The Apicomplexan Parasite, *Eimeria falciformis*, Co-opts Host Tryptophan Catabolism for Life Cycle Progression in the Mouse

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Running title: Subversion of host IDO by an apicomplexan parasite

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Background: The kynurenine pathway catalyzes tryptophan catabolism in mammals.

Results: The rate-limiting indoleamine-2, 3-dioxygenase and other two enzymes of the host kynurenine pathway are required for an efficient development of apicomplexan parasite *E. falciformis* in mouse.

Conclusion: Previously unanticipated pro-parasite function of host Indoleamine-2, 3-dioxygenase.

Significance: Subversion of the host defense (IFNγ, IDO) by an intracellular pathogen for progression of its natural life cycle.

ABSTRACT

The obligate-intracellular apicomplexan parasites e.g. *Toxoplasma gondii* and *Plasmodium species* induce an IFNγ-driven induction of host indoleamine-2,3-dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism in the kynurenine pathway. Induction of IDO1 underlies depletion of subcellular tryptophan in host cells, which is proposed to inhibit the *in vitro* growth of susceptible pathogens. *In vivo* function of IDO during infections, however, is not clear, let alone controversial. We reveal that *Eimeria falciformis*, a monoxenous apicomplexan parasite infecting mouse caecum, induces IDO1 in the epithelial cells of the organ and its expression coincides with the parasite development. The absence or inhibition of IDO1/2 and of two further downstream enzymes in infected animals is detrimental to the parasite growth. The reduction in *Eimeria* oocysts is not due to the immuno-suppressive effect of IDO1 as the parasitized IDO1−/− or inhibited animals are not impaired in their Th1 and IFNγ responses. The parasite development is entirely rescued by xanthurenic acid, a byproduct of tryptophan catabolism inducing exflagellation in male gametes of *Plasmodium* in the mosquito mid-gut. Our data demonstrate a conceptual subversion of the host defense (IFNγ, IDO) by an intracellular pathogen for progression of its natural life cycle. Besides, we show utility of *E. falciformis*, a monoxenous parasite of a well-appreciated host i.e. mouse, to identify *in vivo* factors underlying the parasite-host interactions.

INTRODUCTION

Indoleamine 2,3-dioxygenase 1 (IDO1) is the first and the rate-limiting enzyme of tryptophan degradation via the kynurenine pathway in mammals (1). Other enzymes of the pathway include kynurenine aminotransferase and kynurenine 3-hydroxylase (Fig S1A). Over the last three decades, the tryptophan catabolism has gained an increasing attention because the ensuing depletion of the essential amino acid and accumulation of downstream products can influence multiple and disparate biological processes such as antimicrobial effect, immune-suppression, cellular transformation and...
neuromodulation (2,3). IDO1 is expressed ubiquitously in a variety of tissues (4), and induced by interferon-gamma (IFN-γ) in several cells (5,6).

IDO1 was first reported to play a defensive role against the parasite *Toxoplasma gondii*, supposedly by depleting intracellular tryptophan in IFNγ-treated host cells (7). Likewise, an induction of IDO1 in IFNγ-treated host cells was detrimental to bacteria, *Chlamydia* (8) and *Streptococcus* (9). An elevated *in vitro* expression of the enzyme also inhibited the replication of genital herpes simplex virus 2 (HSV-2) (10) and cytomegalovirus (11). Moreover, the growth inhibition of *T. gondii* and *C. psittaci* could be reversed by exogenous tryptophan (12). Together these reports postulated IDO1 contributing to the host defense by depleting the subcellular tryptophan available to susceptible microbes.

In addition, IDO1 is believed to be an important immuno-regulatory enzyme. The biochemical inhibition of IDO in pregnant mice causes rejection of developing fetus, indicating the importance of this enzyme in pregnancy-associated immuno-tolerance (13). The immuno-suppressive role of IDO1 is thought to be mediated by inhibition of the T-cell proliferation as a consequence of tryptophan starvation (5,6,14) and/or due to an elevated apoptosis inflicted by kynurenine metabolites (15-17). Besides, blocking of IDO exerts no apparent effect on the mouse immune response in a toxoplasmosis model; whereas it is decreased with a concurrent increase in IFN-γ and reduced IL-10 levels in *Leishmania*-infected animals (18). It is, thus, debatable whether an induced expression of IDO is required for the host resistance against the pathogens and/or for anti-proliferative effects to override an escalated immune response.

*Eimeria* species belong to apicomplexan phylum, which comprises obligate intracellular parasites such as *Toxoplasma* and *Plasmodium*. Individual *Eimeria* species infect an exclusive tissue and host-cell type, and complete their life cycle within a single organism offering a particular advantage for *in vivo* and site-specific parasite-host interactions. This study uses *E. falciformis*, a murine parasite, and demonstrates a previously unanticipated subversion of the host IDO1 and tryptophan catabolism by this parasite.

