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# The autoimmune response elicited by mouse hepatitis virus (MHV-A59) infection is modulated by liver tryptophan-2,3-dioxygenase (TDO)

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ABSTRACT

In a previous work we demonstrated that inhibition of mouse indoleamine 2,3-dioxygenase (IDO) by methyltryptophan (MT) exacerbated the pathological actions of mouse hepatitis virus (MHV-A59) infection, suggesting that tryptophan (TRP) catabolism was involved in viral effects. Since there is a second enzyme that dioxygenates TRP, tryptophan-2, 3-dioxygenase (TDO), which is mainly located in liver, we decided to study its role in our model of MHV-infection.

Results showed that *in vivo* TDO inhibition by LM10, a derivative of 3-(2-(pyridyl) ethenyl) indole, resulted in a decrease of anti- MHV Ab titers induced by the virus infection. Besides, a reduction of some alarmin release, i.e, uric acid and high-mobility group box1 protein (HMGB1), was observed. Accordingly, since alarmin liberation was related to the expression of autoantibodies (autoAb) to fumarylacetoacetate hydrolase (FAH), these autoAb also diminished. Moreover, PCR results indicated that TDO inhibition did not abolish viral replication. Furthermore, histological liver examination did not reveal strong pathologies, whereas mouse survival was hundred percent in control as well as in MHV-infected mice treated with LM10.

Data presented in this work indicate that in spite of the various TDO actions already described, specific TDO blockage could also restrain some MHV actions, mainly suppressing autoimmune reactions. Such results should prompt further experiments with various viruses to confirm the possible use of a TDO inhibitor such as LM-10 to treat either viral infections or even autoimmune diseases triggered by a viral infection.

## 1. Introduction

Tryptophan (TRP), an essential amino acid for mammals, is degraded by three oxygenases [1]: tryptophan 2,3 dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO1) and indoleamine 2,3-dioxygenase-2 (IDO2). These enzymes dioxygenate TRP, which is the first step of the pathway leading to kynurenine. The liver is the main source of TDO, which can also be produced, under certain stimulations, by the placenta, the testes and the brain [1]. IDO can be found throughout the body, except in the liver [2]. Methyltryptophan (MT) can inhibit IDO but not TDO. The latter is inhibited by LM10, also named 680C91, a derivative of 3-[2-(pyridyl) ethenyl] indole [3,4].

In addition to liver inflammation, the strain A59 of the mouse hepatitis virus (MHV-A59) causes a transient demyelination and various immune disturbances: involution of the thymus, IgG2a hypergammaglobulinemia [5–7], and the production of autoantibodies (autoAb) to fumarylacetoacetate hydrolase (FAH). MHV induced this autoimmunity in several mouse strains [8]. The antigenic mimicry between FAH and MHV proteins was probably responsible for the autoAb production, which could have been enhanced by an adjuvant effect of alarmins such as uric acid and high-mobility group box1 protein (HMGB1), which are released in the blood during the MHV-infection [9–12].

IDO has been recognize as a central player in an immune regulatory mechanism called “metabolic immune regulation” that participates in a variety of pathological settings like chronic infections, cancer, inflammation and transplantation [1]. The TRP catabolic products produced by IDO-named "kynurenines"- are responsible for triggering different inhibitory or stimulatory immune responses depending on the TRP metabolite, the cell and the physiological context [13]. Some TRP metabolites are known to be pro-inflammatory; such as quinolinic acid (QA), and others have anti-inflammatory functions, such as kynurenic acid (KA) [14–16]. In infectious disease the proinflammatory signals, IFN- γ and IFN- β, induced by the infection enhanced IDO activity

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leading to a robust and sustained TRP degradation [14].

Since IDO has immunomodulatory properties, we expected that blocking its activity would amplify the defense reactions and thus protect mice against the MHV virus. On the contrary, MT significantly increased the virus-induced effects: hypergammaglobulinemia, production of anti-MHV antibodies and anti-FAH autoAb, as well as uric acid and HMGB1 release. The survival of infected mice treated with MT was significantly reduced compared to controls, and hepatic fibrosis was found histologically [17].

