Universidad de la República

Facultad de Ciencias

Programa de Desarrollo de Ciencias Básicas – Biología

Immunoregulatory mechanisms induced by *Fasciola hepatica*

Ph.D. Thesis in Biology

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Montevideo, May 2024

Tesis de Doctorado en Ciencias Biológicas

Programa de Desarrollo de las Ciencias Básicas (PEDECIBA)

Área Biología

Sub-área Biología Celular y Molecular

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Epigraph

"Aos esfarrapados do mundo e aos que neles se descobrem e, assim descobrindo-se, com eles sofrem, mas, sobretudo, com eles lutam."

Paulo Freire (1987)

"Our task is to learn, to become God-like through knowledge. We know so little. You are here to be my teacher. I have so much to learn. By knowledge we approach God, and then we can rest. Then we come back to teach and help others."

Brian Weiss (1988)

Acknowledgments

When it comes to work carried out over many years, as is generally the case with a doctoral thesis, there are always many people to thank. All the people who have come into my life over the last 6 years have contributed somehow to this journey, and I would like to thank them here.

I especially need to thank my advisor, Teresa Freire, for all your support, trust, and friendship over the last few years. I will miss you greatly, but your affection and teachings will always accompany me.

I would not have reached this point without the support of my parents, Januza da Silva and Vitor da Silva Costa. Whenever I look back, I remember that rainy morning when I moved to start college and the warm welcome and encouragement I received. I am incredibly proud to be your daughter.

I found my life partner during this beautiful and challenging journey. Pablo de Cecco, thank you for being my lighthouse on the darkest days. I say it and repeat it: You light up my life every day. I am grateful every day for our family and our little puppies.

As a foreigner, I encountered several difficulties during my journey to a new country. However, my companions always welcomed me with open arms, offered their help whenever needed, and made my days in the lab much easier. I want to express my gratitude to Florencia, Valeria, Miguel, Santiago, Mercedes, Pablo, Eugenia, Ignacio, Guillermina, Juan, Natalie, Hanna, and Alice for being so welcoming and sharing their experiences with me. Thank you so much!

I thank my good friends from my homeland who always listened to me and advised Luiza, Beto, Rebeca, Prediger, Alicia, and Gislene. I Love you. I would also like to thank my psychologist, Patricia Carvalho, for her work with me in recent years. Thank you very much for your commitment and welcoming words.

Last, I would like to express my gratitude to ANII, PEDECIBA, CSIC, INIA, INTA Castelar, Dr. Alejandra Capozzo and Marfrig for their support in developing this thesis.

Abstract

Fasciola hepatica, a highly prevalent helminth parasite in Uruguay, is responsible for significant economic losses linked to livestock production, mainly bovine meat and dairy production. The problem of fasciolosis has increased due to parasite resistance to anthelmintic drugs. Furthermore, despite the availability of drugs, such as triclabendazole (TCZ), for the treatment of fasciolosis, it does not prevent the disease, liver damage, or parasitic reinfection. On the other hand, infected animals present a strong immunoregulation induced by the parasite, making the development of vaccines difficult and increasing susceptibility to secondary infections. One of the alternatives is to identify molecular targets in the host that allow us to associate the severity of the disease with molecules expressed by the natural host. In this sense, our work has sought to identify and evaluate immunoregulatory mechanisms deployed by the parasite. This thesis is divided into two parts: in the first instance, we use an experimental murine infection model, and in the second, we study the infestation in a natural host (bovine).

The murine experimental model has allowed us to analyze the immune system in detail, finding that the parasite regulates the host immune system by inducing Th2 and regulatory T immunity (Treg) through the overexpression of heme oxygenase 1 (HO-1). In peritoneal antigen-presenting cells (APC). HO-1 is involved in heme catabolism and has antioxidant and immunoregulatory properties. Furthermore, HO-1$^+$ APCs also express a receptor of the innate immune system (MGL2) that recognizes carbohydrates of parasitic origin and modulates the induction of the adaptive immune response. By inhibiting HO-1 activity, we detected increased production of reactive oxygen and nitrogen species (ROS/RNS) in peritoneal APCs and decreased mice susceptibility to parasitic infection. As an alternative strategy to evaluate the role of F4/80$^+$ HO-1$^+$ cells in infection, we used MGL2-DTR transgenic mice, which encode the diphtheria toxin receptor in MGL2$^+$ cells. In this study, we demonstrate that peritoneal APCs during infection favor parasite survival, which is mediated by the induction of splenic Tregs *in vivo*. Depletion of MGL2$^+$ cells conferred partial resistance to infection in mice and abrogated the parasite-induced increase in Tregs, demonstrating that MGL2$^+$ (F4/80$^+$ HO-1$^+$) cells are critical for *F. hepatica* infection. and could constitute immunological checkpoints to control parasite infection.

In this thesis, we also characterize the anti-parasitic immune response in experimentally infected cattle and evaluate the effect of the parasite on vaccine-induced immunity. We detected that the

parasite generates significant liver damage, analyzed indirectly through the levels of liver enzymes *in vivo* and by an evaluation score at slaughter. TCZ treatment reduced parasite burden and liver damage but could not eliminate all parasites, and liver damage persisted. In addition, we investigated the cellular response generated by the infection, and we found that at 43 days post-infection (dpi), there is an increase in $CD4^+$ and $CD21^+$ cells in infected animals. The percentage of $CD11c^+$ cells does not differ between the groups. When the humoral and cellular immune response was investigated, we found that specific antibodies against *F. hepatica* remained elevated during the first 12 weeks of infection. However, in the chronic phase of infection, antibody levels decreased considerably. The treatment with TCZ affects the quantity and quality of specific antibodies against *F. hepatica*. The TCZ also increases the levels of IFN-γ, IL-10, and IL-4 in the liver.

Finally, we studied the impact of the parasite on the adaptive immune response generated by vaccination against *Clostridium* spp and foot-and-mouth disease virus (FMDV) during the acute stage and against respiratory pathogens (*Pasteurella multocida* and *Mannheimia haemolytica*) in the chronic stage. At 43 dpi, the animals in the infected group presented a decrease in the levels of IgG antibodies against *Clostridium* spp. We also found that IgG1 levels against FMDV decreased significantly in infected animals at 28 dpi and presented a lower avidity than antibodies from uninfected animals. In the same sense, when evaluating the respiratory vaccine, we found that infected animals showed lower levels of specific antibodies against *M. haemolytica* than those from the control group. Interestingly, TCZ treatment restored vaccine antibody titers. However, *P. multocida*-specific IgG decreased both in the infected group of animals at 213 dpi.

In conclusion, this thesis provides knowledge about the immunoregulatory mechanisms generated by *F. hepatica* in two experimental models of infection, contributing to the identification of strategic targets for the control of infection by this parasite and the importance of the immunomodulation generated by the parasite in vaccine-triggered immunity in cattle.

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List of abbreviations

ABTS.	2,2 -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACK.	Ammonium–Chloride–Potassium
ADCC.	Antibody-dependent cytotoxicity
ALB.	Albumin
ALT.	Alanine-transaminase
APC.	Antigen-presenting cells
AST.	Aspartate aminotransferase
BCA.	Bicinchoninic acid
BHV-1.	Bovine herpesvirus serotype 1
BVD.	Bovine viral diarrhea
BRD.	Bovine respiratory disease
BRSV.	Bovine respiratory syncytial virus
Ca.	Calcium
CAT.	Catalase
CO.	Carbon monoxide
CoPP.	Cobalt-protoporphyrin IX
CLRs.	C-type lectin receptors
DAMPs.	Danger-associated molecular pattern
DBil.	Direct Bilirubin
DC.	Dendritic cells
DEPC.	Diethylpyrocarbonate
DCFDA.	20,70 -dichlorofluorescein diacetate
DHR.	Dihydrorhodamine 123
DNA.	Deoxyribonucleic acid
DT.	Diphtheria toxin
Dpi.	Days post-infection

EDN.	Eosinophil-derived neurotoxin
EDTA.	Ethylenediaminetetraacetic acid
ELISA.	Enzyme-linked Immunosorbent Assay
EPC.	Eosinophil cationic protein
EPG.	Egg per gram
EPO.	Eosinophil peroxidase
FhESP.	*F. hepatica* excretory-secretory products
FhNEJ.	*F. hepatica* newly excysted juveniles
FhTE.	*F. hepatica* Total Extract
FMO.	Fluorescent-minus-one
Foxp3.	Forkhead box P3
GGT.	Gamma-glutamyltransferase
Gpx.	Glutathione peroxidase
Hb.	Hemoglobin
HCT.	Hematocrit levels
HO-1	Heme oxygenase 1
IBR.	Infectious Bovine Rhinotracheitis
IFN.	Interferon
Ig.	Immunoglobulins
IL.	Interleukin
ILC.	Innate lymphoid cells
INIA.	Instituto Nacional de Investigación Agropecuaria
LPM	Large peritoneal macrophage
IU.	International units
FMD.	Foot-and-mouth disease
MCV.	Mean Corpuscular Volume
MGL.	Macrophage Galactose Lectin
MHC.	Major histocompatibility complex

MPB.	Major basic protein
MPV.	Mean Platelet Volume
MR.	Mannose receptor
NLR.	NOD-type receptors
NO.	Nitric oxide
NS.	Non-structural
OD.	Optical density
OPD.	O-phenylenediamine
PAMPs.	Pathogen-associated molecular patterns
PBMC.	Peripheral blood mononuclear cells
PBS.	Phosphate Buffer Saline
PCR.	Rolymerase chain reaction
PCT.	Plateletcrit
PDW.	Platelet distribution width
PLT.	Platelet number
PEC.	Peritoneal exudate cells
PI-3.	Parainfluenza virus serotype 3
PMA.	Phorbol myristate acetate
PRRs.	Rattern recognition receptors
RBC.	Red blood cells
RLR.	RIG-type receptor
RNA.	Ribonucleic acid
RNS.	Reactive nitrogen species
ROS.	Reactive oxygen species
RT.	Room temperature
SD.	Standard deviations
SICCT.	Single intradermal comparative cervical tuberculin test
Sn.	Tin

SnPP.	Sn-protoporphyrin
SOD.	Superoxide dismutase
SPM	Small peritoneal macrophage
TBil.	Total Bilirubin
TCZ.	Triclabendazole
TGF.	Transforming growth factor
Th.	T helper
TLR.	Toll-type receptors
Tm.	melting temperature
TNF.	Tumor necrosis factor
TP.	Total protein
WC1.	Workshop cluster 1
Wpi.	Week post-infection

Introduction

2 Introduction

2.1 Animal Health

To get into this thesis topic, it is essential to understand that animal production rests on four pillars: genetics, nutrition, management, and animal health. Animal health is considered by the Food and Agriculture Organization of the United Nations (FAO) an essential topic in the elimination of hunger, improving the health of people, and producing sustainable food production (Bertoni, 2021), entering the topic into the concept of "One Health."

Worldwide, livestock production currently represents 40% of the gross value of agricultural production and presents an increasing result of population growth (Bruinsma, 2012). In recent decades, human activities such as the increase of urbanization, climate change, and deforestation have brought animals and humans into closer proximity. About 75% of newly emerging infectious diseases come from animals and are responsible for recent outbreaks such as severe acute respiratory syndrome (SARS), Ebola, and Avian Influenza (FAO, 2023).

In undeveloped countries, animal health is a limitation often hindered by health problems such as bacterial, viral, and parasitic diseases (Bertoni, 2021; Tagesu, 2019). Thus, ensuring good health in livestock is crucial to reducing the risk of new pathogens and food-borne diseases (Bertoni, 2021; FAO, 2023; Zinsstag et al., 2011). It also helps prevent economic and socio-economic problems, such as production losses, disruptions to national and international trade, and threats to the livelihoods of the poor populations (Bertoni, 2021).

2.2 Infection diseases of interest in cattle

2.2.1 Viral infections

As mentioned, infectious and parasitic diseases remain significant obstacles to produce profitable livestock in various regions (Bruinsma, 2003). Many of these diseases are caused by viruses, leading to substantial financial losses due to production losses caused by morbidity and mortality (Gebreyes et al., 2020).

The most important viral diseases must be reported to federal agencies and the World Organization for Animal Health (OIE); these diseases include foot-and-mouth disease (FMD), rabies, bluetongue, viral hemorrhagic fever, among others (Gebreyes et al., 2020). An example of the importance of these diseases is that FMD outbreaks cause an estimated annual loss of USD 6.1 billion to 200 billion in endemic countries (Tewari et al., 2020).

In addition, many economically important viruses affecting cattle are not reportable, like enteric and respiratory diseases, which are of high concern. They are multiagent syndromes caused by a mixture of viral and bacterial agents (Gebreyes et al., 2020; Snowder et al., 2006).

In gastrointestinal diseases, the cause of an outbreak is rarely known. Increased mortality and morbidity are often due to the presence of multiple pathogens (Blanchard, 2012; Castells & Colina, 2021). In adult cattle, the most common viral agents of diarrhea are coronavirus, bovine viral diarrhea (BVD), and torovirus. However, diarrhea can also be observed in outbreaks caused by an orthobunyavirus (Castells & Colina, 2021).

The bovine respiratory disease (BRD) is characterized by pneumonia (Fernández et al., 2020). The BRD is a severe problem in feedlots, negatively affecting farm productivity (Snowder et al., 2006). The most common viral agents found in the lungs are BVD, bovine respiratory syncytial virus (BRSV), bovine herpesvirus serotype 1 (BHV-1), and parainfluenza virus serotype 3 (PI-3) (Gagea et al., 2006).

2.2.2 Bacterial infections

Bacterial diseases are a prevalent problem in livestock, affecting young and adult animals and significantly impacting cattle health and productivity. In recent years, the issue of increased antibiotic resistance (AR) has raised concerns about treatment and potential risks to human health (Hernando-Amado et al., 2020).

In adult cows, the gastrointestinal bacterium pathogens of highest concern include *Mycobacterium paratuberculosis* (*M. avium* subsp. paratuberculosis, the cause of Johne's disease) and *Salmonella* spp (Wells et al., 2002). In addition, the bacterial agents commonly found in BRD are *Mycoplasma bovis, Mannheimia haemolytica,* and *Pasteurella multocida* (Gagea et al., 2006).

Another important bacterial agent in livestock is *Clostridium* spp, a Gram-positive, rod-shaped, anaerobic, and spore-forming bacterium, and consists of around 250 species distributed worldwide (Khiav & Zahmatkesh, 2021). Also, *Clostridium* spp is responsible for producing enterotoxaemia, gas gangrene, necrotic enteritis, blackleg, and black disease (Khiav & Zahmatkesh, 2021). *Clostridium* exotoxins can cause damage ranging from mild to severe in the gastrointestinal tract, soft tissues, and nervous system (Carter et al., 2014).

Moraxella bovis is a bacterium that causes bovine keratoconjunctivitis, the most common ocular disease affecting cattle globally (Postma et al., 2018). This Gram-negative coccobacillus bacterium doesn't usually result in fatalities, but it can cause decreased weight gain, reduced milk production, and extra treatment costs. Additionally, it may lead to market discounts due to eye disfigurement and blindness (Postma et al., 2018).

Bacterial diseases can have a significant impact on the reproduction of cattle. Brucellosis, caused by *Brucella abortus*, is a contagious zoonotic bacterial disease that affects the reproductive organs of cattle and generates abortion, infertility, and reduced milk production (Abdelhay Kaoud, 2019). Leptospirosis, another significant bacterial disease, is also zoonotic and can cause reproductive problems such as abortion, stillbirths, and infertility (Abdelhay Kaoud, 2019).

2.2.3 Parasite infections

Parasitic diseases are a significant challenge in cattle health because it can affect animals of all ages, production systems and have zoonotic potential, posing risks to human health. Parasites can be classified into two types: ectoparasites or endoparasites, with or without direct contact with the external environment respectively, respectively (Scheifler et al., 2019). Additionally, parasitic infections can lead to secondary bacterial infections, exacerbating clinical symptoms and complicating treatment strategies (Brady et al., 1999; Corrêa et al., 2020; Howell et al., 2018).

Gastrointestinal nematodes are a type of parasitic worm that can inhabit the digestive tract of animals. Some of the most common species include *Haemonchus contortus, Ostertagia ostertagi, Teladorsagia circumcincta, Cooperia oncophora,* and *Trichostrongylus colubriformi* (Arsenopoulos et al., 2022; Högberg et al., n.d.; Liu et al., 2023). These worms can cause symptoms such as diarrhea, weight loss, anemia, and reduced feed efficiency (Liu et al., 2023).

Liver fluke infection, caused by the trematode parasites *Fasciola hepatica* and *F. gigantica*, is responsible for liver damage, fibrosis, bile duct obstruction, and impaired liver function. Clinical signs include reduced productivity, weight loss, and anemia (M. Costa et al., 2022). Coccidiosis is a disease caused by protozoan parasites belonging to the genus Eimeria. These parasites attack the intestinal tract of cattle, causing enteritis, dehydration, weight loss, reduced growth rates, and bloody diarrhea, especially in young calves (Ashfaq et al., 2023).

Various types of external parasites can transmit pathogens that cause diseases in cattle, some of which are transferable to humans (Pérez de León et al., 2020). These pathogens can result in skin damage, irritation, blood loss, and discomfort, leading to decreased food consumption and weight gain (Pérez de León et al., 2020). Many species of Diptera (two-winged insects) are hematophagous, can cause myiasis at the immature stages or larvae, and invade living soft tissue. In addition, horn flies are mechanical vectors of bacteria causing bovine mastitis, while the face fly is the mechanical vector of *Moraxella bovis* (Pérez de León et al., 2020).

Ticks present an obligate blood-feeding habit; this animal modulate the innate immune response in the host by factors secreted in their saliva to remain attached in the host and parasitizing the same host on multiple occasions (Sonenshine & Roe, 2013; Pérez de León et al., 2020). Ticks are critical vectors of diverse pathogens, such as protozoa, bacteria, and viruses. Bovine babesiosis, anaplasmosis, theileriosis, and heartwater are among the diseases listed as notifiable by the OIE (Pérez de León et al., 2020).

A preventative measure that can be taken to combat many diseases (viral, bacterium, and parasites) is using prophylactic methods, such as following vaccination schedules. However, some diseases currently do not have available vaccines, which can be a limiting factor. Additionally, this strategy often requires cooperation between the general population and public health organizations to plan and prevent disease outbreaks.

2.3 Animal production in Uruguay - main limitations of health

Agricultural production is one of the most important sectors of the economy in Uruguay, representing almost 75% of the country's total exports (DIEA Yearbook, 2023). Livestock production is one of the most practiced activities, where national cattle farming represents 44% of all agricultural production in 2021 (DIEA Yearbook, 2023). The total area of meat farming reaches 13 million hectares, with around 11,6 million cattle and 6,2 million sheep in our country.