**EXPERIMENTAL PROCEDURES**

*Chemical and biological reagents*

The IDO1-knockout mice (Balb/c strain) were a kind donation by Muriel Moser (Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles). The parental control Balb/c and NMRI mice were purchased from the Charles River Laboratories (Sulzfeld, Germany). Animal procedures were performed according to the German Animal Protection Laws as directed and approved by the overseeing authority Landesamt fuer Gesundheit und Soziales (Berlin, Germany). *Eimeria falciformis* oocysts were procured from Bayer (Germany).

*Propagation of Eimeria falciformis*

The natural life cycle of *E. falciformis* was maintained by continuous passages of the parasite oocysts in female NMRI mice. Oocysts in the animal feces were washed in water, sterilized and floated with NaOCl, and quantified using a McMaster counting chamber (19). The purified oocysts were stored in potassium dichromate at 4°C and for the experiments used within 3 months of collection. The 8–12 weeks old animals were orally infected with 100 μl PBS containing indicated amount of oocysts. Infected mice were kept on grids in separate cages, and oocyst yield was evaluated by collecting feces on a daily basis. Individual fecal samples were dissolved in water and diluted in saturated sodium chloride for counting. All animals were weighed every day to monitor their weight loss during the course of infection.

*Transmission electron microscopy*

The female NMRI animals infected with 1500 oocysts were sacrificed on day 1 to day 8 during infection, and their caeca were collected. The parasitized caecal tissues were fixed with 2.5% (v/v) glutaraldehyde and 2.0% (w/v) paraformaldehyde in 100 mM cacodylate buffer (pH 7.4) for 4 hrs at room temperature and for
additional 12 hrs at 4 °C. Tissues were rinsed with 100 mM cacodylate buffer 3x for 15 min, and post-fixed for 4 hrs with 2% (v/v) osmium tetroxide and 3% (w/v) potassium hexacyanoferrate (II) on ice. Samples were rinsed 1x with 100 mM cacodylate buffer for 30 min, and then washed with 5 mM maleate buffer 3x for 15 min. Tissue samples were stained en bloc with 0.5% (w/v) uranyl acetate, washed again with 5 mM maleate buffer (3x for 15 min), dehydrated in an increasing series of ethanol and propylene oxide and embedded in Spurr resin. Thin sectioning was performed with a Reichert Ultra Cut and sections (70-90 nm) were counterstained with 4% (w/v) uranyl acetate followed by lead citrate. All samples were imaged on a transmission electron microscope equipped with a wide-angle CCD camera (Zeiss EM 900, TRS Systems, Moorenweis, Germany).

**Immunohistochemical and western blot analyses**

The caeca of infected or uninfected female Balb/c mice were removed, carefully washed and stored in 4% PFA/PBS for immunohistochemical staining. Tissues embedded in paraffin were cut into 2 µm sections. Samples were treated with xylene, rehydrated in descending ethanol concentrations and then washed with water. Antigen retrieval was achieved by boiling in citric buffer solution (pH 6.0) in a pressure cooker. Slides were rinsed in water, washed with TBS and the primary antibody (rat anti-mouse clone mIDO-48, Biolegend) was added (1:200, 30 min). After washing, the sections were incubated with the secondary antibody (biotinylated rabbit anti-rat, 1:200, 30 min, Dako). Streptavidin-conjugated alkaline phosphatase solution was applied (30 min) onto the sections, which were visualized by Fast RED (Dako). Slides were rinsed in water and then counterstained with hematoxylin. Images were acquired using a LEICA DMIL microscope and LEICA DFC 480 camera, and processed with LEICA Application Suite (v2.8.1).

Immunoblot analysis of tissue homogenates was performed using polyclonal rabbit anti-IDO and rabbit anti-beta tubulin primary antibodies (Genscript) and horseradish peroxidase-labeled anti-rabbit secondary antibody (GE Healthcare). The caeca of uninfected or infected female NMRI mice were removed, washed and snap frozen in liquid nitrogen. Total proteins were prepared in a lysis buffer containing 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF and protease-inhibitor cocktail (Sigma-Aldrich). Samples were homogenized, centrifuged (5 min, 4°C, 12000 rpm) and protein amount in the supernatant was determined by the bicinchoninic acid assay (Pierce). 30 µg of total tissue protein was separated on a 12% SDS-PAGE and blotted onto a nitrocellulose membrane (Applichem). Unspecific binding was blocked by overnight incubation in 5% skim milk powder in TBS containing 0.2 % Tween-20 (TBS-T). The primary antibody (1:1000) was applied in blocking solution for 2 hrs at room temperature, followed by 3x washes in TBS-T and addition of the secondary antibody (1:3000, 45 min at RT). Samples were subjected to 3 washes in TBS-T, and the protein bands were detected by enhanced chemiluminescence (GE Healthcare). The same blots were used to monitor anti-beta tubulin expression (protein loading) using the immunoblot recycling kit (Alpha Diagnostic). Protein expression was quantified by densitometric method using Adobe Photoshop suite.