In the present study, using LM10 to inhibit mouse liver TDO, we asked whether the enzyme could modulate the effects of MHV-infection. Results showed that LM10 reduced levels of Ab to the virus, alarmin release and autoAb to FAH in MHV-infected animals. Besides, mouse survival was not affected. Thus, both TRP oxygenases, TDO and IDO, should act in opposite way regarding MHV activity. Moreover, data suggest that it is worthwhile to explore whether TDO could be a target to control autoimmune and/or viral response in other pathological models.

## 2. Materials and methods

### 2.1. Mice

Female BALB/c mice 8–10 weeks old were purchased at the University of La Plata, Argentina. These specific-pathogen-free (SPF) animals were maintained in isolators and received care according with international legal requirements.

### 2.2. Preparation of MHV stock and viral infection

The NCTC 1469 adherent cell line was used to prepare the MHV-A59 stock as described in [17]. Before using in ELISA assays the virus was UV inactivated [18]. Mice were inoculated intraperitoneally with $10^4$ TCID50 of MHV-A59 and bled at different times.

### 2.3. Treatments

LM10 (a derivative of 3-[2-(pyridyl) ethenyl] indole [3]) was a gift from doctors B. Van den Eynde and L. Pilotte, Ludwig Institute for Cancer Research, Christian de Duve Institute, Université Catholique de Louvain, Brussels, Belgium. Mice were given LM10 (160 mg/ kg/day) *ad libitum* in drinking water 72 h before infection with MHV and for the next 14 days. The animals were bled at 7, 14 and 28 days after MHV-infection (mice named “MHV + LM10”). As controls, other groups of mice were only infected with the virus (“MHV” mice) or treated with LM10 alone (“LM10” mice).

### 2.4. Determination of TDO activity in liver homogenates

According to the method of Elbers et al. [19], livers were homogenized in three times their weight in buffer 50 mM Tris-HCl, 25 % sucrose, 1 mM EDTA pH 7.4. Following, 400 $\mu$l of liver homogenate was added to 1 mL of TDO-buffer (200 mM potassium phosphate buffer pH 7.0, 10 mM ascorbic acid, 5 $\mu$M hematin, and 10 mM L-tryptophan.) and distributed in 48-well microtiter plates. After incubating for 4 h at 37 °C the reaction was stopped using 1/10 v/v 30 % trichloroacetic acid and submitted to centrifugation (400 *g*,10 min). Supernatants were mixed with an equal volume of Ehrlich's reagent and absorbance was detected at 492 nm in a microplate reader.

Kynurenine (Sigma-Aldrich, St. Louis, USA) diluted in TDO-buffer was used as standard. For the calculation of the kynurenine content, linear regression and GraphPad Prism software were employed.

### 2.5. Determination of liver tryptophan concentration

Liver from BALB/c mice treated and controls were removed, soaked in chilled PBS and ground in a Dounce homogenizer at 4 °C with 5 volumes of PBS containing 10 − 3 M phenylmethyl-sulfonyl fluoride (PMSF). The homogenates were centrifuged for 15 min at 400 × g and the clarified extracts kept at −20 °C until used.

