male mice. For quantification of transcripts, the relative method was used to calculate mRNA levels relative to \textit{Cyclophilin B} standard. \textit{WT} levels were set as 1 in the Y-axis. Mean ± SEM; n = 12. **p < 0.01.

**Figure 6. Th1/Th2 equilibrium in the caput epididymidis. A:** Differentiation of the Th1 and Th2 lineages from naïve T cells are mutually exclusive via their respective cytokines. T-bet drives the differentiation of Th1 cells while Gata-3 drives the differentiation of Th2 cells. **B:** Quantitative RT-PCR estimations of T-bet and Gata-3 mRNA accumulation in caput epididymidis samples from \textit{wt} (black bars) and \textit{Ido1} \textsuperscript{−−} (grey bars) male mice. For quantification of transcripts, the relative method was used to calculate mRNA levels relative to \textit{Cyclophilin B} standard. \textit{WT} levels were set as 1 in the Y-axis. Mean ± SEM; n = 12. **p < 0.01. **C:** Histograms show the levels of leptin in caput epididymidis extracts and plasma from 6 month-old \textit{wt} and \textit{Ido1} \textsuperscript{−−} animals. **p = 0.01.

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suggesting that COX1 is actively involved in immunoregulation [29] and that part of its effect is mediated via IL-17 production by Th17 cells [30]. Our data are consistent with these statements since we observed a shift in the Th17/Treg equilibrium towards Th17 cells and an associated increase in the concentration of IL-17, in the caput epididymides of Ido1−/− animals.
Increased levels of IFN-γ and local inflammation in the caput epididymides of \textit{Ido1}−/− animals are expected in the context of a weaker immune-tolerant environment against sperm antigens that should stimulate a Th1-driven autoimmune reaction [40]. In addition, it has been reported that kynurenines including 3-hydroxy-arcachidonic acid (3-OH-AA) and 3-hydroxykynurenic acid (3OHKA) inhibit the actions of Th1 cells and enhances the action of Th2 cells [41]. Other reports have shown that stimulation of IDO activity in dendritic cells diminishes Th1 response [42]. Conversely, it was also reported that 1-methyltryptophan-mediated inhibition of IDO activity enhances Th1-associated inflammation [43]. Therefore, in the absence of IDO1 and its downstream metabolites, as it is the case in the epididymis of the \textit{Ido1}−/− animals [7], one should expect a shift of the Th1/Th2 equilibrium towards Th1. This behaviour in agreement with the hypothesis that the immunosuppressive action of IDO and its role in inducing immune tolerance is ensured by its inhibitory effect on the Th1 subpopulation [40].

**Caput Epididymidis of IDO1-deficient Animals Copes with Increased Local Inflammation and Avoids Compromise of its Immune-tolerant Status Towards Spermatozoa**

We have shown above that IDO1 deficiency promotes inflammation in the caput epididymidis via a shift in both Th1/Treg and Th1/Th2 balances, respectively, in favor of Th17 and Th1 pro-inflammatory T cells. However, this situation does not lead to acute inflammatory conditions such as leukocytospermia and granulomas. This suggests that compensatory mechanisms are at work to maintain the immune balance of the epididymis in \textit{Ido1}−/− animals. In confirmation of this status quo situation, our preliminary investigations via FACS analyses showed that the caput epididymidal leucocyte distribution is not modified when \textit{wt} animals were compared to \textit{Ido1}−/− (Fig. 7). In agreement with previous reports it also showed that most of the lymphoid cells were triple negative (CD3−/CD4−/CD8−) meaning that they could be either dendritic cells (DC) or of the natural killer (NK) lineage and that B cells and CD3+ lymphoid cells were scarce [44]. It is interesting to note that the \textit{CD3+} cells were found to be mostly of the \textit{CD3+} double negative type (devoid of CD4 and/or CD8), the so-called \textit{CD3+DN} or \textit{DN} which have been added recently to the increasing list of T cells that exert T regulatory functions.
similar to those of classical Treg (CD4+CD25+FoxP3+) cells [45–49]. Especially, T<sup>SN</sup> were very recently shown to be involved in the prevention of autoimmune responses engaged in the inflamed epididymis after vasectomy [50]. Other non-exclusive hypotheses can be brought forward to explain the new immune equilibrium found in the caput epididymis of the Ido1-deficient animals and the preservation of the tolerogenic environment towards spermatooza. First, we show here that despite the absence of Ido1 expression and its known inducing effect on the Treg lineage [51], the differentiation of the Treg subpopulation is not dramatically reduced in the caput epididymidis. It is thus possible that a TGF-β family member such as BMP8a might participate in the immune response as expected when autoimmunity is stimulated. Despite the absence of this immunosuppressive player the epididymis of the Ido1<sup>−/−</sup> mice copes with the pro-inflammatory situation and maintains immune-tolerance. Our data suggest that TGF-β signaling, IL-10 up-regulation and impairment of caput epididymis pro tease activity may represent means by which the epididymis of Ido1<sup>−/−</sup> animals restores its immune balance.

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Author Contributions

Conceived and designed the experiments: FS AK LJ HPR RC RJ A NG JR D. Performed the experiments: AJL JHB CDS RG. Analyzed the data: FS AK LJ HPR RG RJ A NG JR D. Contributed reagents/materials/analysis tools: DHM ALM. Wrote the paper: NG JR D.

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