**Isolation of epithelial cells and RNA analysis**

Infected or uninfected caeca were isolated, washed in calcium- and magnesium-free Hank’s BSS, opened longitudinally and cut into 5 mm pieces. To isolate epithelial cells, tissues were transferred into tubes and incubated in Hank’s BSS and DTT (1 mM) for 30 min and 37°C with gentle shaking. Supernatants were discarded. Samples were suspended in Hank’s BSS and EDTA (1 mM), and incubated for 30 min at 37°C with vigorous shaking every 10 min. Subsequent supernatants were passed through a 70 µm cell strainer (BD Bioscience) into fresh tubes and centrifuged (1500 rpm, 5 min). Pellets were resuspended in 1 ml of TRIzol Reagent (Invitrogen) and stored at -80°C.

Total RNA was isolated using the PureLink RNA Mini Kit (Life Technologies). 100 ng RNA was either reverse transcribed into first-strand cDNA (SuperScript III First-Strand
Antigen preparation, cell proliferation and IFNγ assays

Purified oocysts were digested with 0.4% pepsin (pH 3, 37°C, 1 hr; Sigma-Aldrich). The oocyst pellet was mixed with 0.5-mm glass beads (Sartorius) in a 1:1 ratio, and briefly vortexed to release free sporozoites. To excyst the sporozoites, DMEM containing 0.25% trypsin, 0.75% sodium tauroglycocholate, glutamine (20 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml) was added and sample was incubated at 37°C up to 2 hrs. The free sporozoites were collected for IFNγ detection, and the cells were pulsed with 1µCi/well of methyl-[3H]-thymidine (GE Healthcare) for 20 hrs at 37°C. Incorporation of 3H-thymidine was measured by liquid scintillation counting. The IFNγ levels in the supernatants were measured using the mouse ELISA Ready-SET-Go kit (eBioscience).

Pharmacological inhibition of kynurenine pathway

(S)-ESBA ((S)-(4-Ethylsulfonyl)benzoylalanine hydrochloride, Enzo Life Sciences) inhibits kynurenine aminotransferase II (KAT-II) (21), and Ro61-8048 (3,4-dimethoxy-N-[4-(3-nitrophenyl)thiazol-2-yl]-benzenesulfonamide, Tocris Bioscience) is competitive inhibitor of kynurenine 3-hydroxylase (22). 100 mM stock solutions in PBS were prepared for both inhibitors. The animals infected with E. falciformis were treated once daily with 1 mM of Ro 61-8048 or (S)-ESBA diluted in PBS. Inhibitors were applied via oral tube. To inhibit indoleamine 2,3-dioxygenase, mice were given 1-methyl-1DL-tryptophan (5 mg/ml of L- and 2 mg/ml of D-1-MT, Sigma Aldrich) in drinking water supplemented with artificial sweetener, which was changed every two days. All treatments started two days prior to infection and continued until the end of the experiments.

Data and statistical analysis

All graphs represent the mean ± SEM of at least three independent experiments with 5-6 individual mice unless specified otherwise. The two-tailed non-parametric Mann-Whitney test was used for determination of the statistical significance. The p-values below 0.05 were considered as significant.

RESULTS

Eimeria falciformis has a stringently regulated life cycle in its natural host mouse

We first examined a non-lethal natural infection of the inbred and outbred mice via oral inoculation of the purified oocysts of E. falciformis. The NMRI mice were infected with increasing number of oocysts (10-500), and the kinetics of oocyst shedding into animal feces was determined. Irrespective of the inoculums, the first batch of oocyst was detectable on day 7 of infection that continued until day 12 indicating a stringently synchronized
development of the parasite (Fig 1A). The total oocyst output increased at higher doses, and correlated with increments in infection inoculums (Fig 1B). The animals receiving 10 oocysts discharged a total of $1.7 \times 10^6$, and a 10-fold higher dose yielded a 3-fold increase in the oocysts ($5.3 \times 10^6$). A further increment of the initial dose to 500 oocysts resulted in yet another nonlinear enhancement in the oocyst yield ($7.5 \times 10^6$), indicating a saturated infection (Fig 1B). Compared to the outbred NMRI animals, the inbred Balb/c strain receiving equivalent inoculums yielded a similar kinetics of oocyst shedding (Fig S2A). Both strains produced their first batch of oocysts on day 7, and the peak days were not influenced at low and high doses (Fig 1A and S2A) confirming identical pre-patent and patent periods regardless of the animal breeding or inoculums.

The parasitized animals were weighed every day to assess the infection-associated pathology, deduced by loss in their body weight (Fig S2B-C). Infection at all doses resulted in a modest reduction of weight from the day 7 to 10 of infection in two strains, which was subsequently followed by a recovery on day 11 and onwards. Neither NMRI (Fig S2B) nor Balb/c (Fig S2C) infected with 10 oocysts showed a loss of more than 6% of the weight when compared to day 5. Interestingly, a considerably higher dose of 500 oocysts was well tolerated, and animals did not lose more than 10-15% of the body weight. In brief, these results reveal that *E. falciformis* completes its natural life cycle in one-week period irrespective of the inoculums or strains. These data confirmed the suitability of both mouse strains for further assays using considerably variable inoculums of *E. falciformis*.