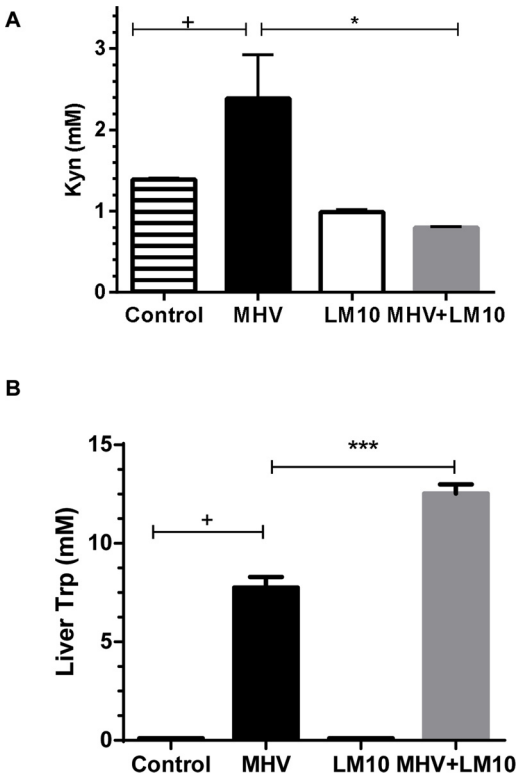


**Fig. 1.** TDO activity and TRP levels in liver homogenates after 14 days of MHV-infection and/or treatment.
Livers were homogenized as described in Materials and Methods, and kynurenine (A) or TRP (B) concentration in supernatants was determined. Bars indicate the mean value ± SEM. Statistical significance of each treatment in relation with MHV * $p \leq 0.05$, *** $p \leq 0001$ or in relation with control values, + $P \leq 0.05$ was determined by one-way ANOVA.

Tryptophan was detected according the colorimetric method decrypted by Bao et al. 2006 [20] with a modification to enhance the accurate and highly specific of detection according Huang et al. 2011 [21]. Essentially, a sample of each liver suspension was heating for 5 h at 50 °C in a solution containing 6 M hydrochloric acid, 18 % formic acid and 0,2 % of DMSO. The colored derivatives 1-formyltryptophan was detected at 580 nm.

### 2.6. Liver fumarylacetoacetate hydrolase (FAH) preparation

Semi-purified enzyme was prepared from rat livers as described previously [8]. In brief, livers were homogenized and submitted to centrifugation at 100,000*g* for 1 h. Later, ethyl alcohol was added to the supernatant to obtain a final concentration of 50 % ethanol. After various steps of centrifugation and precipitation with ethanol or solid ammonium sulfate, the final pellet was resuspended in 20 mM Tris/HCl pH 8.0 and dialyzed against the same buffer. Protein concentration was determined by the method of Bradford [22].

### 2.7. Western-blot assays

#### 2.7.1. Determination of autoAb anti-FAH

As described elsewhere [9,10,17], detection of autoAb to liver FAH was performed by standard western-blot methods. Essentially, semi-purified liver FAH (100 µg) were subjected to 10 % SDS-PAGE and then transferred onto nitrocellulose sheets (GE Healthcare,