Uruguay is among the leading global exporters of bovine meat (FAO, 2018), and the animals are commonly raised in free-range systems (Picasso et al., 2017). However, there has been a recent shift in livestock farming from purely pastoral systems to intensive corral enclosure systems, where the confined animals are adults and generally come from a diet based on forage (Banchero G et al., 2016). Currently, 10% of the total slaughtered animals and approximately 20% of slaughtered steers are fattened in these systems, which implies a potential risk of disease occurrence (Banchero G et al., 2016). A study by Banchero et al. (2016) found that in our country the most important infectious diseases in intensive systems were clostridial diseases, keratoconjunctivitis and respiratory problems.

However, pasture can also serve as a reservoir of many diseases, mainly for younger categories. Many pathogens can survive in nonhost environments for months, and infection of herbivores grazing on pastures is possible (Klose et al., 2022). *Clostridium* spores can live in the soil for many

years (Tagesu, 2019), and cattle can acquire salmonellosis and brucellosis by grazing on contaminated pastures (Allerberger et al., 2003; Aune et al., 2012). In addition, communal pasture increases the risk of transmission of viruses, like BVDV infection in cattle (Braun et al., 1998; Siegwart et al., 2006).

In Uruguay, bacterial diseases pose a threat to both animal and public health, not only due to their zoonotic potential but also because of reports of antibiotic resistance in calves (Casaux et al., 2019; Umpiérrez et al., 2017). Costa et al., (2000) that there is a large spread of BRSV in the bovine population of Uruguay (95% of the samples analyzed). Also, in our country, beef cattle are commonly exposed to BVB and BHV-1 virus, and the animal-level seroprevalence ranges from 69% and 37%, respectively (Guarino et al., 2008).

Importantly, grazing cattle are at high risk of tick, gastrointestinal diseases, and helminth infections (Bishop & Stear, 2003; Lean et al., 2008; Miraballes & Riet-Correa, 2018). The localization and the temperate climate of Uruguay are considered marginal for the development of cattle ticks, which has been an essential problem in Uruguayan livestock farming over the last 100 years (Miraballes & Riet-Correa, 2018).

An epidemiological study on gastrointestinal parasites in beef cattle carried out in Uruguay found that the most common parasites found were *Cooperia* spp., *Ostertagia* spp., *Trichostrongylus axei, Oesophagostomum radiatum* and *Haemonchus placei* (Berdie et al., 1988). *F. hepatica* is also an important helminth in our country; it is present in 18 of the 19 departments and has a prevalence of ~70% to 100% on farms (R. A. da Costa et al., 2019).

2.3.1 Vaccines Used in Uruguay

Preventing infectious diseases in beef cattle is crucial but challenging due to exposure to novel pathogens, stress, and commingling with other animals during different stages of production (Richeson et al., 2019). Vaccinating cattle is a common practice in the livestock sector. However, not all farmers adopt this preventive measure, leaving a significant portion of the cattle population vulnerable to diseases that could have been prevented (Guarino et al., 2008; "Reproductive, Health and Management Characteristics in Dairy Herds in Uruguay," 2021; Richeson et al., 2019).

According to the Ministerio de Ganadería, Agricultura y Pesca, the country is free from the FMDV with vaccination. Cattle population is vaccinated twice a year in animals younger than 2 years old (Costa et al., 2024). It is also important to highlight that Uruguayan legislation does not allow live vaccines (De Brun et al., 2021).

The government recommendation (not mandatory) is vaccination in the first stage of life, mainly against *Clostridium* spp. and symptomatic Carbuncle (*C. chauvoei*). In addition, the use of polyvalent vaccines in reproductive animals, including *Campylobacter* spp., *Leptospira* spp., IBR and BVD, and *Brucella* spp., is also recommended.

Our country's vaccination schedule is not standardized except for the FMDV vaccine. It varies according to the needs of each property and is determined by the responsible veterinarians. For instance, in some feedlots across the country, animals must be vaccinated against respiratory diseases before entering their facilities (Banchero G et al., 2016).

There is scarce data on vaccination adherence by producers in Uruguay. Some studies showed that approximately 50% of the calf population is vaccinated against severe neonatal calf diarrhea (NCD)(Castells et al., 2019). In addition, 97.1% of producers vaccinated their animals against clostridial diseases before entering the feedlot (Banchero G et al., 2016). Another study found that only 3% of beef herds in Uruguay regularly (typically annually) use vaccines against BVD (Guarino et al., 2008). The following table shows the primary vaccines used within Uruguayan territory.

Table 1. Major vaccines used in Uruguay.

Vaccines	Antigens	Mandatory	Immunization age
Foot-Mouth- Disease	A24/ Cruzeiro O1/Campos	Yes	>2 years old
***Clostridium* spp**	*C. chauvoe, C. novyi* *C. perfringens, C. septicum, C. haemolyticum, C. sordellii, C. tetani*	No	>1 year old
Moraxella bovis	*Moraxella bovis*	No	Determined by veterinarian
Respiratory Diseases	IBR, BVD, BRSV, PI3 *Pasteurella multocida* *Mannheimia haemolytica* *Haemophilus somnus*	No	Determined by veterinarian
Reproductive Disease	BHV-1/5, BVD *Leptospira interrogans* *Leptospira borgpetersenii* *Campylobacter fetus*	No	Determined by veterinarian

2.4 *Fasciola hepatica* and Fasciolosis

2.4.1 General information about helminth parasites

Helminth is a term commonly used for worms, and encompasses a diverse group of multicellular eukaryote invertebrate organisms that present bilateral symmetry with elongated, flat, or round bodies (Castro, 1996). Some helminths are parasites and have diverse definitive and intermediate hosts, ranging from mammals to birds, reptiles, mollusks, or arthropods. Helminths can be classified in three groups: cestodes, trematodes and nematodes. Cestodes and trematodes are flatworms (playhelminths) while nematodes are roundworms. *F. hepatica* is a trematode parasite (Castro, 1996).

It is estimated that approximately one-third of people living on less than USD 2.00 per day are infected with one or more helminths in undeveloped regions, like sub-Saharan Africa, Asia, and the Americas (Hotez et al., 2006, 2008). The most common types of helminth infections in humans are ascariasis, trichuriasis, hookworms, schistosomiasis, and filarial nematodes (Hotez et al., 2008).

This means that people living in thousands of poor rural villages in the tropics and subtropics are often infected with multiple parasites over long periods, and children are the group that presents a greater risk of infection by worms (Hotez et al., 2008). Parasitic infections cause delayed growth, decreased physical condition, and impaired memory and cognition in affected people (Crompton & Nesheim, 2002).

In undeveloped countries, livestock is a critical and often the sole source of economic security for poor populations, and helminth infection can affect animal production (Piedrafita et al., 2010). Also, it can provide an interface for transmission to humans (Majewska et al., 2021). *Fasciola* spp. is one of the most important zoonotic helminth infections with a global economic impact on livestock production systems and with a direct effect on human health (Piedrafita et al., 2010).

2.4.2 Fasciolosis

Fasciolosis is a neglected tropical disease caused by *Fasciola hepatica* and *F. gigantica* (Mas-Coma, 2003; Mehmood et al., 2017). These species have a differential distribution globally, while *F. hepatica* can be found in temperate zones such as Australia, Europe, the Americas, and East Africa. *F. gigantica* is found in the tropics such as South-East Asia, India, the Middle East and sub-Saharan Africa (Piedrafita et al., 2010).

In Latin America, fasciolosis is very prevalent in humans and livestock. Bolivia and Peru have a high prevalence (15–66%) of human liver-fluke infection (Carmona & Tort, 2017; Mas-Coma et al., 1999), particularly among indigenous Aymaran people and especially children (Parkinson et al., 2007). However, the prevalence of fasciolosis in humans is often underestimated due to the lack of epidemiological monitoring and difficulties in diagnosis in endemic areas (Cwiklinski et al., 2016a).

The trematode *F. hepatica* can affect all mammals but occurs predominantly in ruminants (cattle, sheep, and goats) (Piedrafita et al., 2010). Fasciolosis has significant economic implications and generates financial losses for farmers, butchers, and consumers (Mehmood et al., 2017). The disease leads to liver condemnation, poor quality carcass, reduced growth rate, decreased conception rate, lower productivity, and even mortality (R. A. da Costa et al., 2019; Mehmood et al., 2017).

In the American continent, fasciolosis was reported in United States, Mexico, Cuba, Peru, Chile, Uruguay, Argentina, Jamaica, and Brazil (Usip et al., 2014). Very high prevalence rate 86% has been documented in cattle from Argentina and in Peru where fasciolosis is recognized as the major problem in cattle production (Mehmood et al., 2017).

In Uruguay, fasciolosis is highly prevalent in livestock and present in most cattle-raising departments (18 of 19 departments) (R. A. da Costa et al., 2019). According to the study of National Animal Health Research Plan (PLANISA, 2009) 81% of the liver condemnations in our country is caused due to *F. hepatica*, estimating a loss of USD 2.64/liver, a total of USD 6,5 million/year.

2.4.3 Causal Agent and Life Cycle of *F. hepatica*

The adult *F. hepatica* has a broad, flat, leaf-shaped body that is usually 20-50 mm long and 6-13 mm wide (Bogitsh et al., 2013; Mas-Coma & Bargues, 1997). Its suckers are relatively small, with the ventral sucker (acetabulum) being slightly larger than the oral sucker (Mas-Coma & Bargues, 1997) (Figure 2.1). It has highly branched testes and intestinal caeca; a short and convoluted uterus; and vitellaria that extend along the lateral edges of the body to the posterior end (Bogitsh et al., 2013).

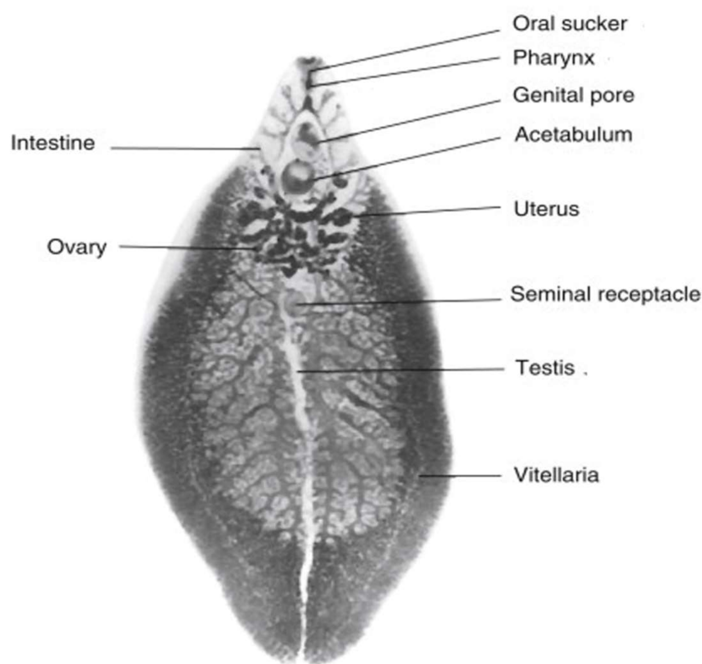

Figure 2–1. Structure and morphology of an adult worm of *F. hepatica.* Extracted and modified from (Bogitsh et al., 2013)

The adult form of *F. hepatica* lives in the bile ducts of mammalian hosts, when it reaches sexual maturity, it releases eggs into the duodenum along with bile. The eggs are then eliminated from the host in feces (Mas-Coma & Bargues, 1997; Moazeni & Ahmadi, 2016). The parasite undergoes an aquatic life cycle where its eggs mature in water. If the climatic conditions are favorable (15-25°C) (Mas-Coma & Bargues, 1997; Moazeni & Ahmadi, 2016), the miracidium will develop

inside the eggs after two to three weeks. In unfavorable climatic conditions, the eggs may remain viable for months (Lapage, 1968; Mas-Coma & Bargues, 1997).

The miracidium hatches and swims rapidly using its cilia until it contacts an amphibious snail of the genus *Lymnaea* which act as intermediate host (Mas-Coma & Bargues, 1997; Moazeni & Ahmadi, 2016). In Uruguay, the main snail that acts as an intermediate host is *Lymnaea neotropica* (Bargues et al., 2017). Miracidia failing to penetrate an appropriate snail die within 24 hours (Olsen, 1986). After penetrating the snail, the miracidium sheds its ciliated covering and develops into a sporocyst (Moazeni & Ahmadi, 2016).

A sporocyst is a cluster of germinal cells, where each germinal cell multiplies and produces a redia. As the redia grow, they break through the sporocyst wall and are released into the snail's digestive gland (liver) (Moazeni & Ahmadi, 2016). The redia is a structure that contains germinal cells. These cells multiply through three generations, giving rise to the cercaria larval stage. The development of cercaria takes about 4-7 weeks after snail infection at a temperature of 20-25°C. However, the development process is slowed at lower temperatures (Mas-Coma & Bargues, 1997; Moazeni & Ahmadi, 2016).

Cercaria swims briefly until it reaches solid support, often water plant leaves above or below the water line, using its long tail to aid in swimming (Mas-Coma & Bargues, 1997; Moazeni & Ahmadi, 2016). Subsequently, the cercaria loses its tail and encysts to form a *metacercaria,* which is the infectious form for definitive hosts (Moazeni & Ahmadi, 2016).

After the ingestion, the *metacercariae* are digested in the host's small intestine in about 1 h, the newly excysted juvenile worms of *F. hepatica* (FhNEJ) penetrate the intestine wall and enter the abdominal cavity in about 2 h, and cross the peritoneal cavity reaching the liver in 4-6 dpi (Moazeni & Ahmadi, 2016). The FhNEJ migrates in the liver parenchyma for 5 to 6 weeks post infection (wpi), causing most of the pathology associated with acute fascioliasis. After 7 wpi maturing flukes enter in the bile ducts (Hoyle & Taylor, 2003; Moazeni & Ahmadi, 2016).

In cattle, the eggs from adult worms are found in the feces approximately 11 wpi, completing the life cycle (Valero et al., 2011), and the adult fluke can remain for up to 1 or 2 years in the bovine host (Moazeni & Ahmadi, 2016). Figure 2.2 shows the biological cycle of *F. hepatica* with a timeline to exemplify the parasite infection process in the bovine host.

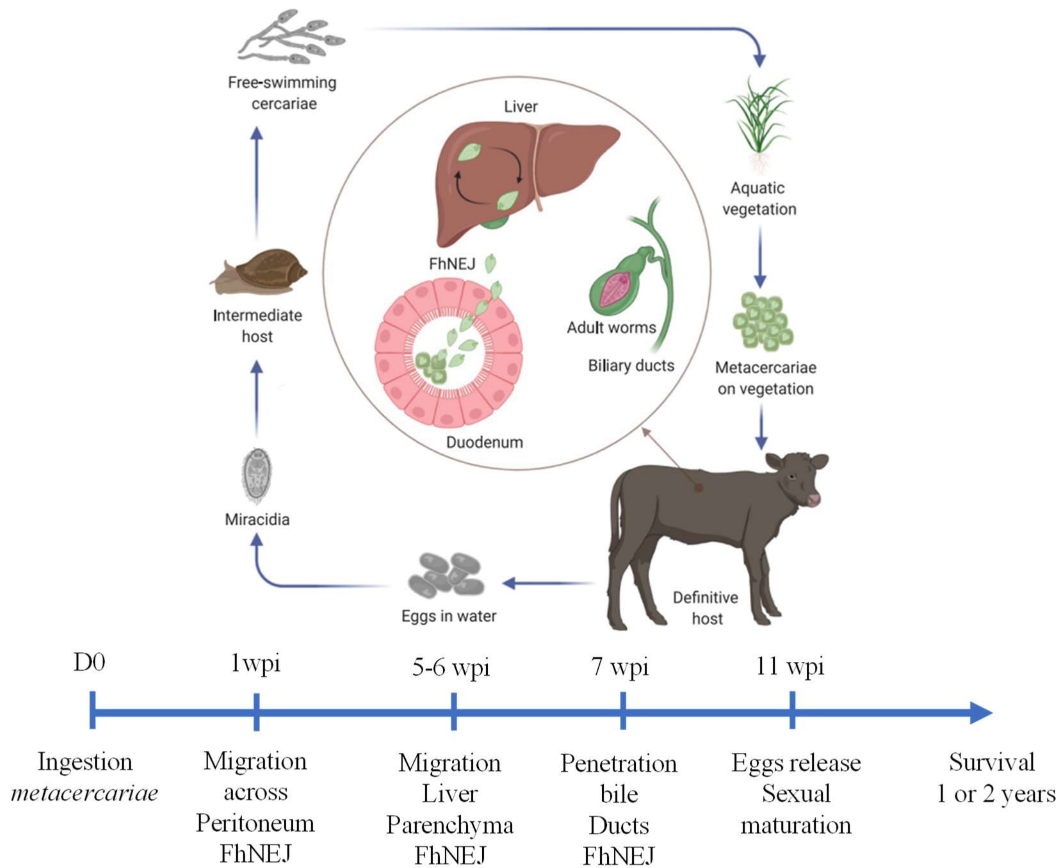

Figure 2–2. Biological cycle of *F. hepatica* and timeline with principal events in the host during the infection. The parasite requires two types of hosts: the intermediate (snails of the genus *Lymnaea*) and the definitive (mammalian). Once the eggs mature in water, they eliminate the miracidium that will infect the snail. Inside the snail, the larva will go through several stages until emerging as cercaria, which then matures to the metacercaria stage and can infect the definitive host. Adapted and modified from: (González-Miguel et al., 2021)

2.4.4 Control and Treatments Currently Available against fasciolosis

Currently, the most effective strategy for combating fasciolosis is the use of anthelmintic drugs, including triclabendazole (TCZ), to eliminate the parasite in the host and reduce the contamination of pastures with eggs (Keiser et al., 2005; Thakare et al., 2019). TCZ is a benzimidazole derivative with a high specific activity against *Fasciola* spp in all stages of infection (Gandhi et al., 2019; Thakare et al., 2019).

TCZ has been widely used to treat livestock since 1983 (McCarthy & Moore, 2014), and its permitted use in humans since the 1990s (Gandhi et al., 2019). However, in recent decades, reports of resistance to TCZ have increased worldwide (Merachew & Alemneh, 2020; Moll et al., 2000; Olaechea et al., 2011). In addition, the treatment does not prevent liver damage, and the animals do not develop protective immunity against *F. hepatica* (Costa. et al., et al., 2022). They can become infected with each new exposure to the parasite.