Asexual and sexual development of *E. falciformis* in mouse caecum

We next performed transmission electron microscopy to investigate *in vivo* progression of *E. falciformis* in NMRI mice (Fig 1C). Animals were infected with a dose of 1500 oocysts for an easier identification of the parasitized sections in infected caeca. As summarized below, *E. falciformis* exhibited asexual and sexual stages within the one week of its life cycle. Infection on days 3–4 revealed immature and mature schizonts in the parasitized epithelial cells of caecum, which indicated the progression of asexual development (Fig 1C, panel d3, d4). The nuclei within the immature schizonts on d6 represent developing merozoites, which were released following the host-cell rupture and detectable in axenic or intracellular form (panel d5). Imaging of early development was not feasible in infected sections collected on d1 and d2. The onset of parasite growth, however, was quite evident on these days as deduced by 18S ribosomal RNA (Fig 1D). The ratio of host and parasite RNA increased over 7 days, and then declined on day 8, indicating a constant increase in parasite burden followed by completion of its life cycle (Fig S1B).

An immature macrogamont and a mature macrogamont with a central nucleus and wall forming bodies were observed on d7 and d8, which marked the initiation and succession of sexual development (Fig 1C, panel d7–d8). Fig 1C (panel d8) shows a microgamont with microgametes adjacent to a macrogamont. The transition to sexual phase on d6 was confirmed by detection of *EfGam56* and *EfGam82* transcripts identified between days 6–8 (Fig 1D). The homologs of *EfGam56* and *EfGam82* proteins are expressed in a macrogamont-specific manner in the avian species of *Eimeria* (23). Taken together, we show the entire natural development of *E. falciformis* in mouse, which consists of distinct asexual and sexual stages in the epithelial cells of host caecum.

*Eimeria falciformis* induces expression of indoleamine 2,3-dioxygenase in mouse caecum epithelia

To examine the expression of IDO1 during *in vivo* development of *E. falciformis*, the naïve control and parasite-infected mice were sacrificed, and samples were isolated for qPCR (Fig 2A) and Western blot (Fig 2B). Compared to uninfected control, the IDO1 transcript was upregulated by 64-fold on the 1st day of infection, and amplified by more than 250-fold on d3. The relative amount of IDO1 mRNA increased further up to 400-fold on day 5, and subsequently decreased to about 230-fold during the late infection (day 7). In accord with qPCR,
immunoblot analysis of uninfected and infected caeca revealed a similar outcome (Fig 2B). Interestingly, IDO1 protein as a 46-kDa band was first detectable on day 2 of infection, which then persisted throughout the parasite’s life cycle. The observed lag in protein expression suggests a post-transcriptional regulation of IDO1. The enzyme expression reached a maximum on day 5 and then declined until day 7, as quantified by immunoblot performed on samples from three independent in vivo infections (Fig 2C). In brief, we show that E. falciformis infection of mouse results in the induced expression of host IDO1 in the parasitized caecum.

To precisely determine the site of IDO expression within the parasitized host tissue, the caeca of control and infected animals were subjected to immunohistochemical staining. As expected from Fig 2B, no IDO staining of the naive tissue was observed. Also, neither epithelial nor cells of the lamina propria appeared stained for IDO protein up to 24 hours of infection (Fig 2D). In contrast, a strong expression of IDO was evident in caecal epithelial cells on day 6 and day 8 of infection. An apparent reduction in IDO expression on day 8 (Fig 2D) corresponds to the protein quantification (Fig 2C), and is likely a consequence of host-cell lysis by the parasite. Collectively, we demonstrate a strong and selective induction of IDO in the epithelial cells of mouse caecum in response to infection with E. falciformis.

**Host indoleamine 2,3-dioxygenase is required for the parasite development**

Induced expression of IDO exerts an antimicrobial effect on susceptible pathogens, likely by depleting the subcellular tryptophan (24). To examine the significance of IDO for in vivo development of E. falciformis, we infected the IDO1+/− (Balb/c) mice and evaluated the entire parasite development as indicated by oocyst formation (Fig 3). Surprisingly, deficiency of IDO reduced the oocyst yield to nearly half of the control group. Whereas the parental mice produced a total of 6.7x10^6 oocysts, the IDO1+/− group shed only 4x10^6 oocysts corresponding to a significant reduction (p <0.0001) of 2.7x10^6 oocysts in transgenic animals (Fig 3A). The kinetics of oocyst shedding remained unaltered in the IDO1−/− group (Fig 3B), and the decline in yield was confined to the peak days of infection (d8, p <0.001; d9 and d10, p <0.0001). The growth of E. falciformis was not completely abolished in the absence of IDO1 that might be attributed to the presence of IDO2. This prompted us to inhibit the total IDO activity using a specific and competitive inhibitor (1-methyltryptophan, 1-MT) (Fig 3C). Inhibition of enzyme activity by 1-MT treatment of the NMRI mice via the drinking water reduced the oocysts yield to 35% (p <0.0001) compared to the untreated control. An equivalent phenotype with 55% fewer oocysts (p <0.001) was obtained when using the Balb/c strain.