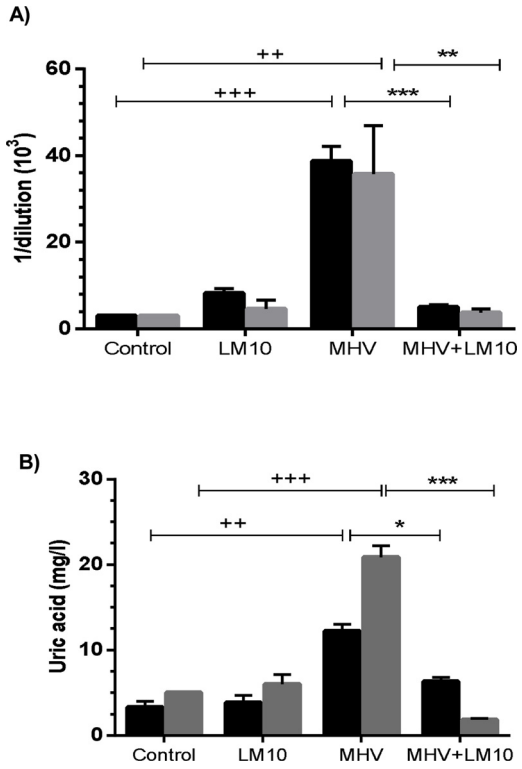


**Fig. 2.** Concentration of anti-MHV Ab (A) and uric acid (B) in sera from mice submitted to the described treatments.
A) ELISA assays were carried out as explained in Materials and Methods. Results are expressed as the mean serum dilution of pooled sera from 10 to 12 mice to reach an optical density value of 1.0.
B) Uric acid was determined with a commercial kit as indicated in Materials and Methods. Results are mean ± SD of three independent determinations performed in each case with pooled serum from seven mice.
Results correspond to 14 (black bars) and 28 (grey bars) days post infection and/or treatment. Normal: animals without any treatment or infection. Statistical significance of each treatment in relation with MHV (*P ≤ 0.05; *** P ≤ 0.001) or in relation with control values (+ + P ≤ 0.01; + + + P ≤ 0.001) was determined by one-way ANOVA.

Buckinghamshire, UK). After blocking non-specific Ab-binding sites the strips were incubated with serum dilutions from control or treated mice. Bound Ab were revealed with peroxidase labeled goat anti-mouse IgG (Santa Cruz Biotechnology, CA, USA) and ECL-plus reagent (GE Healthcare, Buckinghamshire, UK). As a positive control, a pooled serum from FAH immunized mice was used.

#### 2.7.2. Determination of HMGB1 in serum

As already explained [9,13], to detect HMGB1, mouse sera were filtered with Centricon YM-100 (Millipore Corp, USA) and then concentrated 15-fold with Centricon YM-30. Western-blot analysis was carried out as described above, and HMGB1 was revealed with MAb anti-HMGB1 HAP46.5 (Santa Cruz Biotechnology, CA, USA) and peroxidase-labeled goat anti-mouse IgG [9,13].

### 2.8. Determination of anti-MHV Ab

To test anti-MHV Ab by ELISA as already described [13], plates were coated with UV inactivated MHV, blocked with 0.01 M Tris, 0.13 M NaCl, pH 7.4, containing 5 % of fetal calf serum and then incubated with serum from mice submitted to the different treatments. Bound Ab were revealed as described before for Ig assays.

### 2.9. Serum uric acid concentration

The assay kit Uricostat test (Wiener Lab, Rosario, Argentina) was used to determine uric acid concentration in mouse sera [9. 17].

### 2.10. Transaminase determination

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using the GOT (AST) Unitest (Wiener lab., Rosario, Argentina)

### 2.11. Nested PCR analysis

Total RNA was extracted from feces of mice submitted to the different treatments [23]. RNA was transcribed into cDNA using Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit (Life Technologies Ltd, UK) following the manufacturer's instructions. Thus, 0.1-0.5 µg of RNA was added to Oligo (dT) (100 pmol), Reverse Transcriptase (RT) Buffer (4 µl), Ribolock RNase Inhibitor (0.5 µl), dNTP Mix (1 mM, 2 µl), RevertAid Reverse Transcriptase (200 U, 1 µl) and diethylpyrocarbonate (DEPC) water up to 20 µl. The reactants were incubated at 42 °C for 60 min followed by 10 min at 70 °C. Nested PCR was performed as described before [17]. In the first PCR, cDNA (2 µl) were mixed with 10 µM each of 7P10 and 7N998 primers (sequences GTTCCTGGGCAAGAAAATGC and ACCAATTCTAATTTAGATCC, respectively), 2.5 µl of 5x MyTaq Reaction Buffer (Bioline, UK), MyTaq DNA Polymerase (0.06 µl) (Bioline, UK), water in a total volume of 12.5 µl. Amplification was carried out at 94 °C (4 min), 32 cycles of (94 o C for 1 min, 54 o C for 1.5 min, 72 °C for 2 min and final extension step at 72 °C for 5 min.