The development of a vaccine is of great interest in the livestock industry since it would avoid the administration of drugs, reduce parasite resistance, save time and costs, and avoid drug residues in products of animal origin (Dalton et al., 2013). Various technologies, including recombinant, attenuated, subunit, cocktail and nucleic-acid-based, have been tested as vaccines against *F. hepatica* (Beesley et al., 2018; Lalrinkima et al., 2021; Molina-Hernández et al., 2015; Toet et al., 2014). However, none of them have been approved since their effectiveness is limited (Rehman et al., 2023). Additionally, most vaccine candidates have been derived from adult worms; this could be problematic since adult worms are anatomically less accessible to the immune response than juvenile forms (González-Miguel et al., 2021). Another obstacle in the production of *F. hepatica* vaccine is the immunosuppressive response induced by the parasite, which can hinder parasite elimination (Rehman et al., 2023).

2.4.5 Experimental models of fasciolosis

Experimental models are crucial for researching the development of new knowledge about host-pathogen interactions, vaccines, and drugs. To study fasciolosis, commonly used experimental models include sheep, cattle, goats, buffaloes, mice, rats and rabbits (Hussein & Khalifa, 2008).

F. hepatica can affect all mammals, but the degree of susceptibility and parasite burden of infection varies widely among different species (González-Miguel et al., 2021). In laboratory animals such as rats and mice, 5-10 *metacercariae* inoculation is enough to establish *Fasciola hepatica* infections (Hussein & Khalifa, 2008). However, in sheep, chronic fasciolosis occurs when infected with 200-2000 *metacercariae*, and a dose higher than 5000 can be fatal (Chauvin et al., 2001; Hussein & Khalifa, 2008).

Meanwhile, in cattle, a dose between 150-5000 *metacercariae* is tolerated for developing the disease (Hussein & Khalifa, 2008). In addition to experimental models *in vivo*, *ex vivo* and *in vitro* models were developed to study the molecular mechanisms between host-pathogen (Becerro-Recio et al., 2022; González-Miguel et al., 2020).

2.5 Immune response

The immune system is a set of cells and effector molecules responsible for protecting the body from infectious agents, renewing dead or damaged tissue, and regulating immune response in different situations (Fainboim & Geffner, 2005; Tizard, 2017). The immune system can be divided into two large branches: innate immunity and adaptive immunity.

Innate immunity is the first line of defense against pathogens. It is composed by cells and molecules that are ready to act even before infection. Pathogens possess a variety of molecules that the body recognizes as foreign, which are called pathogen-associated molecular patterns (PAMPs) (Bianchi, 2007). Additionally, molecules released from damaged cells also produce alarm signals, called danger-associated molecular pattern (DAMPs)(Bianchi, 2007).

Both PAMPs and DAMPs bind to pattern recognition receptors (PRRs) present on sentinel cells (dendritic cells, DC, and macrophages mainly) throughout the body (Kigerl et al., 2014). The most studied family of PRRs are Toll-type receptors (TLR). However, other types of receptors are of great interest in research, such as C-type lectin receptors (CLRs), NOD-type receptors (NLR), and

RIG-type (RLR) (Fainboim & Geffner, 2005; Sancho & Reis e Sousa, 2012a; Tizard, 2017; Zelensky & Gready, 2005).

The binding of PAMPs and DAMPs to PRRs is required to produce and secrete molecules that trigger inflammation. The nature of the invader determines the type of cells that are recruited, which could be DC, mast cells, eosinophils, neutrophils, monocytes, or innate lymphoid cells (Bianchi, 2007; Tizard, 2017).

Macrophages are essential sentinel cells that recognize tissue damage, stimulate neutrophil and monocyte recruitment, and produce TNF-α, IL-6, and ROS in response to DAMP release (Moreau & Chauvin, 2010). They are divided into two subgroups based on their functions and activation states: M1 and M2. Macrophages M1 type promotes defenses through inflammation producing ROS/RNS, while M2 suppresses inflammation and promotes tissue repair (Chinetti-Gbaguidi et al., 2015).

Three different sub-classes of M2 macrophages have been identified. M2a macrophages are induced by the Th2 cytokines IL-4 and IL-13. Immune complexes in combination with IL-1β or lipopolysaccharide induce subtype M2b. Lastly, M2c macrophages are induced by IL-10, TGF-β or glucocorticoids (Chinetti-Gbaguidi et al., 2015). This polarization is not necessarily permanent; the macrophages can change their phenotype due to the influence of other cytokines and pathogenic products (Chinetti-Gbaguidi et al., 2015; Tizard, 2017).

DCs are specialized antigen-presenting cells that can initiate adaptive immune responses. DCs have three main functions. First, they act as sentinel cells and activate innate defenses in his first encounter with the invaders. Second, they process antigens, thus initiating adaptive immune responses. Third, they regulate adaptive immunity by determining whether an antigen will trigger a response mediated by antibodies or cell-mediated or may even prevent an immune response (tolerance)(Banchereau et al., 2000; Puhr et al., 2015) .

To activate the adaptive immune system, foreign substances must be captured, processed, and presented to T cells. DC are responsible for presenting antigens (Banchereau et al., 2000; Puhr et al., 2015; Tizard, 2017). Once the PAMPs/DAMPs are recognized, DC begin to mature and migrate from the attacked site to secondary lymphatic organs, where they complete their maturation (Palucka & Coussens, 2016).

The recognized molecule undergoes a proteolysis process through which peptides end up being generated, which they will bind to major histocompatibility complex (MHC) class I and II molecules, depending on whether the pathogenic molecule is intracellular or extracellular, respectively (Fainboim & Geffner, 2005; Palucka & Coussens, 2016).

At this stage, T lymphocytes can be categorized into two groups based on their membrane MHC co-receptor: T $CD4^+$ helper and T $CD8^+$ cytotoxic. $CD8^+$ T cells recognize class I peptide-MHC complexes and directly target cells infected by viruses or tumor cells. $CD4^+$ T lymphocytes, on the other hand, recognize class II peptide-MHC complexes on the surface of an APC and differentiate into different effector populations, each with a specific function (Neefjes et al., 2011).

T helper (Th $CD4^+$) cells play a central role in the adaptive immune system by orchestrating the immune response through the production of different types of cytokines. They are classified based on the cytokines they produce. The initial subtypes that were discovered are called Th1 and Th2. Th1 cells produce IFN-γ, while Th2 cells produce multiple cytokines, including IL-4, IL-5, IL-9, and IL-13. Each subtype is differentiated by specific transcription factors, with the T-bet characteristic of Th1 and GATA-3 being the hallmark of Th2 (Peine et al., 2013).

Th1 lymphocytes promote immunity against intracellular microorganisms by mediating cell immune responses and activating macrophages. When Th1 is activated, it produces IL-2, IFN-γ, and TNF-α (Palucka & Coussens, 2016; Stout & Bottomly, 1989). Type 2 responses are linked to improved immunity against certain helminth parasites. Additionally, they regulate the repairing of tissues and wounds after an injury or infection (DeNardo et al., 2009; Tizard, 2017). The activated Th2 lymphocytes play a crucial role in stimulating the growth of B lymphocytes and the production of immunoglobulins (Akkaya et al., 2020; Palucka & Coussens, 2016).

The Th17 subtype is another important T $CD4^+$ population. The development of Th17 lymphocytes is initiated by IL-23, which induces the production of a specific transcription factor called ROR-γt. The Th17 subtype is important to combat extracellular bacterial and fungal infections and produces several cytokines, such as IL-17 (IL-17A and IL-17F), IL-21, and IL-22 (L. Wang et al., 2009).

Regulatory T cells (Treg) are a type of lymphocytes that express CD4 and CD25 (the α chain of the IL-2 receptor) which need the transcription factor FoxP3 to differentiate. Tregs produce suppressor molecules such as TGF-β, IL10, and IL-35, which help in suppressing the responses of T helper cells. (Fehérvari & Sakaguchi, 2004; Tizard, 2017).

Another important type of $CD4^+$ T cells are the follicular helper (Tfh) T cells. They are crucial in generating and maintaining germinal center reactions that produce long-lasting humoral immunity. Tfh cells are identified by the expression of the chemokine receptor CXCR5, the transcriptional repressor Bcl6, and their ability to migrate to the follicle and promote germinal center B cell responses (Hale & Ahmed, 2015).

It is important to note that there are differences in Th populations across species. Much of the information described above pertains to findings in mice and humans. Bovids possess Th1, Th2 and Th17 lymphocytes, and can develop polarized immune responses (Tizard, 2017). The expression of IgG1 is positively regulated by IL-4 and the expression of IgG2 by IFN-γ. Th17 lymphocytes in cattle produce of IL-17A and IL-17F and moderate amounts of IL-22 and IFN-γ (Cunha et al., 2019).

The proportion of T cells with TCR γ/δ (T γ/δ) varies greatly among different mammals. In humans and mice, the number of T γ/δ expressing cells is low (less than 5%). However, T γ/δ cells are expressed in high quantities in pigs and ruminants. For instance, in young cattle, 66% of lymphocytes are T γ/δ^+. Though this percentage decreases with age, it still remains high in adults (8-18%) (Tizard, 2017).

Approximately 50 to 99% of T γ/δ^+ cells in ruminant blood express workshop cluster 1 (WC1), a transmembrane glycoprotein (Hoek et al., 2009; Tizard, 2017). Studies have demonstrated that the T $\gamma\delta^+$ $WC1^+$ is the major bovine regulatory population with the secretion of IL-10 and TGF-β (Guzman et al., 2014; Hoek et al., 2009; Sachdev et al., 2017). Figure 2-3 shows a model for Th cell differentiation from naive CD4 T cells in different species.

B lymphocytes are another crucial component of adaptive immunity. They are typically located in the cortex of lymph nodes, the marginal zone of the spleen, the bone marrow, throughout the intestine, and in Peyer's patches, with a few circulating through the blood. Whenever a B cell comes across an antigen that binds to its receptors, it responds by secreting its receptors

(antibodies) into bodily fluids (Akkaya et al., 2020; Tizard, 2017; Y. Wang et al., 2020). This occurs after processing and presentation of the antigen on MHC II molecules. This allows the interaction of B cells with Tfh cells involving predominantly CD40L–CD40, ICAM1–LFA1 and SLAM family members and by Tfh cell secretion of the cytokines IL-4 and IL-21 (Akkaya et al., 2020).

Antibody molecules are glycoproteins known as immunoglobulins (Ig). There are five structural classes of immunoglobulins, with the most abundant class found in serum being immunoglobulin G (IgG). The second most abundant class in most mammals is IgM. In most mammals, the third most abundant class is immunoglobulin A (IgA); however, IgA is the most prevalent in secretions like saliva, milk, and intestinal fluids. Immunoglobulin D (IgD) is primarily a B cell receptor (BCR) rarely found in body fluids. Immunoglobulin E (IgE) is involved in allergic reactions and helminth infections and is present in low concentrations in serum (Gutman et al., 1981; Moreau & Chauvin, 2010; Tizard, 2017)

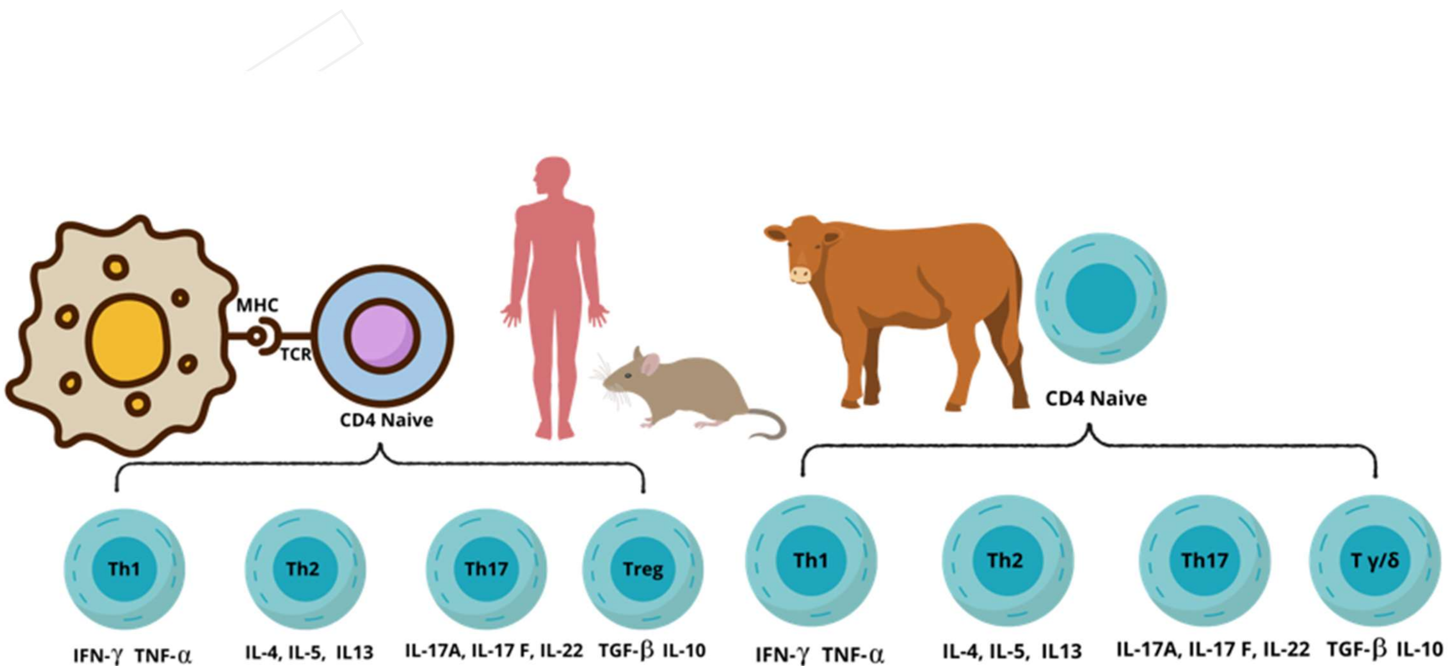

Figure 2–3. Model for Th cell differentiation from naive CD4 1 T cells in different species. Source: Personal archive.

2.5.1 Immunity induced by helminth parasites

The immune response against parasites is complex; they represent one of the most significant challenges to the immune system. They are successful pathogens as they cause chronic infections without killing their hosts, ensuring their survival and transmission (Finlay et al., 2014). Furthermore, *F. hepatica* can infect several species of mammals, resulting in a challenge to gather results and find commonalities in the immune response.

The juvenile stages of *F. hepatica* migrate through tissues and carry out proteolytic processes that depend on cathepsins and other proteases (González-Miguel et al., 2021). The tissue injury induces the release of alarmins, including the three lymphocyte-stimulating cytokines, TSLP, IL-25, and IL-33, from enterocytes (Lekki-Jóźwiak & Bąska, 2024; Tizard, 2017). TSLP activates DCs, while IL-25 and IL-33 stimulate innate lymphoid cells (ILC) to produce type 2 cytokines, help differentiate Th2 lymphocytes, and initiate eosinophil recruitment. To destroy the parasite during the migration the action of eosinophils and inflammatory macrophages is necessary. Both cells have FcεR (CD23) that allows them to bind IgE-coated parasites and destroy them through a process known as antibody-dependent cytotoxicity (ADCC) (Makepeace et al., 2012; Moreau & Chauvin, 2010).

It has been reported that rat macrophages can kill FhNEJ through ADCC by releasing nitric oxide (NO) and ROS (Moreau & Chauvin, 2010; Raadsma et al., 2008). Activated eosinophils discharge cytotoxic granules containing major basic protein (MBP), eosinophil peroxidase (EPO), eosinophilic cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (Lekki-Jóźwiak & Bąska, 2024; Makepeace et al., 2012).

The immune response during the initial stages of fascioliasis is considered a mixed Th1/Th2 response (Ryan et al., 2020). The elevation of cytokines such as IFN-γ, IL-4, IL-10, and TGF-β characterizes this response. In fact, the predominance of Th1 responses during the early stages of infection enhances the host protection against the infection (E. C. B. Araujo et al., 2013; M. Costa et al., 2021). Both IFN-γ and IL-10 are increased during the early stages of bovine *F. hepatica* infection, which supports the idea that the initial immune response is mixed (Clery & Mulcahy, 1998; Ryan et al., 2020).

The parasite migration, associated with the inflammation (Th1 response), causes host oxidative stress, and induces tissue damage (Bottari et al., 2015).The generation of exacerbated ROS/RNS products is associated with severity liver damage (Bottari et al., 2015). An appropriate balance of the oxidative response with redox response is essential to resolve infections and finally the infectious process (Carasi et al., 2017).

The B-cells in infected animals suggest that they play a role in providing protection: there was an increase in the number of B cells in mice and cattle after the infection. In addition, antibody secretion in most experimentally infected animals is detected in 4 weeks of exposure (Beesley et al., 2018).

As the infection advances, the immune response shifts towards a Th2 response, suppressing the Th1 inflammatory response (Ryan et al., 2020). In fact, during the advance of *F. hepatica* infection, the immune response turns out to be a modified Th2 type response (Th2/Treg), accompanied by the production of IL-10 and TGF-β, in addition to the usual cytokines (IL-4, IL-5, IL-9 and IL-13) (Varyani et al., 2017). The development of this response modulates and slows inflammation, which leads to a prolonged illness while at the same time repairing tissue damage caused by the helminths themselves (Varyani et al., 2017). Figure 2.4 exemplifies the immune response against *F. hepatica*.

Helminths co-evolve with the host immune system, allowing them to develop survival strategies, such as producing immunomodulatory molecules that act directly by immunosuppressing innate and adaptive immunity (Drurey & Maizels, 2021; Finlay et al., 2014; Stear et al., 2023). The FhNEJ tegument interacts closely with the host during the migration process (González-Miguel et al., 2021). The more mobile and active the FhNEJ the faster the cycle of shedding the glycocalyx is completed. This mechanism has been postulated as an immune defense strategy to prevent the attachment and function of the host granulocytes on the parasite surface (González-Miguel et al., 2020, 2021).

In addition, several studies have demonstrated that *F. hepatica* excretory-secretory products (FhESP) can evade innate immune mechanisms, generating an immunoregulatory effect linked to the activation of alternative activated macrophage (M2) and inhibit or decreasing the DC activation (Araujo et al., 2013; Carasi et al., 2017; Rodríguez et al., 2015). This leads to the production of ineffective antibodies and increased regulatory cell. For instance, juvenile flukes avoid antibody switching (Moreau & Chauvin, 2010). The resulting IgM are unable to interact with effector cells

such as macrophages and eosinophils(Chauvin & Boulard, 1996). Various immunomodulatory molecules have been identified as the FhESP. Table 2 shows most immunoregulatory molecules produced by the parasite (Ryan et al., 2020).