The pre-patent and patent periods of the parasite growth were not influenced, and the observed effect was due to the decline in oocyst shedding on the peak days of infection in both mouse strains (day 8 to 10; Fig S3A-B). The body weight loss in the parasite-infected IDO1+/− (Fig 3D) or drug-treated animals (Fig S3C-D) during the days from 7 to 10 were comparable to respective controls indicating that the absence of IDO1 or analog inhibition of the total enzyme activity did not affect the morbidity. Taken together, these results suggest that IDO activity is required for an optimal in vivo development of E. falciformis. Notably, this observation contrasts with the reported defensive function of IDO against other pathogens, in vitro.

**Parasite-infected IDO1−/− and 1-MT-treated mice are not impaired in Th1 immune response**

Induction of IDO activity is also known to have an immunosuppressive effect on T-cell proliferation and on IFN-γ production (5,15,16,25). Inhibition or absence of IDO in the parasitized 1-MT-treated or IDO1−/− animals, therefore, has potential to augment the immune response and reduce the oocysts production. To test this notion, we infected IDO1−/−, 1-MT-treated and control animals with E. falciformis, and sacrificed them on day 6 and 8, which correspond to a prominent infection and coincide with the induced expression of IDO. The measurement of Th1-cell proliferation and...
IFN-γ responses in the mesenteric lymph nodes (mLN) of intestine as well as in the spleen revealed a comparable parasite-specific proliferation in both assay groups compared to the wild type control (Fig S4A-B). In addition, no notable differences in levels of IFN-γ were detectable following E. falciformis infection (Fig S4C-D). Briefly, the parasite-infected IDO1-knockout and 1-MT-treated animals mount a normal Th1 and IFN-γ response against the parasite. Hence, a decline in parasite growth in the IDO-deficient mice is not likely due to an amplified immune response.

Inhibition of kynurenine pathway enzymes is detrimental for parasite development

We reasoned whether a downstream metabolite of the tryptophan catabolism via the kynurenine pathway underlies the observed effect on E. falciformis. To test this conjecture, we focused on two enzymes, kynurenine aminotransferase II (KAT-II) and kynurenine 3-hydroxylase (Kyn-3OH), for which specific inhibitors with proven efficacy in mouse model are available. KAT-II and Kyn-3OH are selectively inhibited by (S)-4-[(ethylsulfonyl)benzoylalanine hydrochloride (S-ESBA.HCl) (21) and 3,4-dimethoxy-N-[4-(3-nitrophenyl)-2-thiazolyl]benzenesulfonanamide (Ro61-8048) (22), respectively (Fig S1A). The mouse can tolerate relatively high doses of S-ESBA.HCl (5 mM) (26) and Ro61-8048 (200 mg/kg bodyweight) (27) via different application routes without apparent signs of mortality and morbidity. We applied them orally at much lower doses (S-ESBA.HCl, 1 mM; Ro61-8048, 2 mg/kg), and monitored the oocyst output in comparison to the control carrier-treated animals (Fig 4A). Notably, as observed for IDO1−/− and for 1-MT-treated mice, the use of S-ESBA as well as of Ro 61-8048 reduced the total oocyst yield. Blocking of KAT-II and Kyn-3OH resulted in a decline by 50% (p <0.0001) and 40% (p <0.001), respectively. Yet again, application of the inhibitors yielded a normal kinetics of oocyst shedding indicating no apparent influence on the pre-patent and patent periods (Fig 4B). In addition, exposure to drugs had no effect on morbidity as scored by the loss in body weight of experimental animals compared to respective control groups (Fig 4C). Consistent with above findings, we show that perturbation of three distinct enzyme activities along the kynurenine pathway exerts a similar phenotype strongly suggesting the requirement of a downstream metabolite for an efficient parasite development.

**Xanthurenic acid can restore the decline of oocyst yield in IDO1−/− mice**

Xanthurenic acid (XA) is an end-metabolite of the tryptophan catabolism (Fig S1A). The urinary amount of XA is increased 3-fold in response to induction of IDO in mouse (4). Moreover, sequential catalysis by IDO, Kyn-3OH and KAT-II, whose inhibition is detrimental to E. falciformis growth, merges in synthesis of XA. Therefore, we questioned whether XA is required by the parasite, and its exogenous application can counteract for the IDO deficiency. To assess the notion, the metabolite was administered to the parasite-infected IDO1−/− animals, and the oocysts were enumerated. As expected, the absence of IDO1 reduced the amount of oocyst to nearly half compared to wild type control (Fig 5A). And, the treatment with XA completely rescued the oocyst decline in transgenic animals (p <0.0001). In fact, the XA-treated animals yielded a 32% higher oocyst count (7x10⁶) compared to untreated wild type (5.3x10⁶), which, however, did not meet statistical significance. Fig 5B shows that the course of infection was not affected in response to the metabolite exposure as judged by a natural kinetics of oocyst release. Also, XA did not influence the infection-associated pathology (Fig 5C), which further signified a specific and positive effect on the parasite development. In brief, these results demonstrate that XA, a byproduct of tryptophan degradation, is indeed required for an optimal progression of E. falciformis in mouse.