To achieve the second PCR, 2 µl of the first PCR product was used following the same amplification conditions and reaction components with the primers pair 7P148 and 7N722 (sequencesAAGCAGACTGCA ACTACTCA and ACAAGAGCAGCAATTTCTTC, respectively). The PCR products (target 575 bp fragment) were electrophoresed on agarose gel 1 %. Visualization was done using ethidium bromide.

### 2.12. Statistical analysis

Statistical significance between experimental values was calculated using the one-way ANOVA test. To compare the differences in mouse mortality rates between groups, the Kaplan–Meier method was utilized. All statistical analysis were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. LM10 administration inhibited liver TDO activity

Since kynurenine is the final product of the TRP degradation elicited by TDO, the enzyme activity is directly related to the concentration of that metabolite [19]. Thus, kynurenine levels in liver homogenates from control and treated mice showed that MHV-infection significantly augmented TDO activity over basal values, whereas LM10 administration did not produce any effect (Fig. 1A). By contrast, increase of TDO activity induced by the viral infection was notably reduced by LM10 treatment (Fig. 1A). Also, liver TRP levels were elevated in infected mice, and these values augmented after LM10 treatment (Fig. 1B)

### 3.2. LM10 administration did not affect transaminase (ALT/GOT and AST/GPT) values

It is largely accepted that MHV-infection yields an important raise of ALT and AST values in serum [6,9,17]. Accordingly, we found that "MHV" animals exhibited elevated ALT serum levels after 7 and 14 days of infection (87.0 ± 4.0 and 92.0 ± 13.0 U/l, respectively, whereas