FhESP interacts with several molecules in immune system cells to carry out immunoregulatory mechanisms in the host. Furthermore, the immune system can develop and produce immunoregulatory mechanisms to avoid further tissue damage. One of the molecules produced by the body to attenuate immune responses is the Heme oxygenase 1(HO-1). This stress-responsive enzyme protects against oxidant-induced injury during inflammatory processes (E. C. B. Araujo et al., 2013). However, the HO-1 also plays an essential role in evading the host immune response in bacterial diseases (Abdalla et al., 2015; Mitterstiller et al., 2016).

Helminths express carbohydrate-containing glycoconjugates on their surface, and the immune system recognizes these molecules through the interaction of CLRs. The CLRs are a group of proteins that recognize and bind carbohydrates (lectins) derived from molecules of viral, bacterial, fungal, parasitic, or even tumor, origin in a Ca^{+2}-dependent manner (Sancho & Reis e Sousa, 2012b; Zelensky & Gready, 2005). These transmembrane receptors are involved in various immune processes, such as antigen uptake and presentation, cell adhesion, apoptosis, and T-cell polarization (Hewitson et al., 2009; Loukas & Maizels, 2000).

DCs express abundant CLRs; the family includes the mannose receptor (MR), DEC-205, DC-SIGN, DCIR, and MGL(Singh et al., 2009). Several works have reported that the interaction of glycans in different helminth parasites with CLRs expressed in DC mediates an immunomodulatory host's immune response (Hewitson et al., 2009; Loukas & Maizels, 2000; Van Die & Cummings, 2010). The following sections will further explore the functions and role of HO-1 and the Macrophage Galactose Lectin (MGL) in helminthic infections. Table 3 describes some of the essential immunoregulatory molecules for understanding this thesis.

The immunosuppression generated by the infection in cattle can generate significant problems for animal production. They include impacts on detecting Tuberculosis in herds, as infected animals do not respond to diagnostic single intradermal comparative cervical tuberculin test (SICCT). This test measures the hypersensitivity response to the tuberculin and is dependent on the functional capacity of Th1 cells to secrete IFN-γ (Claridge et al., 2012).

In addition, helminth infections increase the susceptibility to secondary diseases, such as tuberculosis, hemorrhagic diarrhea (*E. coli* O157), and HIV. They also impair the vaccine efficacy in many species, including humans (Elias et al., 2006; Howell et al., 2018b; Steenhard et al., 2009; Urban Jr et al., 2007; Varyani et al., 2017; Walson et al., 2009; Wammes et al., 2010). Understanding the immunological response and protective mechanisms against *F. hepatica* is vital to develop disease control strategies in many species.

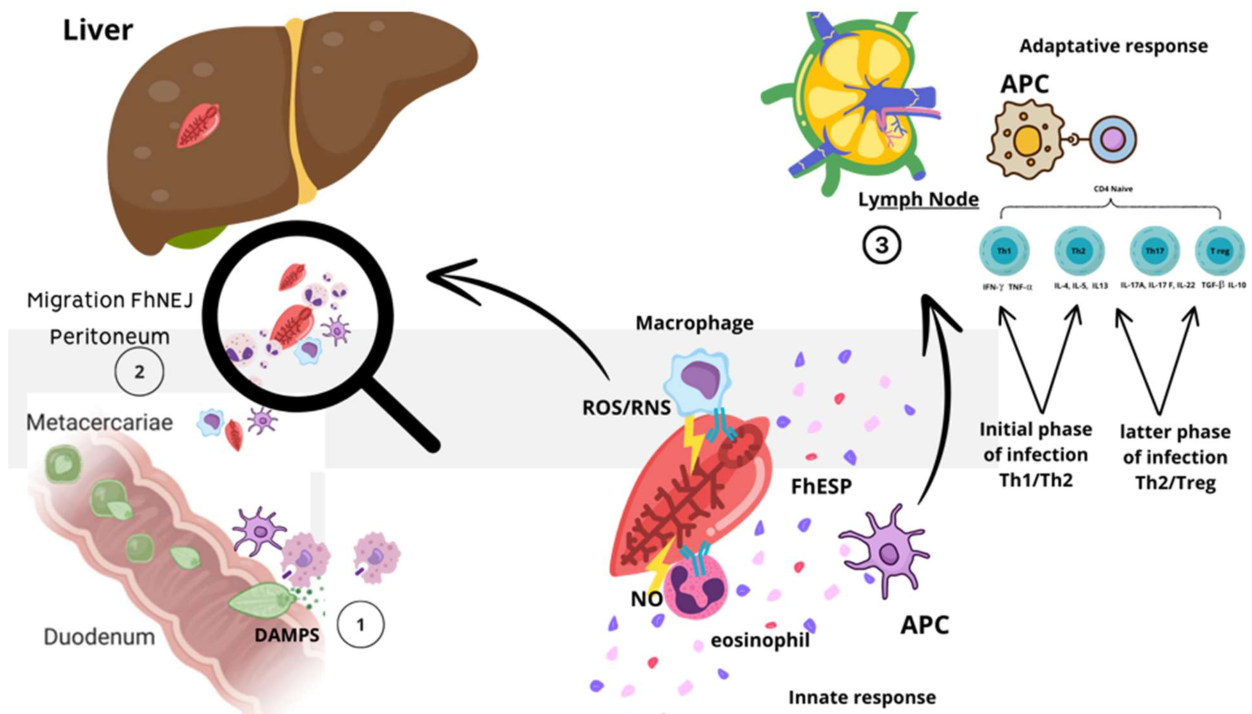

Figure 2–4. Immune response against *F. hepatica*. 1) Invasion and tissue damage generated by FhNEJ; 2) FhNEJ migration in the peritoneal cavity and mechanism of innate immune response; 3) Beggin of adaptive immune response after presenting antigen by APC cells and types of Th responses generates by the parasite.

Table 2. *F. hepatica*-derived products with immunomodulatory capacity

FhESP	Specie investigated	Action	Reference
Fatty acid binding protein	Mice	Reduction of pro-inflammatory cytokines Inhibits TLR4	(Martin et al., 2015; Ramos-Benítez et al., 2017; Robinson et al., 2009)
Cysteine Proteases	Mice	Prevention of eosinophil attachment; decrease of IFN-γ response	(Brady, O'Neill, et al., 1999; Carmona et al., 1993; Donnelly et al., 2010)
Helminth defense molecule	Mice Human	Prevention of antigen presentation by macrophages; reduction of inflammation	(Alvarado et al., 2015; Robinson et al., 2012; Tanaka et al., 2018)
Glycans	Mice	Modulation of DC function that induces a Th2/Treg and suppresses Th1	(M. Costa et al., 2022; Guasconi et al., 2011, 2018; Rodríguez et al., 2015, 2017)
TGF-like molecule	Bovines	Induction of M2 profile	(Sulaiman et al., 2016)
Kunitz-type molecule	Mice Human	Decrease in inflammatory cytokine secretion by DCs	(Falcón et al., 2014)
Glutathione S-transferases	Mice	Suppression of Th1 responses; downregulation of NF-κβ pathway	(Aguayo et al., 2019; Dowling et al., 2010)
Thioredoxin Peroxidase/Peroxiredoxin	Bovine	Detoxification of ROS; induction of M2 macrophages	(Dorey et al., 2021)

adapted from: (Ryan et al., 2020)

Table 3. Immunoregulatory molecules in immune cells

Molecule	Cell type	Action	interaction	Specie	reference
MGL2	APC	Treg proliferation; modulates DC function	Glycans by *F. hepatica*	Mice	(Costa et al., 2022; Rodríguez et al., 2017)
ICOSL	APC	Treg proliferation; B cell differentiation	ICOS (T cells)	Mice	(Costa, et al., 2021, 2022; Oliveira et al., 2021)
PDL-1	APC	inhibits T cells	PD-1 (T cells)	Mice	(Guasconi et al., 2011, 2015, 2018)
PDL-2	APC	inhibits T cells	PD-1 (T cells)	Mice	(Guasconi et al., 2011, 2015, 2018)
CTLA-4	Treg	inhibits T cells; decreases IL-2	CD80/86 (APC)	Mice	(Aldridge & O'Neill, 2016a; M. Costa et al., 2021)
HO-1	APC	Treg proliferation Detoxification of ROS		Mice	(Biswas et al., 2014; Carasi et al., 2017b; M. Costa et al., 2021)

2.5.2 Role of HO-1 in the immunomodulation

As mentioned in the previous section, HO-1 has an important role in protecting against exacerbated immune responses and also participates in the evasion of the immune system in some diseases. HO is an evolutionarily conserved intracellular enzyme that catabolize heme into Fe^{2+}, carbon monoxide (CO), and biliverdin (Gozzelino et al., 2010).

There are two HO isoenzymes, namely HO-1 and HO-2 (Gozzelino et al., 2010). HO-1 expression is induced in response to oxidative stress, whereas HO-2 is constitutively expressed and not inducible (Gozzelino et al., 2010). HO-1 is one of the major acute-phase proteins and is up-regulated by various inducers such as hydrogen peroxide, prostaglandins, endotoxin, and cytokines (IL-1 and TNF) (Paul et al., 2005).

HO-1 is expressed by macrophages, whose primary function is protection and homeostasis against free heme-induced toxicity (Vijayan et al., 2018). Furthermore, HO-1 has been shown to functionally switch these cells to an M2 phenotype, limiting inflammatory cytokines (Naito et al., 2014). HO-1 can also be expressed by DCs, where it inhibits the maturation and production of ROS, induces the production of IL-10, and inhibits T proliferation (Chauveau et al., 2005).

Several studies confirm that HO-1 plays an essential role in different infectious diseases and can have both beneficial and detrimental consequences for the host immunity against pathogens (Carasi et al., 2017a). For instance, HO-1 can promote *Plasmodium* sp. liver infection (Epiphanio et al., 2008) and susceptibility to bacterial diseases (Abdalla et al., 2015; Mitterstiller et al., 2016), whereas it plays a favorable role in the host during cerebral malaria (Pamplona et al., 2007).

A widely used strategy to study the activity and effects of HO-1 is through pharmacological manipulation of this enzyme (Gerbitz et al., 2004). HO-1 expression can be induced by using synthetic metal porphyrins such as cobalt-protoporphyrin IX (CoPP) and zincprotoporphyrin IX (ZnPP) (Gerbitz et al., 2004). On the other hand, its activity can be inhibited by using the synthetic heme analog tin (IV)-protoporphyrin (Sn-protoporphyrin - SnPP) (Sardana & Kappas, 1987).

In mice infected with *F. hepatica*, HO-1 is upregulated on APC in both the peritoneal cavity and liver. The pharmacological induction of HO-1 has been found to increase clinical signs and liver damage. On the other hand, the inhibition of HO-1 activity has been shown to protect the animals against infection (Carasi et al., 2017). Thus, HO-1 could be a potential therapeutic target for modulating the host immune response during fasciolosis.

2.5.3 Role of MGL in immunoregulation

The Macrophage Galactose Lectin is a receptor member of the C-type lectin family expressed in tolerogenic DCs and macrophages (Pirro et al., 2018). In humans, there is a single variant of MGL (hMGL); in mice, there are two isoforms, mMGL1 and mMGL2, which have differences in their ligand specificity. mMGL2 is the one that has a specificity similar to human MGL (Singh et al., 2009).

hMGL and mMGL2 recognize tN-acetylgalactosamine (GalNAc) and galactose, including the Tn-antigen (GalNAc-Ser/Thr), TF (Gal-GalNAc-Ser/Thr) and Core 2 (Singh et al., 2009). Studies on MGL effects on the immune system have shown mixed results. Some studies suggest that MGL can stimulate the activation of $CD4^+$ or $CD8^+$ T lymphocytes (Zizzari et al., 2015). In contrast, others suggest it can affect the maturation of dendritic cells, promoting the production of cytokines that regulate the immune response (Phongsisay, 2016; van Kooyk et al., 2015).

For instance, MGL recognizes the Tn antigen expressed on the CD45 membrane molecule in human effector T cells (van Vliet et al., 2006a; Van Vliet et al., 2013) and reduces T cell proliferation and production of inflammatory cytokines, eventually leading to T cell apoptosis (van Vliet et al., 2006a; Van Vliet et al., 2013). Furthermore, it has been reported that MGL could be involved in the retention of immature DCs since the migration of DCs was substantially enhanced or favored by blocking MGL with a specific antibody (van Vliet et al., 2008).

Several reports have shown that MGL (human and murine) can recognize glycoconjugates in helminth parasites like *Schistosoma mansoni*, *Taenia crassiceps* and *Trichuris suis* (Klaver et al., 2013; C. A. Terrazas et al., 2013; van Liempt et al., 2007). Rodríguez and collaborators (2017)

demonstrated that the Tn antigen expressed by *F. hepatica* can modulate TLR2-induced maturation of human monocyte-derived DCs by up-regulating the production of IL-10 and TNFα in a process mediated by hMGL. In bovines, the function of C-type lectins has yet to be studied. The MGL receptor was described in only one work, where a lectin array was developed to study interactions with pathogens (Jégouzo et al., 2020). However, the importance of this receptor in helminth infections in cattle remains unknown.

2.6 Hypothesis

Considering the immunomodulatory properties of *F. hepatica*, this thesis hypothesizes that certain molecules expressed by the host during *F. hepatica* infection, such as MGL2 and HO-1, promote an immunoregulatory response that favors parasite survival. This response could, in turn, affect the quantity or quality of the immunity induced by bacterial and viral vaccines.

Objectives

3 General aim

Considering the immunoregulatory mechanisms generated by *F. hepatica* in various hosts, this thesis aims to investigate the role of immunoregulatory strategies in APCs during infection in mice. Additionally, we will explore how fasciolosis impacts on biochemical parameters, cellular and humoral immune response and in the immune response generated by bacterial and viral vaccines in cattle. The study will focus on understanding the role of immunoregulatory strategies in these hosts.

3.1 Specific objectives

1. To elucidate the role of HO-1 expression in APC cells and the effects in the immune response during *F. hepatica* infection in the murine experimental model;

2. To clarify the importance of $MGL2^{+}$ cells in the induction of Treg during *F. hepatica* infection in the murine experimental model;

3. To investigate the relationship between liver damage, hematological and circulating leukocyte parameters caused by *F. hepatica* infection in the bovine experimental model;

4. To characterize the cellular and humoral immune response *F. hepatica*-infected cattle;

5. To explore the impact of fasciolosis on immunity induced by viral (FMDV) and bacterial (*Clostridium ssp, P. multocida,* and *M. haemolytica*) vaccines in the bovine host.

General Methodology

4 General methodology

In the first two years of this project, we performed experimental activities *in vivo* with mice infected with *F. hepatica.* The experimental strategy was based on studying the anti-oxidant and immunoregulatory properties caused by HO-1 and the MGL2 receptor in F4/80^{+} cells during the parasite infection.

Over the past three years, our thesis involved conducting *in vivo* experiments with cattle infected with *F. hepatica*. The experimental strategy was to evaluate the impact of this parasite on various aspects such as hematological parameters, liver function, innate immunity, and adaptive immune memory against viral (FMDV) and bacterial (*Clostridium* ssp*, P. multocida,* and *M. haemolytica*) vaccines.

4.1 General methodology associated with infections in an experimental mouse model

4.1.1 Mice

Female BALB/c or C57BL/6 mice (six- to eight-week-old) were purchased from División de Laboratorios Veterinarios (DILAVE, Uruguay). Gp91phox-knockout and MGL2-DTR mice were purchased from Jackson Laboratory (USA) and maintained at UATE, Institut Pasteur Montevideo.

Six to eight BALB/c, Gp91phox-ko, and C57BL/6 littermates (controls for gp91phox and MGL2-DTR mice) were used per experiment. In a controlled environment, the experiments were carried out in the Unidad de Reactivos para Biomodelos de Experimentación (URBE, Facultad de Medicina), with temperatures between 19 and 21 ºC and cycles with 14 h of light and 10 h in darkness. The animals were allocated to cages with filters, and they received water and a sterile ration *ad libitum*.

Mouse handling, care, and experiments were carried out in compliance with institutional guidelines and regulations from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, https://www.cnea.gub.uy/, accessed on 12 November 2021, National Law 18.611, Uruguay). Procedures involving animals were approved by the Universidad de la República's Committee on Animal Research (Comisión Honoraria de Experimentación Animal, CHEA Protocol Number 07153-000817-18, 070153–000,811-19).

4.1.2 Infection

Our laboratory had previously characterized and fine-tuned the murine experimental infection model (Carasi et al., 2017; Frigerio et al., 2020; Rodríguez et al., 2015). The infection was carried out by administering 10 *F. hepatica* metacercariae per animal (Montevideo, Uruguay) orally. Mice were bled and euthanized by cervical dislocation, peritoneal exudate cells (PECs), spleens, and livers were removed after 1, 3-, 7-, 15-, and 21-days post-infection (dpi) at the same time, non-infected-control-animals. Red blood cells (RBC) were lysed with ammonium chloride potassium buffer.

PECs were obtained by injecting 10 ml of cold Phosphate Buffered Saline (PBS, Phosphate Buffer Saline, NaCl 0,137 M, KCl 0,0027 M, Na_2HPO_4 0,01 M, and KH_2PO_4 0,0018 M) in the peritoneal cavity from infected animals and then extracted. The obtained organs and cells were subsequently processed for analysis by flow cytometry, cell culture, or quantitative PCR (qPCR). A clinical score was used to determine the severity of the infection (Carasi et al., 2017), detailed in Table 4. The minimum score is 0, while the maximum is 10 points.

Ascites	**Spleen**	**Number of Lesions/ hepatic lobe**	**Liver lobes**
0 – none (normal cell content)	0 – Normal	0 – None	0 – Healthy
1 – mild (medium cell content)	1 – Splenomegaly (<2x)	1 – <3 Lesions	1 – 1 hepatic lobe affected
2 – Moderate (High cell content)	2 – Splenomegaly (>2x)	2 – >3 Lesions	2 – > 2 hepatic lobes affected
3 –Severe (High cell and blood content)		3 – Complete affection of lobes	

4.1.3 Inhibition and induction of the HO-1

To modulate HO-1 activity, the infected and control mice received intraperitoneal injections of either vehicle (sterile PBS, 100 µL), CoPP (20 mg/kg), SnPP (40 mg/kg), or CoPP plus SnPP. CoPP and SnPP were within a range of doses used in studies describing the upregulation of HO-1 by CoPP and inhibition of the enzyme's activity by SnPP (Sardana & Kappas, 1987).

Considering that CoPP can also induce the expression of other proteins and taking into account that SnPP only inhibits HO-1 activity, the group treated with both substances simultaneously was added. The animals were injected one day before infection, one day after infection, and every four days until the end of the experimental protocol (between 7, 19, and 21 dpi).