To gain further insight into the action of XA on E. falciformis development, we infected IDO1−/−, XA-treated transgenic and parental animals, and performed quantitative PCR of caecum-derived RNA samples. As shown above (Fig 1D, S1B), Ef18S rRNA was used to score asexual growth from d3 to d5, and the presence of EfGam56 and EfGam56 (d6-8) indicated the macrogametocyte development (Fig 6). Specific amplification of
individual ESTs and their expected sizes was confirmed by gel electrophoresis (Fig 6A). Next, we compared the abundance of each transcript amongst the three parasitized mice samples (Fig 6B-D). No apparent and significant change was observed in relative abundance of Ef18S rRNA (Fig 6B), EfGam82 (Fig 6C) and EfGam56 (Fig 6D) ESTs in the IDO1+/−-derived caeca samples, when compared to the parental samples and also irrespective of the XA treatment. These results show that the aforementioned effect of host IDO and XA on the parasite life cycle is not mediated via their action on asexual or macrogametocyte development.

DISCUSSION

Eimeria falciformis shows a strict tropism for epithelial cells in the mouse caecum i.e. the parasite is host-, tissue- and cell- specific. These features along with non-pathogenic nature for humans and a short life cycle make this parasite a decent model for investigating localized host responses at the site of infection as also shown recently by Stange et al (28). The parasite development in mouse, a well-established model host, offers a particular advantage for identifying host determinants governing the parasite interactions. This work shows that induction of IDO1 is co-opted by E. falciformis for an efficient progression of its natural life cycle demonstrating an unanticipated and conceptual exploitation of a key immune (IFNγ signaling) and a biochemical (kynurenine) pathway by an apicomplexan parasite.

Three enzymes can initiate the kynurenine pathway degrading most of the cellular tryptophan into various metabolites collectively termed as kynurenines. IDO1 is constitutively expressed (4,29,30), and induced in a variety of cell types e.g. dendritic cell, macrophage, endothelial cells and fibroblasts (1,5,31,32). The second enzyme tryptophan 2,3-dioxygenase (TDO) is expressed mainly in the liver, induced by the amino acid itself and regulates tryptophan homeostasis (33). Finally, IDO2, sharing ~43% homology with IDO1, is present mainly in kidney, epididymis, testis and liver (34). Unlike IDO1, IDO2 is not inducible by IFNγ and expressed despite the absence of cytokine in IFNγ-knockout mice (34). Its function during infection is questionable. IFNγ, required for limiting parasitic infections including of Eimeria species (35-37), is a potent inducer of IDO1 (38-40). In line, expression of IDO1 is undetectable in IFNγR-knockout mice caeca parasitized with E. falciformis (not shown). In wild type mice, IDO1 expression is restricted to the caecum epithelium probably due to strict tropism of E. falciformis for these host cells, and exerts a previously unanticipated beneficial effect on the in vivo parasite development.

In vivo blocking of IDO increases the burden of parasites such as T. gondii (18) and Trypanosoma cruzi (41) and of fungi (Candida albicans, (42)) in mice, which seems consistent with its antimicrobial role, in vitro. In contrast, inhibition or deficiency of enzyme during other parasitic (Leishmania donavoni, (18); Trichuris muris, (43)) and bacterial (Citrobacter rodentium, (44)) infections reduced the pathogen burden in animals. Moreover, 1-methyltryptophan (1-MT), a competitive inhibitor of IDO, had none (HSV-1) to a negative (BM5) influence on the virus replication in mice (18,45), which occasionally differs with in vitro findings (46). Likewise, IDO1 as a positive determinant of Eimeria growth does not reconcile with canonical antimicrobial function notwithstanding a potential depletion of tryptophan in the infected host cell. It must be noted that a fair comparison of different models is not feasible even within a parasite phylum (i.e. Apicomplexans) due to dissimilar setups, let alone with other pathogens as mentioned elsewhere. Typically, however, the in vivo function of IDO1 during infection with intracellular pathogens appears to be rather pathogen- and/or stage-specific.