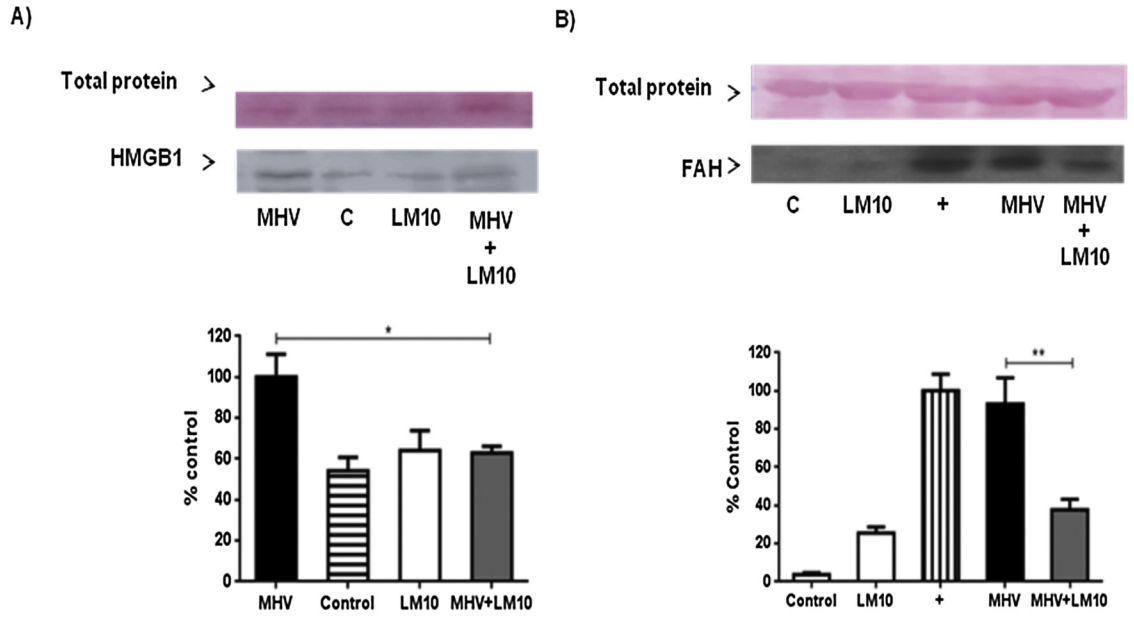


**Fig. 3.** Effect of LM10 on HMGB1 release (A) and autoAb to FAH (B) induced by MHV- infection. A) Pools of sera from six mice submitted for 14 days to the indicated treatments were used to detect HMGB1 by Western-blot assays as described in Material and Methods. Arrow shows the HMGB1 position.
B) Ab to mouse liver FAH were determined by Western-blot assays as described in Materials and Methods using pools of sera from six mice. Arrow shows the position of the bands corresponding to FAH. Positive control corresponds to an anti-FAH serum.
Normal: mice without any treatment. Both tests were repeated at least three times with similar results. Total protein was determined by Ponceau S stain and the intensity of each band was quantified by Gel-Pro Analyzer and is presented as percentage of MHV-infected serum (A) or FAH immunizated serum (B). Statistical significance was determined by one-way ANOVA (* $P \leq 0.05$ or ** $P \leq 0.01$).

control value was 38.3 ± 4 U/l). In addition, LM10 treatment did not change those figures (data not shown). Similar results were shown for AST determination (data not shown).

### 3.3. LM10 administration decreased anti-MHV Ab titers

Anti-MHV Ab titers were determined by ELISA and calculated as the serum dilution to obtain an OD = 1.0. Results showed that Ab titers significantly augmented over control after 14 and 28 days of MHV-infection (Fig. 2A). Moreover, “MHV + LM10” animals showed a strong reduction of Ab to MHV, whereas in “LM10” mice Ab titers did not differ from controls (Fig. 2A).

### 3.4. LM10 treatment diminished uric acid and HMGB1 release

Elevated concentrations of uric acid and HMGB1 were found in plasma from MHV-infected mice [9]. Present results revealed that LM10 treatment significantly reduced levels of plasmatic uric acid produced by MHV action, whereas “LM10” animals displayed uric acid levels similar to controls (Fig. 2B).

Furthermore, after 14 days of LM10 treatment, there was a remarkable decrease of plasmatic HMGB1 levels in “MHV + LM10” animals compared with “MHV” mice (Fig. 3A). Serum from “LM10” mice showed a band similar to controls (Fig. 3A).

### 3.5. LM10 administration suppressed the expression of autoAb to liver FAH

AutoAb to liver FAH, and their relationship with alarmin liberation, were described previously in MHV-infected mice [8,9]. Here we show that after 14 days of LM10 treatment the expression of autoAb in serum from MHV-infected animals significantly decreased (Fig. 3B). Conversely, administration of LM10 alone did not produce any effect (Fig. 3B).

### 3.6. Detection of MHV by nested PCR

In order to determine whether LM10 could produce the inhibition of MHV replication, detection of MHV in feces from infected mice treated or not with LM10 was performed as indicated in [19]. Results did indicate the presence of MHV in feces of infected mice under LM10 treatment at the different times tested (Fig. 4).

### 3.7. Effect of LM10 treatment on survival of MHV-infected mice

Results indicated that, under our experimental conditions, neither MHV-infection nor LM10 administration affected mouse survival during 28 days, the time of the assays (Fig. 5). Besides, LM10 administration to MHV-infected mice slightly induced mouse mortality, the percent of survival being 85 % at the end of the assays, but results were not significantly different from “MHV” mice according to Kaplan-Meier test (Fig. 5).

## 4. Discussion

Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) managed the majority of TRP degradation to kynurenine in mammals [24,25]. TDO regulates systemic TRP levels [18] and plays a fundamental role in the physiological regulation of TRP flux in the human body. Hepatic TDO was involved in stress-induced depression-related behavior in the rat, because the enzyme leads to the production of neuroactive metabolites through TRP catabolism [26]. More recently, Lanz et al. [27] demonstrated that TDO deficiency is associated with subclinical neuroprotection in a mouse model of multiple sclerosis. In this paper, we described a new action of TDO, *i.e.*, its property to modulate a viral infection.