4.1.4 IL-10 receptor-blocking

To neutralize the IL-10 receptor (IL-10R), BALB/c mice (n=6–8/group) received an intraperitoneal injection with 15 µg of monoclonal rat IgG2a anti-IL-10R (clone 1B1.3A from BioXcell, Lebanon, NH, USA) or an isotype-matched control antibody (clone HRPN from BioXcell, Lebanon, NH, USA), at the day before and after infection with *F. hepatica* and every three days until animal sacrifice at 20 d.p.i.

4.1.5 MGL2- DTR

To evaluate parasite infection while depleting $MGL2^{+}$ cells, 10 metacercariae were administered on day 0 into MGL2-DTR mice that were previously injected i.p. with diphtheria toxin (DT, 0.5 µg/mouse) (The Native Antigen Company, USA) or PBS on day -1 and every 2 or 3 days until the end of the experiment. Each experimental group contained at least six mice.

4.1.6 Hepatic injury

The liver damage caused by *F. hepatica* was measured by quantifying the activity of alanine-transaminase (ALT) in serum, according to instructions provided with the commercial kit (SpinReact). The solution was prepared with four volumes of reagent 1 (R1) and 1 volume of reagent 2 (R2). In a 96-well plate, 20 µL of serum was added to each well, followed by 200 µL of the use solution (R1 and R2). The samples proceeded to the absorbance measurement at 340 nm, and five absorbance measurements were performed consecutively with a space of 1 min between each measure. The difference between absorbance and the average of the differences in absorbance per minute (ΔA/min) was calculated. The specific activity was calculated using the following equation: U/L of ALT = ΔA/min x 1750.

4.1.7 Cell suspensions of splenocytes and hepatic leukocytes

The spleens were mechanically disrupted with sterilized glass slides to isolate splenocytes from infected and naive animals. The cell suspension was washed with PBS, and the RBC were lysed with Ammonium–Chloride–Potassium (ACK) buffer containing 154.4 mM NH_4Cl, 10 mM $KHCO_3$, 0.1mM EDTA, pH 7.4.

After this procedure, two additional washes were performed with PBS and centrifugation at 1500 rpm for 5 min, using a Sorvall centrifuge ST16R (Thermo Scientific). Cell counting was carried out using a Neubauer chamber.

The livers were perfused with PBS in the abdominal vena cava and cut in the suprahepatic vein until the livers were cleared to isolate hepatic leukocytes. After this, livers were mechanically disaggregated with a tissue homogenizer and left to decant for 15 min on ice. The supernatant was then transferred to another tube and centrifuged at 1300 rpm for 7 min. Subsequently, the supernatant was discarded, and the pellet was resuspended in 6 ml of 35% Percoll. The cell suspension was centrifuged at 1300 rpm for 20 min at 20°C without acceleration or brake. After removing the supernatant, RBC were lysed, and the cells were washed with PBS, proceeding to their counting.

4.1.8 Proliferation Assay and Cell Culture

4.1.8.1 Parasite Protein Extract (FhTE)

F. hepatica total extract (FhTE) was prepared from live adult flukes obtained from infected bovines. Flukes were washed for 1h at 37°C with PBS, pH 7.4, sonicated, and then centrifuged at 40,000 × g for 60 min (Rodríguez et al., 2015, 2017). FhTE protein concentration was measured using the bicinchoninic acid (BCA) assay (Sigma, St. Louis, MO, USA). To remove endotoxin contamination, FhTE was applied to a column containing endotoxin-removing gel (detox-gel, Pierce Biotechnology, Waltham, MA, USA). The endotoxin levels were quantified using the Limulus Amebocyte Lysate kit Pyrochrome (Associates of Cape Cod, East Falmouth, MA, USA) and found to be lower than 0.05 EU/mL.

4.1.8.2 Protein quantification

The determination of protein concentration was performed using the BCA method (Smith et al., 1985). The reaction was carried out by adding 10 µL of the sample in different dilutions in PBS and 200 µL of the reaction mixture, its composition being the reagent BCA (Sigma Aldrich, US) and copper sulfate, in a 50:1 parts ratio. Subsequently, it was incubated for 30 min at 37°C, and the absorbance at 570 nm was measured.

4.1.8.3 Splenocyte proliferation

Splenocytes (0.5×10^6/well) from infected mice or uninfected mice were cultured for five days at 37°C and 5% CO_2 in RPMI-1640 with 400 µg/mL of glutamine (Capricorn, Ebsdorfergrund, Germany) complete medium containing: 10% heat-inactivated fetal bovine serum (FBS, Capricorn Scientific, Ebsdorfergrund, Germany), 50 mM of 2-mercaptoethanol, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin (Merk, Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of FhTE (75 µg/mL).

4.1.9 IFN-γ quantification by specific sandwich ELISA assay

A specific sandwich ELISA was performed to assess the IFN-γ levels in culture supernatants from splenocyte proliferation and cultivated cellular experiments. NuncF Maxisorp ELISA plates were sensitized with primary antibody anti-IFN-γ (BD Bioscience) in 0.1 M phosphate buffer pH 9.6 overnight at 4 °C. The next day, the plates were blocked with 1% PBS Gelatin for 1 h at 37°C, followed by incubation with samples. A calibration curve was included using each recombinant cytokine as a standard. Next, the biotin-conjugated secondary antibody anti-IFN-γ was added to each well and incubated for 1 h at 37°C. Streptavidin-peroxidase was incubated for 45 min at 37°C. Finally, color development was performed with 0.5 mg/mL o-phenylenediamine (OPD) and 0.12% H_2O_2 in 0.1 M pH 5 citrate-phosphate buffer. Plates were read photometrically at 492 nm in an ELISA auto-reader (Labsystems Multiskan MS, Finland).

4.1.10 Leucocytes Analyzed by Flow Cytometry

Cell suspensions from PECs, livers, and spleens were washed twice with PBS containing 2% FBS and 0.1% sodium azide (FACS buffer), stained with specific membrane antibodies for 30 min at 4 ◦C (Frigerio et al., 2020).The cell suspensions were fixed with Cytofix (Biolegend) for 20 min at room temperature (RT) for intracellular molecules. After this procedure, the cells were permeabilized with Permwash (Biolegend) and incubated for 1 h with the HO-1 (ab13248) specific antibody diluted in Permwash in RT.

The samples were incubated with Brefeldin (3 µg/mL), Ionomycin (0,1 µg/mL), and phorbol myristate acetate (PMA) (0,1 µg/mL) for 6 h at 37°C to analyze cytokine production. ROS/RNS produced by $F4/80^+$ cells were determined with 20,70 -dichlorofluorescein diacetate (DCFDA, Merk, Kenilworth, NJ, USA) probe, a fluorogenic dye that is oxidized into the fluorescent 20,70-dichlorofluorescein. Briefly, cells were incubated in PBS for 30 min at 37°C with DCFDA. Subsequently, the usual protocol was explicated in the superior paragraph. Analyses were performed using a BD Accuri C6 Plus cytometer and software. The antibodies against corresponding membrane molecules are present in Table 6.

Table 4 Antibodies used in mice in flow cytometry

Specify	Clon	Company
	Membrane antibodies	
Sirpα	P-84	Biolegend
CD11c	N418	Biolegend
CD86	GL1	BD Biosciences
Siglec-F	E50-2440	BD Biosciences
F4/80	BM8	Biolegend
CD11b	M1/70	BD Biosciences
CD40	HM40-3	Biolegend
CD80	16-10A1	BD Biosciences
I-A/I-E	M5/114.15.2	Biolegend
CD4	RM4-5	Biolegend
CD8	53-6.7	Biolegend
CD3	17A2	Biolegend
CD25	PC61	Biolegend
ICOSL	HK5.3	Biolegend
CCR2	SA203G11	Biolegend
PDL-1	10F9G2	Biolegend
DCFDA		Sigma Aldrich
	Intracellular Antibodies	
HO-1	ab13248	Abcam
FOXP3	MF14	eBioscience
TNF- α	MP6-XT22	Biolegend
IL-10	JES5-1E3	Biolegend

4.1.11 Determination of Oxidative and Antioxidative Genes by qRT-PCR

4.1.11.1 RNA extraction

Total RNA extraction from BALB/c mice PEC was performed with Tri-Reagent (Merk, Kenilworth, NJ, USA). The protocol stored the cells in 500 µl of Tri-reagent at -20ºC. For processing, the samples were incubated in the reagent at room temperature for 5 min. Subsequently, 125 µL of chloroform was added, vortexed for 15 s, and incubated for 15 min at RT. The mixture was allowed to stand for 10 min and centrifuged at 2400 rpm for 15 min. After centrifugation, two phases were obtained.

The upper phase was transferred to another Eppendorf, and 500 µL of isopropanol was added for each mL of Tri-reagent used. It was incubated for 10 min at RT, then centrifuged for 10 min at 12,000 g at 4°C. The pellet obtained at this stage corresponds to the RNA. The RNA was washed with 1 mL of 75% ethanol (prepared with H_2O treated with diethylpyrocarbonate DEPC), vortexed, and centrifuged at 7500 g for 5 min at 4°C. The pellet was allowed to dry and resuspended in 30 µL of H_2O-DEPC previously heated to 55°C.

RNA quantification was performed by spectroscopy by measuring absorbance at 260 nm. The purity of the extracted RNA was determined by analyzing the ratio between the absorbance at 260 and 280 nm. In the case of RNA, this ratio must be greater than 1.7. The quantification and degree of purity measurements were performed in Nanodrop equipment (Thermo Scientific).

4.1.11.2 Synthesis of cDNA

The SensiFAST cDNA Synthesis kit from Bioline was used to synthesize cDNA. First, a reaction mix was made that included TransAmp Buffer, the Reverse Transcriptase enzyme at 200 U/µL, and 1 µg of RNA, calculated according to the concentration obtained from Nanodrop. The cDNA was made in a T Gradient Thermocycler of the Biometra brand, using the following program: 25°C for 10 min, 42°C for 15 min, 48°C for 15 min, and 85°C for 5 min.

4.1.11.3 q-PCR

The gene expression of Nrf2, catalase (CAT), glutathione peroxidase (gpx) 1 and 2, superoxide dismutase (sod) 1 and 2, and NADPH-oxidase subunits p47phox and gp91phox mRNA was detected using the Eco real-time PCR System (Illumina, San Diego, CA, USA) and Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). Standard amplification conditions were 10 min at 95 ◦C, 40 thermal cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension of 10 min at 72°C.

Results were expressed as the ratio between each gene under study and GAPDH expression. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. All reactions were performed with at least five biological replicates. Table 7 shows the primers and Tm conditions used.

Table 5 Gene names, primes and Tm conditions used in mice

Gene	Oligo Sense	Antisense Oligo	Tm
Nrf2	CAGCATGTTACGTGATGAGG	GCTCAGAAAAGGCTCCATCC	56°C
Gpx1	GGGACTACACCGAGATGAACGA	ACCATTCACTTCGCACTTCTCA	60°C
Gpx2	GAGGAACAACTACCCGGGACTA	ACCCCCAGGTCGGACATACT	60°C
Sod1	TGGGTTCCACGTCCATCAGTA	ACCGTCCTT TCCAGCAGTCA	60°C
Sod2	ATTAACGCGCAGATCATGCA	TGTCCCCCACCATTGAACTT	60°C
Catalase	GCGTCCAGTGCGCTGTAGA	TCAGGGTGGACGTCAGTGAA	60°C
p47phox	GAGGCGGAGGATCCGG	TCTTCAACAGCAGCGTACGC	56°C
Gp91phox	CCAGTGAAGATGTGTTCAGCT	GCACAGCCAGTAGAAGTAGA	56°C
GAPDH	ATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGG CCATGTAGG	56°C

4.1.12 Statistical Analysis

The results of the experiments were expressed as mean ± SEM. GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Depending on the experiment, the results were analyzed using one-way ANOVA followed by Tukey's test or the two-tailed Student's t-test. Significant differences shown by an asterisk were considered when $p < 0.05$.

4.2 General methodology associated with infections in an experimental bovine model

4.2.1 Characterization of the cattle infection experiments

Six to eighteen-month-old male Aberdeen Angus (n=36) castrated calves were used in this experiment. In three previous tests, the animals were negative for *F. hepatica* exposure, as determined by the sedimentation technique (Giovanoli Evack et al., 2020). The animals were drenched on arrival with Detomax® 1% (Doramectin 1g/100 ml) with the recommended dose (1 ml/50 Kg body weight). They remained free of gastrointestinal parasites (as determined by the modified McMaster technique (Escribano et al., 2019) to detect fecal egg counts per gram (EPG) until *F. hepatica* experimental infection.

Steers were housed indoors and fed high-quality pasture and water *ad libitum* at Estación experimental INIA La Estanzuela, Colonia, Uruguay. After 115 days post-infection (dpi), animals have transported to an intensive animal farming facility (feedlot) in Fray Bentos, Río Negro, Uruguay.

4.2.2 Cattle infection

Experimental infections (n=24) were carried out with 500 metacercariae (Ridgeway Laboratories, England) spread in saline solution, inserted into gelatine capsules (Torpac®), and delivered orally using a dosing gun. As a control, non-infected steers (n=12) were maintained under the same conditions as infected groups.

Animal handling and experiments were carried out by strict guidelines and regulations from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, http://www.cnea.org.uy/, National Law 18.611, Uruguay). INIA's Committee on Animal Research (CNEA Protocol Number: 0009/11).

4.2.3 Cattle Vaccination

Before experimental infection, all animals were vaccinated against *Clostridium* ssp and FMDV in the first year of life. Vaccination against *Clostridium* spp was performed using a commercial vaccine (Clostridium 9+T, Virbac) administered according to the manufacturer's recommendations. Cattle were vaccinated twice against FMDV with the full dose (5 ml) Oleolauda bivalent vaccine from Paraguay (series 5967700A) formulated with A24/Cruzeiro and O1/Campos strains administered intramuscularly in the first six months of age. The vaccination was performed in the context of the compulsory national program of vaccination against FMDV in Uruguay.

After experimental infection (115 dpi), all animals were vaccinated against respiratory diseases, using a commercial polyvalent vaccine (Vac-sules Feedlot, Microsules), administered according to the manufacturer's instructions.

4.2.4 Sample Collection

Fecal and plasma samples were collected on day 0 and every 14 days during the acute stage, and after every 28 days during the chronic phase of infection. *F. hepatica* EPG in feces were determined individually using the sedimentation technique (Giovanoli Evack et al., 2020) before the infection (day 0) and at 15, 28, 43, 59, 71, 87, 115, 157, 193 and 213 dpi. After 115 dpi, infected animals were divided into two groups: infected (n=12) or infected and TCZ-treated (n=12). TCZ treatment (12 mg/kg, Fasimec®, Novartis) was performed according to the recommendations of the drug supplier laboratory. At the end of the experiment, animals were transported to an abattoir (Marfrig S. A. Tacuarembó), and sample collection, liver examination, and fluke recovery were carried out.

4.2.5 Sedimentation technique

We performed the protocol developed by Giovanoli Evack et al., 2020; Faeces were manually extracted from the rectums of all animals used in the experiment. 5g of feces was mixed with 30 ml of saturated water and homogenized. The faeces were allowed to sediment for 15 min, after which the supernatant was decanted. The sedimenting steps were performed three times for a total of 45 min of exposure to water. Methylene blue was added to the sediment to identify *F. hepatica* eggs.

4.2.6 Fluke Recovery and liver damage

Livers, bile ducts, and gallbladders were examined for *F. hepatica* parasites (Roberts et al., 1997). Fluke recovery was determined by the number of adult flukes from each processed liver. Livers were weighed and given a score by a veterinary pathologist according to the macroscopic liver damage (ranging from 0 to 3) at dissection, where 0 represented the absence of tissue necrosis and liver damage, 1 represented less than 30% (slight), 2 represented between 30 and 70% (moderate), and 3 represented more than 70% (severe) of the liver surface. Fibrosis, capsule, consistency, calcification in the biliary ducts and abscesses, as well as atrophy of hepatic lobes, were also

determined by a similar score: 0 (absence), 1 (slight), 2 (moderate), and 3 (severe) (Marcos et al., 2007).

4.2.7 Hemogram and circulating leukocyte counting

Animals were bled for a complete hemogram before the infection (day 0) and after 43-, 87-, 157-, and 213-dpi. Blood samples were processed to assess hematocrit. Mean Corpuscular Volume (MCV) and Mean Platelet Volume (MPV) were determined using Counter 19 from the Weiner lab. Total counts of leukocytes were determined in a microscope using a Neubauer Haemocytometer. Thin smears were prepared on individually labeled microscope slides using one or two drops of blood. Smears were air-dried, fixed in absolute methanol, and stained with Giemsa to analyze leukocyte and lymphocyte counts. Sera were collected to quantify transaminase activity levels.

4.2.8 Hepatic synthetic functions and transaminase determination

Plasma albumin levels, total protein, and hepatic enzyme activity were determined using an automated spectrophotometer (Dimension RxL Max integrated chemistry system; Siemens). Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Gamma-glutamyltransferase (GGT) activities were expressed as international units per liter (IU/l), Total Bilirubin (TBil), and Direct Bilirubin (DBil) determinations were expressed in mg/dl, and Albumin (ALB) and Total Protein (TP) determinations were expressed in g/dl.

4.2.9 Evaluation of systemic cellular immune response

4.2.9.1 PBMC isolation

The blood was collected in a 105 mM sodium citrate buffer solution and used to purify peripheral blood mononuclear cells (PBMC). After obtaining the plasma, the buffy coat was extracted, placed on 6 ml of Histopaque density 1.077 g/ml, and centrifuged at 2500 rpm at 20ºC for 30 min using a Sorvall centrifuge ST16R (Thermo Scientific) with intermediate acceleration and without brake. Subsequently, the cell suspension corresponding to the PBMCs was extracted, and three washes were carried out with PBS. Cells were stored in the Fetal Bovine Serum with 10% DMSO at -80ºC until use.

4.2.9.2 PBMC analyses by flow cytometry

Cell suspensions from PBMC were washed twice with PBS containing 2% FBS and 0.1% sodium azide (FACS buffer) and stained with specific membrane antibodies for 1 h at 4°C (Table 8). Intracellular ROS/RNS levels in PBMC cells were measured using Dihydrorhodamine 123 (DHR). The cells were incubated with DHR in PBS at 37°C for 30 min.

The cell suspensions were fixed with Cytofix (Biolegend) for 20 min at R.T. for intracellular molecules. After this procedure, the cells were permeabilized with Permwash (Biolegend) and incubated for 1 h with the HO-1antibody diluted in Permwash in RT. Analyses were performed using a BD Accuri C6 Plus cytometer and software. The antibodies against corresponding membrane molecules are present in Table 8.

Table 6. Specific antibodies used in bovine PBMC for flow cytometry.