Induction of IDO was shown to inhibit T-cell proliferation by a cell cycle arrest, anergy and/or apoptotic events (5,6,14). Eimeria-infected IDO1-knockout animals are not impaired in local or systemic Th1 and IFNγ responses. Instead, a downstream metabolite of tryptophan catabolism, xanthurenic acid, underlies this finding. Xanthurenic acid is produced from 3-hydroxykynurenine as a byproduct of detoxification process (47,48). More importantly, it is known as a gametocyte-
activating factor for *Plasmodium*, which facilitates its sexual development by promoting exflagellation of the male gametes in the midgut of mosquito (49). The restoration of the parasite growth in IDO1-knockout mouse shows that host-derived xanthurenic acid is also required for an efficient progression of *Eimeria*, which underlies host IDO1 and kynurenine pathway. Accordingly, the development of *Plasmodium* species is impaired in the mosquito mutant (kh* Aedes aegypti*), which lacks kynurenine 3-hydroxylase (50). Our data do not support the prospect that the asexual growth and macrogametocyte development of *E. falciformis* are influenced by IDO1 and XA. Instead, we suggest that the effect of XA on *E. falciformis* development in mouse is similar to *Plasmodium* in mosquito host i.e. on the maturation of male gametes. Exploitation of tryptophan catabolism by both apicomplexan parasites in their respective host appears to be evolutionarily conserved despite a switch of two- to/from one-host life cycle. Whether *Plasmodium* species induce the only counterpart of mammalian IDO (i.e. TDO) in mosquito to co-opt the tryptophan catabolism remains to be determined.

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**FOOTNOTES**

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Abbreviations: 1-MT, 1-methyltryptophan; IDO, Indoleamine-2, 3-dioxygenase; IFNγ, Interferon gamma; KAT-II, Kynurenine aminotransferase II; Kyn-3OH, Kynurenine 3-hydroxylase; Ro61-8048, 3,4-dimethoxy-N-[4-(3-nitrophenyl)-2-thiazolyl]benzenesulfonamide; S-ESBA.HCl, (S)-4-(ethylsulfonyl)benzoylalanine hydrochloride; TDO, Tryptophan 2,3-dioxygenase; XA, Xanthurenic acid

Data deposition: NCBI accession IDs of the sequences derived from this research: *Ef*Gam56, JK730356; *Ef*Gam82, JK730357

**FIGURE LEGENDS**

Fig. 1: *Life cycle of Eimeria falciformis in the natural host mouse.* (A) NMRI mice were infected with indicated amounts of oocysts. The parasite yield was evaluated from d6 until d14 post-infection. (B) Total shed oocyst in response to varying inoculums. (C) NMRI mice infected with 1500 oocysts were dissected from d3 to d8 of infection, and parasitized caeca tissue samples were subjected to transmission electron microscopy. Only the representative images are shown in the panel. Bars: d3 and d5-8, 2.5 μm; d4, 1 μm. (D) PCR Detection of the parasite’s growth and transition to sexual development. Total RNA was isolated from the parasitized NMRI animals infected with 50 oocysts, and subjected to standard PCR for indicated transcripts. The *Ef*18S and *Mm*18S rRNA was detectable throughout the course of infection. Gam56 and Gam82 were first detectable on d6 post-infection Bars represent the mean ± SE of two independent assays each with 3-5 animals per group. Abbreviation: iSz, immature schizont; mSz, mature schizont; m, merozoite; maGam, macrogametocyte; miGam, microgametocyte and; wfb, wall forming body.

Fig. 2: *Eimeria falciformis induces indoleamine 2,3-dioxygenase in the mouse caecum epithelium.* (A) Quantitative PCR of IDO transcript in the parasitized and enriched epithelial cells. The fold induction was normalized to the *Mm*18S rRNA. Bars show the mean ± SE of three independent experiments. (B) Immunoblot of the caeca tissue collected on various days of infection. *Mm*IDO1 was detected as a single band of 46-kDa, and β-tubulin (50-kDa) was included as a loading control. (C) The tissue homogenates were subjected to anti-*Mm*IDO1 and anti-*Mm*β-tubulin immunoblot analysis (panel B). Expression of IDO was calculated by densitometric means and normalized to β-tubulin. Three independent blots were used and the mean ± SE are shown. (D) Immunohistochemical staining of IDO in the caecum of naïve and infected (d1, d6, d8) Balb/c mice. EC, epithelial cell; Lu, lumen; LP, lamina propria and; Sm, Submucosa

Fig. 3: *IDO is required for the in vivo development of Eimeria falciformis.* (A) WT Balb/c and IDO1−/− mice were infected with 50 oocysts and the parasite development was quantified by counting the number of oocysts shed in feces. (B) Kinetics of oocyst release from the d6-14 post-infection. Bars show the mean ± SE of three independent assays each with 8 animals/group. (C) Effect of 1-methyl-DL-tryptophan (1-MT) treatment on the in vivo development of *E. falciformis.* Outbred NMRI or inbred Balb/c animals were infected with 50 oocysts and the oocyst yield in comparison to untreated controls was determined.
Bars represent mean ± SE of two independent experiments with 5 to 6 animals per group. (D) Bodyweight loss in WT Balb/c and IDO1−/− mice from the panel A. * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.0005.

**Fig. 4:** Inhibition of 3-kynurenine hydroxylase and kynurenine aminotransferase impairs the life cycle of *Eimeria falciformis*. Balb/c mice infected with 50 oocysts were treated with S-ESBA or Ro61-8048. Total oocyst yield (A) and output kinetics (B) were quantified in treated and control animals. Bodyweight of animals (C) was monitored to deduce any signs of morbidity in groups from the panel A. Graphs show mean ± SE of two independent experiments each with 5 mice per group. *** p ≤ 0.0005.