LM10 was designed to specifically inhibit TDO in order to be used in tumor rejection. The lack of IDO inhibition by LM10 was demonstrated at the same time [4]. Under our experimental conditions, liver kynurenine concentration strongly augmented over control after MHV-infection, and significantly diminished after LM10 mouse treatment.

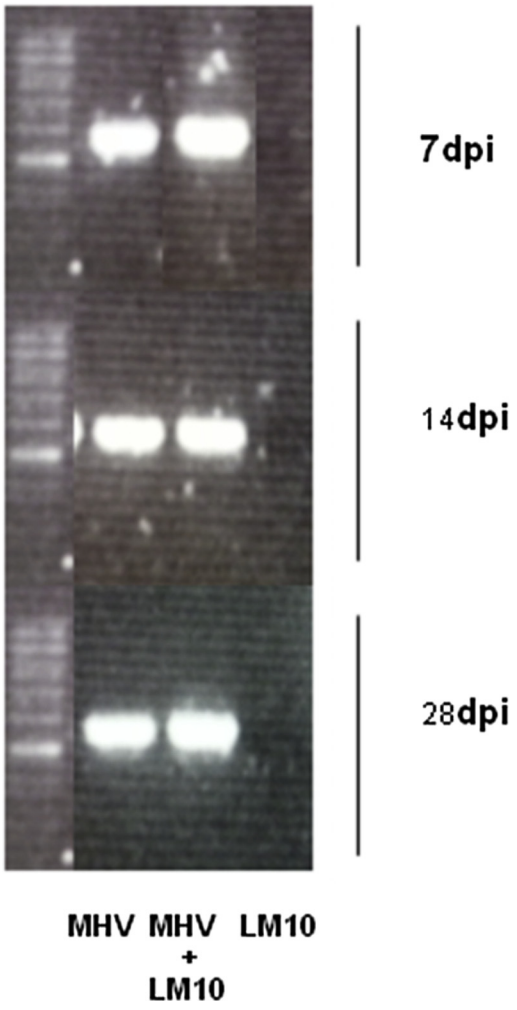


**Fig. 4.** Fecal excretion of MHV in mice submitted to different treatments. Nested PCR was performed as indicated in Material and Methods. PCR products at the indicated times and treatments were detected by ethidium bromide. dpi: days post-infection and/or treatment.

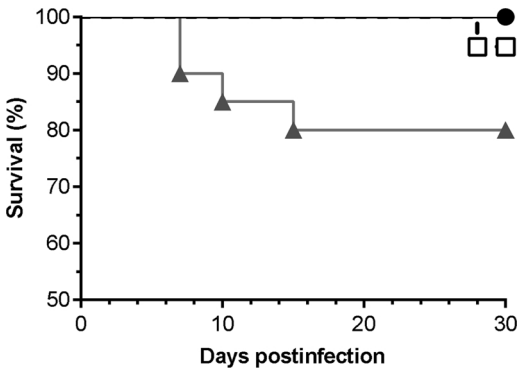


**Fig. 5.** Effect of LM10 administration on survival of MHV-infected mice. Data are taken from five independent experiments. Symbols are as follows: (●) "MHV" mice (N = 26), (□) "LM10 mice" (N = 20), and (▲) "MHV + LM10" mice (N = 24). The Kaplan–Meier test was utilized to compare the differences in mortality rates between groups.

These data indicated that TDO expression was stimulated by the virus infection, being subsequently inhibited by LM10 administration.

We have demonstrated a strong association between the high concentration of uric acid and HMGB1 protein in plasma and the production of autoAb to liver FAH [9]. In addition, it was shown that IDO inhibition by MT induced large alarmin liberation with the concomitant generation of autoAb [17]. By contrast, herein we showed that LM10 treatment inhibited uric acid and HMGB1 liberation as well as the induction of autoAb to FAH. In this way, the effect of TDO inhibition is analogous to those of allopurinol and ethyl-pyruvate that we have described before [9]. Actually, it was proposed that allopurinol should inhibit TDO by preventing the conjugation of the enzyme with its cofactor [28].