Specify	**Clon**	**Company**
	Antibodies	
CD4	CC8	BioRad
WC1	CC101	BioRad
CD21	CR2	BioRad
CD11c	BAQ153A	BioRad
DHR123		Invitrogen
HO-1	ab13248	Abcam

4.2.10 Analysis of the specific immunological response of *F. hepatica*

4.2.10.1 Determination of Total IgG

Total IgG Antibodies in sera were determined by sandwich ELISA using a commercial kit following the manufacturer's instructions (Bovine IgG ELISA Kit, ab205078, Abcam, United States).

4.2.10.2 Determination of parasite-specific antibodies

The levels of IgG against *F. hepatica* in serum were determined by an in-house ELISA. Ninety-six-well microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 µg/well of *F. hepatica* lysates (FhTE) in 50 mM carbonate buffer (pH 9.6). After blocking with 1% gelatine in PBS, three washes with PBS containing 0.5% Tween-20 were performed. Serially diluted sera samples in PBS-Tween were added in duplicates to the wells overnight at 37°C. One of the wells was washed with PBS, and the other was washed with PBS containing 7 M Urea to detach low-avidity binders. After washing, wells were incubated for 1 h at 37°C with sheep anti-cattle IgG peroxidase-conjugate (Biorad), and o-phenylenediamine-H_2O_2 was then added. As the substrate, plates were read photometrically at 492 nm in an ELISA auto-reader (Labsystems Multiskan MS, Finland).

4.2.11 Cytokine quantification by specific sandwich ELISA assay

A sandwich ELISA was performed to evaluate cytokine levels in sonicated bovine tissue extract (liver and spleen). NuncF Maxisorp ELISA plates were sensitized with primary antibodies mouse anti-bovine IL-4, IL-10, and IFN-γ (BioRad) in 0.1 M phosphate buffer pH 9.0 overnight at 4°C.

The next day, the plates were blocked with 1% PBS-Gelatin for 1 h at 37°C followed by incubation with samples at different dilutions in PBS-Tween 0.05% according to the cytokine studied for 1 h at 37°C. A calibration curve was included using each recombinant cytokine as a standard. Next, the biotin-conjugated secondary antibody was added to each well and incubated for 2 h at 37°C.

Streptavidin-peroxidase was incubated for 1 h at 37°C. Finally, color development was carried out with OPD as described previously.

To measure the total cytokine, we divided the obtained cytokine concentration adjusted to the work sample's dilution factor by the total protein concentration of the same sample (measuring by BCA); the result is expressed in pg/mg. Exemplification of the formula used:

$$\frac{Cytokine\ (\frac{pg}{mL}) \times Dilution\ Factor}{total\ protein\ concentration\ (\frac{mg}{mL})} = [\ \ ]\ pg/\text{mg}$$

4.2.12 qPCR

Total RNA extraction and synthesis of cDNA from the liver, and spleen was performed using the same protocol described in mice. The gene expression (Table 9) was detected using the Eco real-time PCR System (Illumina, San Diego, CA, USA) and Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). Standard amplification conditions were 5 min at 95°C, 40 thermal cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C, with a final extension of 10 min at 72°C. Results were expressed as the ratio between each gene under study and HPRT expression. Relative gene expression levels were calculated using the 2−ΔΔCT method and normalized to HRPT.

Table 7. Primers used in cattle samples.

Gene	Oligo Sense	Antisense Oligo	Tm
IL-10	TGTTGACCCAGTCTCTGCTG	GGCATCACCTCTTCCAGGTA	60°C
TGF-β	CTGCTGTGTTCGTCAGCTCT	TCCAGGCTCCAGATGTAAGG	60°C
IFN- γ	TTCTTGAATGGCAGCTCTGA	TTCTCTTCGGCTTTCTGAGG	56°C
IL-12	CAGGCCTGTTTACCACTGGA	CTCATAGATACTTCTAAGGCACAG	60°C
HPRT	GGGACTTGAATCACGTGTGTGT	CCACAGAACAAGAACATTGGATCA	60°C

4.2.13 Determination of vaccine-induced bacterial-specific antibodies

The levels of IgG against *Pausterella multocida, Mannheimia haemolytica*, and *Clostridium* spp in serum were determined by ELISA. Ninety-six-well microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 µg/well of bacterial lysates in 50 mM carbonate buffer (pH 9.6). After blocking with 1% gelatine in PBS, three washes with PBS containing 0.5% Tween-20 were performed. Serially diluted sera samples (1/800) in PBS Tween were added to the wells for 1 h at 37 °C. After washing, wells were treated for 1 h at 37°C using sheep anti-cattle IgG peroxidase-conjugate (Biorad), and OPD-H_2O2 as previously described.

4.2.14 FMDV-specific antibodies and avidity

A24/Cruzeiro-specific bovine total IgG and IgG avidity were determined using a single dilution indirect ELISA as described by Lavoria et al. (2012) using 146S purified viral particles as capture antigens (incubation overnight, 4°C). Briefly, the plates were blocked with PBS Tween 0,5% with 10% serum equine and washed with PBS Tween 0.5%. The serum samples, diluted in 1:50 proportion, were run in duplicates. One of the wells was washed with PBS, and the other was washed with PBS containing 6 M Urea to detach low-avidity binders. Specific antibodies were revealed using peroxidase-conjugates against bovine total IgG (Biorad, CA) and 2,2´azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the chromogen. Optical density (OD) values for samples and controls were corrected by subtracting mean blank OD values (cOD). The ratio between Urea and PBS-treated samples (cOD) multiplied by 100 estimated the avidity index.

4.2.15 ELISA FMDV-specific IgG subtypes

As described previously, ELISA FMDV-specific IgG subtypes A24/Cruzeiro-specific bovine IgG isotypes (IgG1 and IgG2) were detected by indirect ELISA. Serum samples were run in two-fold serial dilutions starting at 1:50. Plates were revealed using anti-IgG1 and -IgG2 peroxidase conjugate antibodies (Biorad, CA). The isotype antibody titers were expressed as the highest serum dilution reaching an optical density (OD) equal to the mean OD obtained from all pre-immune negative sera ± 2 standard deviations (SD).

4.2.16 Antibodies against non-structural (NS) protein FMDV

Antibodies against the highly conserved 3B non-structural protein were determined using a commercial kit following the manufacturer's instructions (PrioCHECK™ FMDV NS Antibody ELISA Kit, ThermoFisher Scientific).

Results

5 Role of HO-1 expression in APC cells and the effects in the immune response in *F. hepatica*-infected mice

This section details the findings related to objectives specifics 1.

The main results of this part I of the chapter are contained in the attached publication (Art 1).

Costa, M., da Costa, V., Frigerio, S., Festari, M. F., Landeira, M., Rodríguez-Zraquia, S., Freire, T. (2021). Heme-Oxygenase-1 attenuates oxidative functions of antigen presenting cells and promotes regulatory T cell differentiation during *Fasciola hepatica* infection. *Antioxidants*, *10*(12), 1938

5.1 Heme-Oxygenase-1 decrease oxidative functions in F4/80^{+} cells and generates Treg during *F. hepatica* infection

In this first study, we focused on the function of the enzyme HO-1, known for its antioxidant and anti-inflammatory properties. We investigated how the expression of HO-1 in APCs affects the immune response during the experimental murine model of *F. hepatica* infection.

Previous studies have demonstrated that *F. hepatica* can alter the host's immune response by inducing regulatory DC (Cwiklinski et al., 2016b; Dowling et al., 2010; Moazeni & Ahmadi, 2016; Rodríguez, Kalay, et al., 2017) and alternatively activated macrophages, as well as promoting the adaptive immune response with Th2 and Treg-associated cytokine's (Donnelly et al., 2008; Flynn & Mulcahy, 2008; O'neill et al., 2000; Walsh et al., 2009). Macrophages and DC are two types of myeloid cells with high heterogeneity that specialize in the presentation of antigens. Macrophages are recognized for their great degradative capacity and high production of oxidative mediators. DC play an indispensable role in the adaptive immune response, orchestrating the activation of naïve T cells and inducing their differentiation into effector T cells (Banchereau et al., 2000; Puhr et al., 2015). Additionally, both DC and macrophages (F4/80^{+} cells) can trigger inflammation by releasing reactive oxygen or nitrogen species (ROS/RNS) and pro-inflammatory cytokines.

HO-1 is an inducible enzyme that is critical in various processes, such as cryoprotection, protection from heme-induced toxicity, and regulates the host inflammatory response(Carasi et al., 2017; Davies et al., 2013; Jenkins & Allen, 2021; Vijayan et al., 2018) . HO-1 plays an important role in

preventing the maturation of DC and the production of ROS (Kutty & Maines, 1981). Furthermore, HO-1 can effectively reduce the inflammatory capacity of both macrophages and DC. In fact, when these cells express HO-1, it promotes the secretion of interleukin 10 (IL-10), which in turn suppresses the proliferation of T-cells. Furthermore, the expression of IL-10 is associated with the presence of anti-inflammatory M2c macrophages that produce HO-1(Sierra-Filardi et al., 2010). This association is particularly observed in macrophages that express a hemoglobin scavenger receptor (CD163) (Philippidis et al., 2004). Furthermore, different studies have shown that HO-1 can have positive or negative effects on the host immune response against various pathogens (Chung et al., 2009). It is worth mentioning that Mitterstiller et al. (2016) and Abdala et al. (2015) found that HO-1 promotes bacterial survival inside macrophages by decreasing the production of ROS. However, the importance of antioxidant function of HO-1 in infections caused by helminth parasites is still poorly understood.

Our research group previously reported that the anti-inflammatory effects of HO-1 induction can be harmful in *F. hepatica* infection in mice and is associated with the generation of a Th2/Treg immune profile (Carasi et al., 2017). However, we have not studied the antioxidant mechanism of HO-1 during *F. hepatica* infection. In this part of the thesis, we investigated the role of ROS/RNS production by F4/80$^+$ HO-1$^+$ cells during *F. hepatica* experimental infection in mice and characterized the adaptive cell immune response.

5.1.1 Results

5.1.2 HO-1 expression in F4/80$^+$ peritoneal cells decrease the production of ROS/RNS

First, we analyzed the recruitment of HO-1-expressing cells to the peritoneal cavity (PEC) of *F. hepatica*-infected mice. To do so, we infected mice with 10 mertacercariae per animal and evaluated PEC at different time points: 1, 5, 8, 15, and 21 dpi. To determine the severity of the infection, we used the protocol previously used in our laboratory (Carasi et al., 2017) that takes into account the degree of cells in the peritoneal cavity, the presence of hemorrhage, damage hepatic, and splenomegaly. The identification of HO-1$^+$ cells was performed by flow cytometry.

As shown in Figure 1A, the clinical score increased with the infection, whereas ALT levels in serum significantly increased only after 21 dpi. ALT is an indicator of liver

dysfunction. Interestingly, we detected a significant increase of HO-1^{+} cells in the peritoneal cavity, as shown in Figures 1B and C. These cells were mainly composed of F4/80^{+} cells, as depicted in Figure 1D and E. F4/80 is a commonly used marker to identify monocyte-macrophage cells in mice but it can be also expressed on some subsets of DC (Vermaelen & Pauwels, 2004). CD11c is usually used to characterize DC, although other cells, such as alveolar macrophages can express both F4/80 and CD11c molecules (Osterholzer et al., 2009). The increase of F4/80^{+} cells was found to be directly proportional to the advanced stages of the infection (after 15 dpi). It could be explained by the recruitment of monocytes or the rapid proliferation of the tissue-resident population(Jenkins et al., 2011).

The expression of HO-1 increased slightly in F4/80^{+} cells after 1 dpi, but considerably increased over the days of infection as shown in Figure 1F. We also observed that the expression of HO-1 in peritoneal F4/80^{+} cells inversely correlated with the production of ROS/RNS: while the production of ROS/RNS increased significantly only at 1 dpi, it decreased during infection (Figure 1G).

Pharmacological agents and genetic probes that induce or inhibit HO-1 are widely used tools that allow to study and explore its role in infections, pathological systems, and immune regulatory properties (Carasi 2017). In this work, we used the HO-1 inducer CoPP (Cobalt protoporphyrin) and the inhibitor SnPP (tin protoporphyrin) to study the role of HO-1. To confirm the inverse correlation between HO-1 expression and ROS/RNS production in F4/80^{+} cells, RAW 264.7 macrophages were incubated with parasite components (FhTE) in the presence of CoPP or SnPP, and the production of ROS/RNS by these cells was analyzed.

The treatment with FhTE slightly increased the production of ROS/RNS, whereas CoPP and SnPP significantly decreased and increased the production of ROS/RNS, respectively, as shown in Figure 1H. On the other hand, FhTE induced the expression of ROS/RNS, which could be the outcome of an active respiratory burst, like that observed in F4/80^{+} cells from PECs of infected mice at 1 dpi (Figure 1G).

In summary, these results suggest that *F. hepatica* triggers the expression of HO-1 in F4/80^+ cells that are recruited to the peritoneal cavity, which inhibits the production of ROS/RNS during the course of infection.

5.1.3 Presence HO-1^+ in APC peritoneal Cells is associated with increased of splenic CD4^+ CD25^+ and CD8^+ CD25^+ cells

Given that HO-1 can induce regulatory T cells. Then, we examined the presence of CD4^+ and CD8^+ cells in the spleens of infected mice. Although we could not find any significant differences in the percentage of CD4^+ and CD8^+ cells during the infection, we did observe a significant increase in the number of these cells after 15 dpi as shown in Figure 2B and Supplementary Figure S2.

In our analysis, we examined the existence of splenic CD25^+ CD4^+ (Figure 2B and Supplementary Figure S2) or CD8^+ (Figure 2C and Supplementary Figure S2) T cells. We found no significant differences in the percentage of these cells during the infection. However, their number significantly increased after 15 dpi.

We also analyzed T cells in the livers of infected animals and we found no difference in the number or percentage of CD4^+ T cells (Figure 2D and Supplementary Figure S3). In advanced infected mice, the number, but not the frequency, of hepatic CD25^+ CD4^+ T cells was increased. Further analysis showed that the number of splenic CD25^+ CD4^+ and CD25^+ CD8^+ cells positively correlated with the number of peritoneal HO1^+ cells (Figure 2E, Supplementary Figure S3, and Figure 2F).

5.1.4 HO-1 activity decreases the Production of ROS/RNS in F4/80^+ cells and increases of splenic regulatory CD4^+ T Cells

To investigate the mechanism behind HO-1-mediated inhibition of ROS/RNS production, we administered the HO-1 inhibitor SnPP to infected mice. We used a dose of 40 mg/kg of SnPP to inhibit the enzyme activity of HO-1, while sterile PBS (200 µL) was used as a control (Paul et al., 2005; Sardana & Kappas, 1987). Mice received intraperitoneal injections of SnPP 1 day before

infection, 1 day after infection, and every 4 days until the end of the experimental protocol (between 19 and 21 dpi).

Figure 3A shows that the treatment with SnPP inhibited the increase of clinical score in infected mice. Importantly, SnPP treatment of infected mice affects the rise in the number and frequency of HO-1$^+$ cells induced by the infection since no significant difference was found in infected mice with respect to the control group with SnPP treatment (Figure 3B and Supplementary Figure S4). Interestingly, SnPP-treated infected mice showed an increased number of F4/80$^+$ cells that produced higher levels of ROS/RNS than control-infected mice despite expressing similar levels of HO-1 (Figures 3D and 3E). The SnPP acts as a competitive inhibitor of HO-1 both in vitro and in vivo (Fernández-Fierro et al., 2020; Schulz et al., 2012). The inhibitory activity of SnPP on HO-1 suggests that its effects are caused by the catabolizing activity of heme rather than by its expression and function as a transcription factor. This could be the reason why PEC from SnPP-treated mice did not show a decrease in HO-1 expression despite a significant increase in ROS/RNS production.

Peritoneal F4/80$^+$ cells from infected mice treated with SnPP presented a reduced expression of ICOSL with respect to infected mice (Figure 3F). ICOSL is expressed on the surface of many APCs and is responsible for inducing Treg proliferation (Y. L. D. C. Oliveira et al., 2021). Of note, the treatment with SnPP did not result in an increase in the count of CD4$^+$ T cells or CD4$^+$/CD25$^+$ T cells in the spleen (as shown in Figure 3G and Figure 3H, respectively). However, in the absence of SnPP treatment, these cells expressed higher levels of CTLA4 (as shown in Figure 3H). CTLA4 expression can down-regulate T cell activation and it is constitutively expressed by CD4$^+$CD25$^+$ T cells (Salomon & Bluestone, 2001; T. Takahashi et al., 2000), suggesting a role of CTLA4 in the function and maintenance of these cells.

Taken together, these results suggest that the activity of HO-1 reduces the production of ROS/RNS during fasciolosis. This reduction is associated with an increase in splenic regulatory CD4$^+$ T cells, which may involve the ICOSL protein in antigen-presenting cells or the expression of CTLA4 in Tregs.

Next, we evaluated how SnPP treatment affected the recruitment or phenotypical characteristics of peritoneal F4/80^{+} cells in the peritoneal cavity at early *F. hepatica* infection stages (1 dpi) and compared them with both non-treated infected and control mice. Two different cell populations were observed based on F4/80 expression (Figure 4A and Supplementary Figure S1), which were called F4/80^{hi} and F4/80^{int}.

In the peritoneal cavity, there are two macrophage subsets that coexist in adult mice (Bou Ghosn et al., 2010). The resident macrophages in the healthy peritoneal cavity are characterized by the expression of high levels of F4/80 and low levels of MHC II (F4/80^{hi} MHCII ^{low}) and are sometimes referred to as large peritoneal macrophages (LPM)(Bain & Jenkins, 2018; Bou Ghosn et al., 2010). These F4/80^{hi} cells possess characteristic macrophage morphology, including abundant cytoplasmic vacuoles, and are highly phagocytic (Bain & Jenkins, 2018; Bou Ghosn et al., 2010; Jenkins et al., 2011; P. R. Taylor et al., 2003).

Other subsets described in the peritoneal cavity are the inflammatory macrophages that show a phenotype F4/80^{low} MHCII ^{hi} and are called small peritoneal macrophages (SPM). These cells can produce NO in response to LPS stimulation *in vivo* (Bain & Jenkins, 2018; Bou Ghosn et al., 2010). In an inflammatory environment, the LPMs rapidly disappear from the peritoneum cavity, leading to an increase in SPMs in the same time (Bou Ghosn et al., 2010).