**Fig. 5:** Xanthurenic acid can completely restore the impaired parasite development in IDO1−/− mice. Total parasite yield (A) as well as the kinetics of oocyst shedding (B) was measured in indicated groups. Bodyweight of animals (C) was monitored to deduce any signs of morbidity in groups from the panel A. Bars show mean ± SE of two independent experiments with 5 animals/group. * p ≤ 0.05, *** p ≤ 0.0005.

**Fig. 6:** Asexual and macrogametocyte development of *E. falciformis* are not influenced in IDO1−/− mice. The animals were infected with 1500 oocysts and treated with xanthurenic acid (XA), as indicated. Total RNA derived from the parasitized caeca was subjected to quantitative PCR as described in materials and methods. (A) Specific detection of the indicated parasite (*Ef*18S rRNA, *Ef*Gam82 and *Ef*Gam56) and host (*Mm*18S rRNA) ESTs by qPCR followed by gel electrophoresis. To evaluate the relative abundance (dCt) of individual transcripts, *Ef*18S rRNA (B), *Ef*Gam82 (C) and *Ef*Gam56 (D) in each animal group, their Ct values were normalized to respective host samples (*Mm*18S rRNA). Error bars represent the mean ± SE of two independent experiments.
**Figure 1**

A

![Graph showing Oocyst Yield (x 10^6) vs. days post-infection for different Oocyst Inoculum levels: 10, 100, and 500.](image)

- **Y-axis**: Oocyst Yield (x 10^6)
- **X-axis**: Days post-infection (6 to 14)

B

![Bar chart showing Total Oocysts (x 10^6) for different Oocyst Inoculum levels: 10, 100, and 500.](image)

- **Y-axis**: Total Oocysts (x 10^6)
- **X-axis**: Oocyst Inoculum (10, 100, 500)

C

- **Day 3**: mSz
- **Day 4**: iSz
- **Day 5**: m
- **Day 6**: iSz
- **Day 7**: maGam
- **Day 8**: maGam, wfb

D

![RT-PCR gel showing amplification of **Mm18S rRNA**, **EfGam56**, **EfGam82**, and **Ef18S rRNA** genes.](image)

- **Mm18S rRNA**: 131 bp
- **EfGam56**: 142 bp
- **EfGam82**: 148 bp
- **Ef18S rRNA**: 185 bp

**Legend**

- naïve
- 1, 2, 3, 4, 5, 6, 7, 8
- (day p.i.)
Figure 2

A

![Graph showing IDO1 Transcript (Fold Induction) versus days post-infection. Bars represent expression levels at different time points.

B

![Western blot images for naïve and infected samples. M. tuberculosis (Mtb) proteins IDO1 and β-tubulin are shown at 46-kDa and 50-kDa bands, respectively.

C

![Graph showing IDO1 Expression (Arbitrary Units) versus days post-infection. Bars represent expression levels at different time points.

D

![Immunohistochemistry images for naïve and infected samples. Anti-Mtb IDO1 antibodies highlight cellular structures, with images showing the distribution of Mtb proteins at different time points post-infection.]
Figure 3

A

Total Oocysts (x 10^6)

WT (n = 23)  IDO1^-/- (n = 24)

B

Oocyst Yield (x 10^6)

WT  IDO1^-/-

C

Total Oocysts (x 10^6)

NMRI (n = 10)  Balb/c (n = 11)

D

Bodyweight [%]

1-Methyltryptophan

-  +  -  +

days post-infection

WT  IDO1^-/-
Figure 4

A

Total Oocysts (x $10^6$)

- w/o (n = 10)
- S-ESBA (n = 9)
- Ro 61-8048 (n = 9)

B

Oocyst Yield (x $10^6$)

- WT
- S-ESBA
- Ro 61-8048

C

Bodyweight [%]

days post-infection

- WT
- S-ESBA
- Ro 61-8048
Figure 5

A

![Graph showing total oocysts (x 10^6)]

<table>
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<tr>
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<th>WT (n = 10)</th>
<th>IDO1^-/- (n = 10)</th>
<th>IDO1^-/- (+XA) (n = 8)</th>
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B

![Graph showing oocyst yield (x 10^6)]

C

![Graph showing bodyweight [%]]
Figure 6

A

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<td>(+ XA)</td>
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*Ef18S rRNA* 185 bp

*Mm18S rRNA* 131 bp

Asexual Development

*EfGam82* 148 bp

*EfGam56* 142 bp

Macrogametocyte Development

*Ef18S rRNA* [dCt]

B

![Graph showing relative abundance of Ef18S rRNA](image)

C

![Graph showing relative abundance of EfGam82](image)

D

![Graph showing relative abundance of EfGam56](image)
The apicomplexan parasite, Eimeria falciformis, Co-opts host tryptophan catabolism for life cycle progression in the mouse
Manuela Schmid, Maik J. Lehmann, Richard Lucius and Nishith Gupta

J. Biol. Chem. published online April 25, 2012

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