Furthermore, results showed that TDO inhibition decreased the Ab to MHV. These effects could be related to the lesser extent of alarmin liberation described above, although this fact was not tested in our previous paper [9]. Besides, TDO inhibition did not affect neither mice survival nor liver histopathology. Even if these last observations could indicate that the virus was eliminated from the host, PCR results did show the presence of MHV in mouse fecal extracts during the time of experimentation.

It has been proposed that TRP catabolism, performed in tumor tissues by IDO and/or TDO, is an important microenvironmental factor that suppresses anti tumor immune responses [29]. Thus, inhibitors of both enzymes are now published [2,4,30] and some of them have been tested in clinical trials in patients with solid cancer [31,32].

The TRP/kynurenine pathway is also involved in non-cancer models. For example, in a nephrotoxic serum glomerulonephritis model, inhibition of IDO by MT enhanced Th1 response whereas IgG1 isotypes decreased and IgG2a isotype rose, respectively. This change of IgG subclass was consistent with splenocyte cytokine production [33]. Furthermore, Scott et al. [34] found that inhibiting IDO activity had the unexpected consequence of ameliorating, rather than exacerbating, arthritis symptoms. MT treatment led to decreased autoAb titers, reduced levels of inflammatory cytokines, and an attenuated disease course [34]. Additionally, IDO inhibition at the time of vaccination decreased humoral immune response to hepatitis B surface [35].

A main distinction between TDO and IDO is that their expression is modulated by different situation and, in addition, each of these enzymes has a particular tissue distribution. TDO is located primarily in the liver and is induced by TRP, metabolic steroids and insulin. On the contrary, IDO is present in many extra-hepatic tissues, and is induced by IFN-γ during an immune response [13]. In our model of MHV-infection, IDO and TDO inhibition originated opposite results. In fact, whereas MT treatment amplified and aggravated the condition of mice infected with MHV [17], inhibition of TDO by LM10 administration ameliorated the mouse pathological signs of MHV-infection. Consequently, results suggest that the solely kynurenine decrease should not be responsible of the various effects on viral activity. Thus, the differential regulatory mechanisms controlling TRP metabolism suggested previously [13], could clarified the opposite responses observed after TDO or IDO inhibition in MHV-infected mice. TRP flux down the Kyn pathway is determined by the levels of plasma TRP and by hepatic TDO activity. After TDO activity inhibition, TRP catabolisms in the liver diminish, leaving TRP available for IDO-mediated production of Kyn metabolites on immune cells. Thus, LM10 treatment on MHV-infected mice, allows the use of TRP catabolism to regulate immune response against MHV. Consequently, IDO-mediated immune response modulation is required to respond to MHV infection, while TDO activity should have mainly metabolic role in this scenario. As it has been proposed in the "tryptophan -utilization hypothesis" the importance of TRP metabolism in infection is associated to the production of different Kyn metabolites that regulate immune cells response mediated by IDO, rather than with the limitation of TRP as a regulatory mechanism [13,36].

In an outstanding publication, Plotz [37] suggested that auto-antigen (autoAg) structural characteristics in addition to their location and catabolism could explain the complexity of the autoimmune repertoire. Since restraint of alarmin release almost eliminated the autoimmune response to FAH produced by MHV, we have proposed that the liberation of these endogenous adjuvants induce the compromised dendritic cells to "confound" FAH molecule with a foreign Ag because

its similitude with some MHV proteins [9].

Nevertheless, results presented in this work suggest that specific TDO blockage could suppress autoimmune reactions, Ab production and alarmin liberation produced by MHV. Thus, LM10 could be considering a good candidate to study its effect on some viral infections and/or autoimmune pathologies.

## Declaration of Competing Interest

No conflict of interest were disclosed by MDV, JLA, MFM and LAR.

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