At 1 dpi the treatment of SnPP increased both the frequency and number of SPM, while it decreased LPM cells in the peritoneal cavity of infected mice (as shown in Figure 4B). However, regardless of SnPP treatment, SPM cells expressed similar levels of HO-1 and ROS/RNS (as shown in Figures 4C and 4D). The peritoneal F4/80^{int} (SPM) cells showed elevated levels of CCR2 (as shown in Figure 4E). However, only those cells from infected mice treated with SnPP demonstrated significantly increased levels of IL-33R (as shown in Figure 4F). This could indicate the initiation of an early immune response against the parasite. CCR2 is a receptor responsible for recruiting monocytes to sites of inflammation. Previous studies have demonstrated that deficiency in CCR2 can compromise cytokine secretion by both Th1 and Th2 cells (Bakos et al., 2017). Bakos and collaborators also demonstrated that during inflammation, high levels of CCR2 can induce a profile Th1/Th17 and reduce their differentiation to Tregs. Our results would suggest that SnPP treatment induces the presence of F4/80^{int} IL-33R^{+} cells in the peritoneal cavity, which

in turn protects mice from infection. IL-33 is a protein that plays a role in the immune response to helminth infections and initiates a specific type of immune cell called innate lymphoid cell type 2. However, the role of IL-33 during *F. hepatica* infection in mice is still unknown.

5.1.5 Effect of HO-1 inhibition by SnPP treatment in the gene expression of antioxidant molecules

To investigate the relationship between *F. hepatica* infection and HO-1 expression in peritoneal cells, we analyzed the gene expression of different molecules involved in the oxidative and antioxidative responses. These were Nrf2, catalase (CAT), superoxide dismutase (SOD) 1 and 2, glutathione peroxidase (GPX) 1 and 2, and NADPH-oxidase subunits p47phox and gp91phox.

Nrf2 is a transcription factor that induces the gene expression of a wide variety of antioxidants, including HO-1 (Biswas et al., 2014). SOD (1 and 2), CAT and GPX (1 and 2) are the most important enzymes of the cell antioxidant defense system (Ighodaro & Akinloye, 2018). NADPH oxidase is an important generator of ROS in macrophages and DC, which consists of two transmembrane components (gp91Phox and p22Phox) associated with three cytosolic components (p47Phox, p67Phox and p40Phox) (Nauseef, 2008).

We observed a significant reduction in Nrf2 transcription factor mRNA levels in PECs from infected mice treated with SnPP (Figure 5A). These results correlated with decreased mRNA levels in antioxidant enzymes, including cat, gpx2, and sod2 (Figure 5B). However, there were no significant differences in the gene expression of gpx1, while an increase in sod1 expression was observed (Figure 5B). With SnPP treatment of infected mice, there was an unexpected decrease in mRNA levels of the NADPH oxidase subunits gp91phox and p47phox (Figure 5C).

5.1.6 Deficiency of NADPH oxidase protects mice from liver damage, limiting the IL-10 production

As shown previously in Figure 5C, treatment with SnPP protected mice from parasite infection, leading to reduced levels of gp91phox mRNA in PECs from infected mice. Subsequently, we analyzed the infection in gp91phox knockout mice.

Deficiency of gp91phox reduced the clinical signs and liver damage caused by *F. hepatica* infection (Figure 6A and Supplementary Figure S5). This partial protection was linked to a lower increase of HO-1^{+} peritoneal cells, both in terms of frequency and number (Figure 6B). In addition, HO-1^{+} cells from gp91phox knockout infected mice showed reduced expression of MHCII (Figure 6C) and CD40 (Figure 6D), but not CD80 (Figure 6E), when compared to cells from wildtype mice. This suggests that NADPH oxidase may have a role in both the immune response and the pathogenesis caused by *F. hepatica*.

Further analysis of peritoneal cells from these mice showed that F4/80^{+} cells were not increased in the absence of gp91phox (Figure 7A) while ROS/RNS production was reduced (Figure 7B). In addition, it was observed that these cells had lower levels of Sirpα (Figure 7C), ICOSL (Figure 7D), and IL-10 (Figure 7E). Furthermore, the number of CD4^{+} cells (Figure 7F and Supplementary Figure S6) and CD4^{+}/CD25^{+}FoxP3^{+} Tregs (Figure 7G and Supplementary Figure S6) was found to be lower in gp91phox knockout infected mice as compared to wild-type mice. There were no significant differences in IFN-γ production by splenocytes stimulated with FhTE between gp91phox knockout and wild-type infected mice (Figure 7H).

5.1.7 IL-10 plays a crucial role in the HO-1 expression in *F. hepatica* infection

Interleukin 10 is a type of cytokine that plays an important role in regulating the immune system. It is produced in large quantities by macrophages and DCs and results in the inhibition of alloreactive T-cell proliferation. (Carasi et al., 2017; Rodríguez et al., 2015). Thus, we investigated the potential correlation between IL-10 signaling and HO-1 expression in the context of *F. hepatica* infection. For this purpose, we administered a neutralizing antibody specific for the IL-

10 receptor (IL-10R) (2 μg/mouse in PBS) intraperitoneally on day -1 and day +1, +4, +8, +12, +17, to the infected mice. The obtained results indicate that IL-10R blockade resulted in the alleviation of the clinical symptoms associated with the parasite infection (Figure 8A).

Although IL-10R neutralization did not affect the recruitment of F4/80+ cells in the peritoneal cavity (Figure 8B), the HO-1 expression demonstrates a tendency to decrease when induced by *F. hepatica* infection. It is worth noting that neutralizing the IL-10 receptor reduced the frequency of $CD4^+$ (Figure 8D and Supplementary Figure S7) and $CD4^+$ $CD25^+$ T cells (Figure 8E) in the spleen of infected animals but did not affect their overall number. These findings suggest that IL-10 signaling could be important for HO-1 expression of $F4/80^+$ cells during *F. hepatica* infection, which in turn could affect the differentiation of regulatory T cells.

5.1.8 Discussion

In our study, we examined how positive HO-1 cells in fasciolosis correlate with the differentiation or expansion of Tregs through cellular and molecular mechanisms. Our findings agree with the bibliography that HO-1 expression in $F4/80^+$ cells decrease the production of ROS/RNS in the inflammatory environment (Araujo et al., 2012; Campbell et al., 2021; Wu et al., 2011). This suggests that HO-1 has a protective effect on the oxidative function of APCs.

It has been found that inhibiting HO-1 can help to control *M. tuberculosis* infection in mice by promoting IFN-γ and NOS2-mediated responses (D. L. Costa et al., 2021). Additionally, previous studies have shown that HO-1 plays a role in suppressing pro-inflammatory Th1 immune responses in experimental colitis. It has also been reported that sickle cell alloimmunization provides protection against atherosclerosis (Takagi et al., 2018; Zhong et al., 2014).

HO-1, even when enzymatically inactive, is capable of providing protection against hydrogen peroxide-induced toxicity (Hori et al., 2002; Vijayan et al., 2018). This is likely due to its ability to promote the gene expression of antioxidant proteins. However, the mechanisms behind these effects are not yet fully understood. It is possible that HO-1 acts as a transcription factor, but further investigation is needed since the nuclear localization of HO-1 in $F4/80^+$ cells derived from *F. hepatica*-infected mice with or without SnPP treatment has not been studied. It is unlikely that the

protective effect of SnPP treatment is a direct consequence of its impact on *F. hepatica*. This is because the degree of infection and pathological effects induced by the parasite were related to an increase in Tregs, indicating that HO-1 activity has an impact on the host adaptive immunity *in vivo*.

Our study shows that the increase in mRNA levels of Nrf2, a transcription factor responsible for regulating cellular redox balance and protecting against oxidative stress (Loboda et al., 2016; Pibiri et al., 2018), is linked to an increase in some antioxidant enzyme genes. This suggests that the infection, HO-1, Tregs, and the Nrf2 master regulator work together as a complex axis of antioxidant and immunoregulatory properties in *F. hepatica* infection. It is necessary to determine the role of these enzymes in order to confirm their antioxidant function during *F. hepatica* infection. Additionally, murine macrophages that are activated by heme exhibit anti-inflammatory properties that are dependent on the enzymatic activity of HO-1 (Hualin et al., 2012).

It should be noted that the detrimental role of oxidative mechanisms in the parasite and host surroundings due to helminth parasite infections is not well-established (R. B. de Oliveira et al., 2013; Derda et al., 2004; Sanchez-Campos et al., 1999). Some reports suggest that *Strongyloides papillosus* infection induces oxidative/nitrosative stress in sheep, but its effect on the parasite remains unknown (Dimitrijević et al., 2012). On the other hand, *Schistosome* infection leads to immense oxidative stress in the host that is insufficient to control the infection (Masamba & Kappo, 2021).

According to a recent report, rabbits infected with *F. gigantica* have shown a high oxidative status in their serum and liver. Additionally, there was a decrease in the expression of SOD and CAT genes, as well as enzyme activity in the serum of these infected animals. These findings are different from the results of our research on mice infected with *F. hepatica*. After conducting their study, the authors concluded that *F. gigantica* disrupts the antioxidant and detoxification cascades in the host, which can cause a pathogenic response (Rehman et al., 2021).

In our experiment, we used a DCFDA fluorescent probe that does not differentiate between ROS and RNS. Therefore, these studies should be supplemented with others employing ROS-specific

probes like DHE or specific inhibitors of nitric oxide production. In order to analyze ROS produced by NADPH-oxidase, we used gp91phox knockout mice instead. These mice have a deficiency in NADPH oxidase function, which results in a significant decrease in ROS production. They were somewhat protected against *F. hepatica* infection. This suggests that the timing of ROS production by NADPH oxidase may be critical in limiting the damage caused by *F. hepatica* (see Figure 9B).

Uncontrolled ROS production induced by a pro-inflammatory immune response can harm leukocyte function and viability and damage the immune system (Rehman et al., 2021). Therefore, the excessive production of ROS by F4/80$^+$ cells could benefit the parasite rather than the host. F4/80$^+$ cells derived from wild-type mice showed increased levels of ICOSL and IL-10 compared to those from gp91phox knockout mice. This difference could be linked to the differentiation or expansion of a larger number of splenic Tregs expressing higher levels of CTLA4. Both ICOSL (D.-Y. Li & Xiong, 2020) and CTLA4 (Sobhani et al., 2021) play a crucial role in the differentiation of Treg cells. Further analysis of the role of IL-10 produced by APC during the infection demonstrated that the presence of splenic Tregs and peritoneal F4/80$^+$ expressing HO-1 were both dependent on IL-10 activity. However, the production of IL-10 by the host also provides protection to host cells during the acute pro-inflammatory immune response.

During the early stages of *F. hepatica* infection, the production of ROS/RNS is partially effective against the parasite (Figure 9B). In order to gain a better understanding of the early events that occur during *F. hepatica* infection in mice, we examined the expression of HO-1 and the recruitment of F4/80$^+$ cells at 1 dpi. We found that there were two distinct populations of cells expressing different levels of F4/80 present in the peritoneal cavity. Moreover, the cells elicited in SnPP-treated mice exhibited higher levels of IL33R, as shown in Figure 9B.

In summary, this study shows that the enzyme HO-1 plays an essential role during *F. hepatica* infection by reducing the oxidative functions of APCs and promoting the differentiation of Tregs. These findings suggest that HO-1 could be a potential therapeutic target for modulating this parasite's immune response during infection.

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Heme-Oxygenase-1 Attenuates Oxidative Functions of Antigen Presenting Cells and Promotes Regulatory T Cell Differentiation during *Fasciola hepatica* Infection

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Abstract: *Fasciola hepatica* is a fluke that infects livestock and humans causing fasciolosis, a zoonotic disease of increasing importance due to its worldwide distribution and high economic losses. The parasite regulates the host immune system by inducing a strong Th2 and regulatory T (Treg) cell immune response through mechanisms that might involve the expression or activity of heme-oxygenase-1 (HO-1), the rate-limiting enzyme in the catabolism of free heme that also has immunoregulatory and antioxidant properties. In this paper, we show that *F. hepatica*-infected mice upregulate HO-1 on peritoneal antigen-presenting cells (APC), which produce decreased levels of both reactive oxygen and nitrogen species (ROS/RNS). The presence of these cells was associated with increased levels of regulatory T cells (Tregs). Blocking the IL-10 receptor (IL-10R) during parasite infection demonstrated that the presence of splenic Tregs and peritoneal APC expressing HO-1 were both dependent on IL-10 activity. Furthermore, IL-10R neutralization as well as pharmacological treatment with the HO-1 inhibitor SnPP protected mice from parasite infection and allowed peritoneal APC to produce significantly higher ROS/RNS levels than those detected in cells from infected control mice. Finally, parasite infection carried out in gp91^{phox} knockout mice with inactive NADPH oxidase was associated with decreased levels of peritoneal HO-1^{+} cells and splenic Tregs, and partially protected mice from the hepatic damage induced by the parasite, revealing the complexity of the molecular mechanisms involving ROS production that participate in the complex pathology induced by this helminth. Altogether, these results contribute to the elucidation of the immunoregulatory and antioxidant role of HO-1 induced by *F. hepatica* in the host, providing alternative checkpoints that might control fasciolosis.

Keywords: helminth; heme-oxigenase-1; immunoregulation; antigen presenting cell; regulatory T cell; ROS/RNS

Citation: Costa, M.; da Costa, V.; Frigerio, S.; Festari, M.F.; Landeira, M.; Rodríguez-Zraquia, S.A.; Lores, P.; Carasi, P.; Freire, T. Heme-Oxygenase-1 Attenuates Oxidative Functions of Antigen Presenting Cells and Promotes Regulatory T Cell Differentiation during *Fasciola hepatica* Infection. *Antioxidants* **2021**, *10*, 1938. https://doi.org/10.3390/antiox10121938

Academic Editors: Elias Lianos and Maria G. Detsika

Received: 2 October 2021
Accepted: 10 November 2021
Published: 3 December 2021

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1. Introduction

Fasciolosis is a major parasitic disease of livestock caused by the trematode *Fasciola* spp. [1]. Nowadays, the number of infected people around the world is increasing, which makes fasciolosis an emerging zoonosis [1]. The World Health Organization (WHO) estimates that approximately 180 million are at risk of infection and 17 million people are infected, with a high prevalence in humans in Africa and South America [2]. Moreover, the economic losses caused by fasciolosis are estimated at around 3 billion US dollars per year due to livestock infection [1].

During infection, *Fasciola hepatica* modulates the host immune response characterized by the presence of regulatory dendritic cells (DC) [2–5], alternative activated macrophages [6], and

an adaptive immune response characterized by Th2 and regulatory T cell (Treg)-associated cytokines [7–10]. Macrophages and DC comprise a highly heterogeneous myeloid cell population specialized in antigen presentation. DC play a crucial role in orchestrating the adaptive immune response by activating naïve T cells and inducing their differentiation into different effector T cells depending on the pathogen [11,12]. Furthermore, both macrophages and DC can promote inflammation by secreting pro-inflammatory cytokines and reactive oxygen or nitrogen species (ROS/RNS), although they can also inhibit inflammation through anti-inflammatory cytokine production [13–15]. Thus, they exhibit functional plasticity that enables them to adapt to various local conditions in order to restore homeostasis after inflammation [11,12,16].

Heme-oxygenase-1 (HO-1) is the inducible rate-limiting enzyme involved in the catabolism of free heme. It participates in several processes, by providing cytoprotection, protecting from heme-induced toxicity, and regulating the host inflammatory response [14,17]. In fact, HO-1 acts as a stress-responsive enzyme induced by the nuclear factor NF-E2-related factor 2 (NRF2), to provide defense against oxidative-induced injury during inflammatory processes [14]. HO-1 also limits the secretion of pro-inflammatory cytokines [18,19] and promotes anti-inflammatory cytokines [20,21], in a process triggered, at least in part, by ROS in macrophages, although its effects depend on the model of study [14]. In the same line, expression of HO-1 inhibits DC-maturation and the production of ROS [22]. In addition, HO-1 expression by DC induces the production of IL-10, an anti-inflammatory cytokine, that inhibits T-cell proliferation [23]. Additionally, IL-10-producing anti-inflammatory M2c macrophages express HO-1 [24], especially on those that express CD163, a hemoglobin scavenger receptor, that in fact mediates IL-10 production and HO-1 synthesis [25]. Therefore, HO-1 acts as a key mediator of anti-inflammatory pathways in both macrophages and DC.

Several reports demonstrate that HO-1 can have both beneficial and detrimental effects for the host immunity against different pathogens [26]. Furthermore, the antioxidant role of HO-1 in infectious diseases is still unclear, especially in helminth parasites. Interestingly, bilirubin, one of the enzymatic byproducts of HO-1, suppresses the killing of bacteria by reducing the neutrophil burst via its antioxidant activity [27]. In addition, HO-1 promotes bacteria survival inside macrophages by decreasing ROS production, as demonstrated for *Salmonella typhimurium* [28] and *Mycobacterium abscessus* [29] via its ROS-diminishing properties. Recently, we showed that the anti-inflammatory effects of HO-1 induction are detrimental in *F. hepatica* infection, which is dominated by a Th2/Treg differentiation profile [17]. However, the antioxidant mechanisms induced by HO-1 during *F. hepatica* infection have not been approached so far.

Thus, in this work, we investigated the role of ROS/RNS production by myeloid HO-1^{+} cells during *F. hepatica* experimental infection in mice, and characterized the adaptive cell immune response. Our results indicate that HO-1 expression by myeloid cells during *F. hepatica* infection negatively correlates with the production of ROS or RNS and the increase of antioxidant molecules. Furthermore, the pharmacological inhibition of HO-1 by a well-characterized inhibitor of HO-1 enzymatic activity, tin protoporphyrin IX (SnPP), in *F. hepatica*-infected mice was associated with lower levels of Tregs, in a process that was mediated by IL-10 biological activity. However, the gene expression of the NADPH oxidase subunit gp91^{phox} was decreased when SnPP was administrated to infected mice. Moreover, parasite infection carried out in gp91^{phox} knockout mice with inactive NADPH oxidase showed decreased levels of peritoneal HO-1^{+} cells, splenic Tregs, and partially protected mice from the hepatic damage induced by the parasite, revealing that a more complex molecular mechanism involving ROS production participates in the intricate pathology induced by this helminth. Altogether, these results contribute to the elucidation of the immunoregulatory and antioxidant role of HO-1 during *F. hepatica* infection, providing interesting molecular checkpoints that might control fasciolosis.

2. Materials and Methods

2.1. Mice

Female BALB/c mice (six- to eight-week-old) were purchased from DILAVE Laboratories (Uruguay). Gp91^{phox-} knockout (B6.129S-Cybbtm1Din/J) mice were purchased from Jackson Laboratory (USA) and maintained at UATE, Institut Pasteur Montevideo. Six to eight BALB/c, gp91^{phox-}, and C57BL/6 littermates (controls for gp91^{phox-} mice) were used per experiment. Animals were kept in the animal house (URBE, School of Medicine, UdelaR, Uruguay), with water and food supplied *ad libitum*. Mouse handling, care, and experiments were carried out in compliance with institutional guidelines and regulations from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, https://www.cnea.gub.uy/, accessed on 12 November 2021, National Law 18.611, Uruguay). Procedures involving animals were approved by the Universidad de la República's Committee on Animal Research (Comisión Honoraria de Experimentación Animal, CHEA Protocol Number 07153-000817-18).

2.2. Parasite Protein Extract (FhTE)

FhTE was prepared from live adult flukes obtained from infected bovines. Flukes were washed for 1 h at 37 °C with phosphate buffered saline (PBS), pH 7.4, sonicated, and then centrifugated at 40,000× *g* for 60 min [4,30]. Finally, protein lysates were dialyzed against PBS. FhTE protein concentration was measured using the bicinchoninic acid assay (Sigma, St. Louis, MO, USA). In order to remove endotoxin contamination, FhTE was applied to a column containing endotoxin-removing gel (detoxi-gel, Pierce Biotechnology, Waltham, MA, USA), and endotoxin levels were quantified using the Limulus Amebocyte Lysate kit Pyrochrome (Associates of Cape Cod, East Falmouth, MA, USA) and found to be lower than 0.05 EU/mL. Furthermore, at the used concentrations, FhTE did not induce the production of pro-inflammatory cytokines such as IL-12 or IL-6 [4,30]. The concentration of *F. hepatica* extracts used in the in vitro experiments did not modify cell viability, as evaluated by the MTT (2-[4,5-dimethyl-2-thiazolyl]-3,5-diphenyl-2H-tetrazolium bromide) assay [4,17,30].

2.3. *F. hepatica* Infections

2.3.1. Parasite Infections, Animal Treatments, and Sample Obtention

The infection was achieved by orally administrating 10 *F. hepatica* metacercariae (Montevideo, Uruguay) per mouse. Mice were bled and peritoneal exudate cells (PECs), spleens, and livers were removed after 1, 8, 15, and 21 days post-infection (dpi), while non-infected animals were used as controls. Each experimental group contained at least four mice. PECs and hepatic leukocytes were obtained as already described [31]. Red cells were lysed with ammonium chloride potassium buffer.

HO-1 activity was inhibited using SnPP (40 mg/kg), and vehicle (PBS, 200 µL) was used as a control. The SnPP dose was within a range of doses used in previous works [32,33]. Mice received intraperitoneal injections of SnPP 1 day before infection, 1 day after infection, and every 4 days until the end of the experimental protocol (between 19 and 21 dpi). When gp91^{phox-} and non-infected littermates were used (n = 6–8/group), infections were performed in the same conditions as previously described. In order to neutralize IL-10 receptor (IL-10R), BALB/c mice (n = 6–8/group) were intraperitoneally injected with 15 µg of monoclonal rat IgG2a anti-IL-10R (clone 1B1.3A from BioXcell, Lebanon, NH, USA) or an isotype-matched control antibody (clone HRPN from BioXcell, Lebanon, NH, USA), the day before and after infection with *F. hepatica* and every 3 days until animal sacrifice at 20 dpi. Blood samples were obtained, and PECs, spleens, and livers were removed. The infection severity was assessed with a defined clinical score according to the following parameters: presence or absence of peritoneal hemorrhage, presence of macroscopic liver damage and splenomegaly, and the amount of cell content in the peritoneal cavity [17], where the minimum score was 0 and the maximum was 10. The alanine aminotransferase

(ALT) activity in sera was used to quantify liver damage, determined with a commercial kit (Spinreact, Girona, Spain) according to the manufacturer's instructions.

2.3.2. Proliferation Assay and Cell Culture

Splenocytes (0.5×10^6/well) from infected mice or uninfected naïve mice (control group) were cultured for 5 days at 37 °C and 5% CO_2, in RPMI-1640 with 400 µg/mL of glutamine (Capricorn, Ebsdorfergrund, Germany) complete medium containing 10% heat-inactivated fetal bovine serum (FBS, Capricorn Scientific, Ebsdorfergrund, Germany), 50 mM of 2-mercaptoethanol, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin (Merk, Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of FhTE (75 µg/mL), as previously described [30]. An IFNγ-specific sandwich ELISA assay (Biolegend, San Diego, CA, USA) was used to quantify IFNγ levels in culture supernatants.

RAW264.7 macrophages were cultured at 0.5×10^6/mL in complete RPMI medium in the presence or absence of the HO-1 inductor (CoPP) and inhibitor (SnPP) (50 and 100 µM, respectively) or FhTE (75 µg/mL) overnight at 37 °C. Afterwards, the ROS/RNS production was determined as described in the following section.

2.4. Flow Cytometry

Cell suspensions from PECs, livers, and spleens were washed twice with PBS containing 2% FBS and 0.1% sodium azide (FACS buffer), stained with specific antibodies for 30 min at 4 °C as previously published [31]. The following antibodies were used: anti-Sirpα (P-84), -CD11c (N418), -CD86 (GL1), CD8 (53-6.7), -Siglec-F (E50-2440), -F4/80 (BM8), -CD11b (M1/70), -CD40 (HM40-3), -CD80 (16-10A1), and I-A/I-E (M5/114.15.2). Expression of FoxP3, HO-1, and IL-10 was analyzed by intracellular staining. Cells in which IL-10 was analyzed were incubated with Brefeldin-A for 6 h at 37 °C and phorbol myristate acetate (PMA, 200 nM) (Merk, Sigma-Aldrich, USA). After two washes with FACS buffer, cells were incubated with the following antibodies: anti-CD3 (17A2), -CD4 (RM4-5), -CD8 (53-6.7), or -F4/80 (BM8). After permeabilization with Cytofix and Perm wash buffers (Biolegend, USA), cells were incubated with IL-10 (JES5-1E3), FoxP3 (MF14), and HO-1 (clone ab13248 from Abcam, Waltham, MA, USA) specific antibodies. ROS/RNS produced by F4/80$^+$ cells were determined with 2′,7′-dichlorofluorescein diacetate (DCFDA, Merk, Kenilworth, NJ, USA) probe, a fluorogenic dye that is oxidized into the fluorescent 2′,7′-dichlorofluorescein. Briefly, cells were incubated in PBS for 30 min at 37 °C with DCFDA, washed with FACS buffer, and fluorescence was measured in a flow cytometer. Analyses were performed using a BD Accuri C6 Plus cytometer and software (BD-Biosciences). Antibodies were obtained from Biolegend (USA). Analyses were performed with Accuri C6 Plus software.

2.5. Determination of Oxidative and Antioxidative Genes by qRT-PCR

Nrf2, *catalase*, glutathione peroxidase (*gpx*) 1 and 2, superoxide dismutase (*sod*) 1 and 2, and NADPH-oxidase subunits p47^{phox} and gp91^{phox} mRNA levels were detected using the Eco real-time PCR System (Illumina, San Diego, CA, USA) and Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). ARN purification was performed with Tri-Reagent (Merk, Kenilworth, NJ, USA) of PECs obtained from BALB/c mice at 20 dpi, SnPP-treated or untreated, as previously described [17]. Standard amplification conditions were 10 min at 95 °C, 40 thermal cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, with a final extension of 10 min at 72 °C. The following primers were used: *nrf2*-F: 5′-CAGCATGTTACGTGATGAGG-3′, *nrf2*-R: 5′-GCTCAGAAAAGGCTCCATCC-3′, *gpx1*-F: 5′-GGGACTACACCGAGATGAACGA-3′, *gpx1*-R: 5′-ACCATTCACTTCGCACTTCTCA-3′, *gpx2*-F: 5′-GAGGAACAACTACCCGGGACTA-3′, *gpx2-R*: 5′-ACCCCCAGGTCGGACATACT-3′, *sod1*-F: 5′-TGGGTTCCACGTCCATCAGTA-3′, *sod1*-R: 5′-ACCGTCCTT TCCAGCAGTCA-3′, *sod2*-F: 5′-ATTAACGCGCAGATCATGCA-3′, *sod2*-R: 5′-TGTCCCCCACCATTGAACTT-3′, *catalase*-F: 5′-GCGTCCAGTGCGCTGTAGA-3′, *catalase*-R: 5′-TCAGGGTGGACGTCAGTGAA-3′, *p47phox*-F: 5′-GAGGCGGAGGATCCGG-3′, *p47phox*-R: 5′-TCTTCAACAGCAGCGTACGC-3′, *gp91phox*-F: 5′-CCAGTGAAGATGTGTTCAGCT-3′, *gp91phox*-R: 5′-GCACAGCCAGTAGA

AGTAGA-3′, *gapdh*-F: 5′-ATGACATCAAGAAGGTGGTGAAG-3′, *gapdh*-R: 5′-TCCTTGGAGG CCATGTAGG-3′. Results were expressed as the ratio between each gene under study and GAPDH expression. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. All reactions were performed with at least five biological replicates.

2.6. Statistical Analysis

Results of the experiments were expressed as mean ± SEM. GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Results were analyzed using one-way ANOVA followed by Tukey's test, or the two-tailed Student's *t*-test, depending on the experiment. Significant differences shown by an asterisk were considered when $p < 0.05$.

3. Results

3.1. HO-1 Expression in F4/80$^+$ Peritoneal Cells Inversely Correlate with ROS/RNS Production

In order to confirm the recruitment of PECs expressing HO-1 to the peritoneum of *F. hepatica*-infected mice, we identified HO-1$^+$ cells by flow cytometry at different time points of the infection. As seen in Figure 1A, the clinical score increased upon infection, although ALT in serum significantly increased only after 21 dpi, demonstrating liver dysfunction. In addition, HO-1$^+$ cells significantly increased in the peritoneal cavity during the infection (Figure 1B,C and Supplementary Figure S1). These cells were mainly composed by F4/80$^+$ cells (Figure 1D,E and Supplementary Figure S1), and their increase also correlated with the advanced stages of the infection (after 15 dpi). The expression of HO-1 in F4/80$^+$ cells slightly increased after 1 dpi, while it considerably increased during the infection (Figure 1F). On the other hand, the production of ROS/RNS was significantly increased only at 1 dpi, and decreased during infection (Figure 1G), suggesting that the expression of HO-1 in peritoneal F4/80$^+$ cells inversely correlated with the production of ROS/RNS. In order to provide more evidence in this regard, we incubated RAW 264.7 macrophages with parasite components (FhTE) in the presence of CoPP or SnPP, and analyzed the production of ROS/RNS by these cells. FhTE slightly increased the production of ROS/RNS, while CoPP and SnPP significantly decreased and increased the production of ROS/RNS by FhTE-treated macrophages, respectively (Figure 1H). Of note, FhTE *per se* induced ROS/RNS expression, which could be the result of an active respiratory burst, such as that seen in F4/80$^+$ cells from PECs of infected mice at 1 dpi (Figure 1G). Altogether, these results might indicate that *F. hepatica* induces the expression of HO-1 in F4/80$^+$ cells recruited to the peritoneum, inhibiting ROS/RNS production during the course of the infection.

3.2. The Presence of Peritoneal HO-1$^+$ Cells Associates with Increased Splenic CD4$^+$ CD25$^+$ and CD8$^+$ CD25$^+$ Cells during Infection

Considering that HO-1 can induce regulatory T cells [34–37], we analyzed the presence of both CD4$^+$ and CD8$^+$ cells in spleens of infected mice. Although we could not find any significant differences between the percentage of CD4$^+$ and CD8$^+$ cells during the infection, we did observe that they significantly increased in number after 15 dpi (Figure 2A and Supplementary Figure S2). We also analyzed the presence of splenic CD25$^+$ CD4$^+$ (Figure 2B and Supplementary Figure S2) or CD8$^+$ (Figure 2C and Supplementary Figure S2) T cells. Again, no significant differences were found in the percentage of these cells during the infection, while their number significantly increased after 15 dpi. Finally, we analyzed the presence of CD4$^+$ T cells in livers from infected animals and did not find any difference in their percentage nor their number (Figure 2D and Supplementary Figure S3). However, the number, but not the frequency, of hepatic CD25$^+$ CD4$^+$ T cells was increased in advanced infected mice (Figure 2E and Supplementary Figure S3). Further analyses demonstrated that the number of splenic CD25$^+$ CD4$^+$ and CD25$^+$ CD8$^+$cells positively correlated with the number of peritoneal HO1$^+$ cells (Figure 2F).

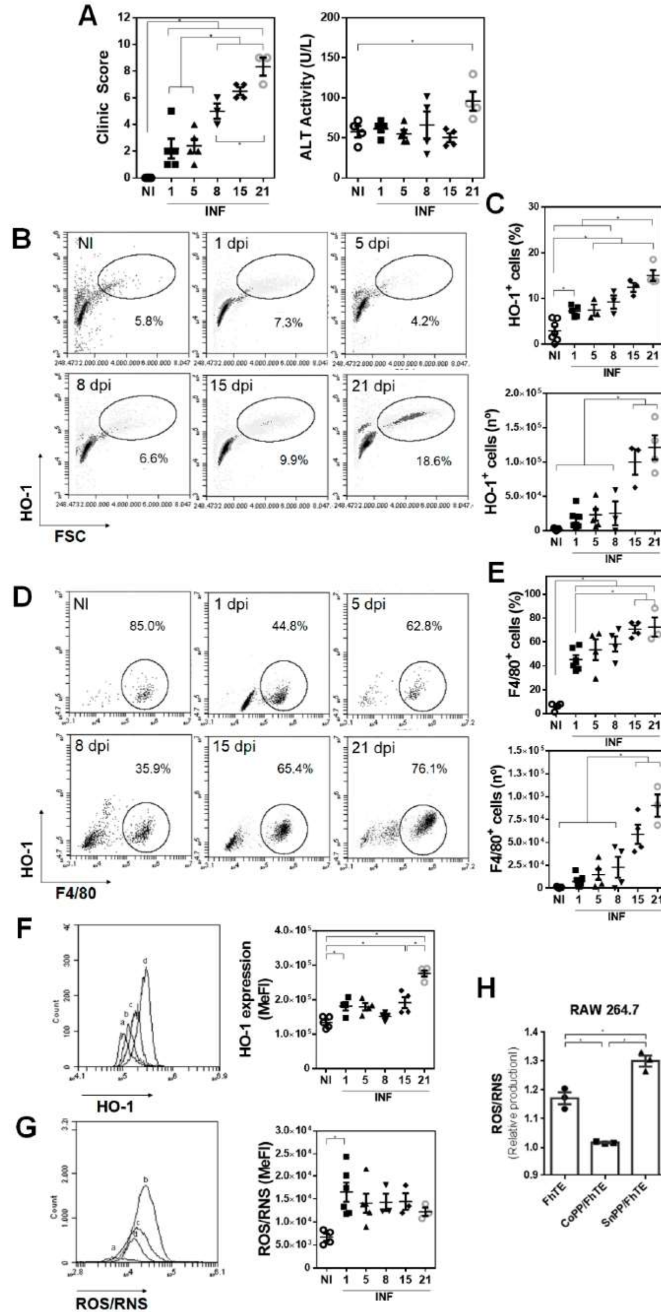

Figure 1. HO-1 expression in F4/80⁺ peritoneal cells inversely correlates with ROS/RNS production. Mice (n = 4–6) were infected with 10 metacercariae and sacrificed at 1, 8, 15, and 21 dpi. Non-infected

mice were used as control (NI). (**A**) Clinical signs including hemorrhage, splenomegaly, and macroscopic liver damage were assessed to evaluate the severity of the disease [17]. ALT activity in sera was used as a marker of liver damage. (**B**) Analysis by flow cytometry of HO-1$^+$ cells in PEC from infected and control (NI) mice. (**C**) Frequency and cell number of HO-1$^+$ cells in the peritoneal cavity of infected and control (NI) animals by flow cytometry. (**D**) Analysis by flow cytometry of F4/80$^+$ HO-1$^+$ cells in PEC from infected and control (NI) mice. (**E**) Frequency and cell number of F4/80$^+$ in the peritoneal cavity of infected and control (NI) animals by flow cytometry. (**F**) HO-1 expression in F4/80$^+$ in the peritoneal cavity of infected and control (NI) mice. Letters on histograms correspond as follows: a: NI, b: 1 dpi, c: 15 dpi, d: 21 dpi. Median fluorescence intensity is shown (MeFI) in the plot. (**G**) ROS/RNS quantification in F4/80$^+$ in the peritoneal cavity of infected and control (NI) mice using the DCFDA probe by flow cytometry. Letters on histograms correspond as follows: a: NI, b: 1 dpi, c: 15 dpi, d: 21 dpi. Median fluorescence intensity is shown (MeFI) in the plot. (**H**) Murine RAW264.7 macrophages were cultured in the presence of 75 µg/mL of FhTE or CoPP (100 µM/mL) and SnPP (50 µM/mL) overnight at 37 °C. Then, cells were collected and incubated for 30 min at 37 °C in PBS with the DCFDA probe and analyzed by flow cytometry. The RNS/ROS levels are shown as the ratio between FhTE/medium (FhTE), CoPP + FhTE/CoPP (CoPP/FhTE), and SnPP + FhTE/SnPP (SnPP/FhTE). Representative experiments are shown. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

3.3. HO-1 Activity Decreases the Production of ROS/RNS by F4/80$^+$ Cells and Correlates with an Increase of Splenic Regulatory CD4$^+$ T Cells Induced by F. hepatica Infection

In order to evaluate whether HO-1 interferes with the production of ROS/RNS, we treated *F. hepatica*-infected mice with the HO-1 inhibitor SnPP. SnPP treatment was associated with a decrease in the clinical signs of infected mice (Figure 3A). In addition, SnPP treatment of infected mice abrogated the increase of HO-1$^+$ cells, both in frequency and number, induced by the infection, since no significant difference was found in infected mice with respect to the control group with SnPP treatment (Figure 3B and Supplementary Figure S4). Surprisingly, a significant increase in F4/80$^+$ cell number, but not frequency, was found in both SnPP-treated and non-treated infected mice (Figure 3C and Supplementary Figure S4). Indeed, the F4/80$^+$ cell number was higher in SnPP-treated infected mice. Nevertheless, F4/80$^+$ cells of SnPP-treated infected mice produced higher levels of ROS/RNS than control infected mice (Figure 3D), although they expressed similar levels of HO-1 (Figure 3E). Of note, the expression of ICOSL in peritoneal F4/80$^+$ cells of infected mice was significantly reduced with SnPP treatment (Figure 3F). Lastly, SnPP treatment during *F. hepatica* infection did not induce an increase in the number of splenic CD4$^+$ T cells (Figure 3G) or CD4$^+$/CD25$^+$ T cells (Figure 3H), although these cells expressed higher levels of CTLA4 in the absence of SnPP treatment (Figure 3H). Altogether, these results suggest that HO-1 activity inhibited by SnPP decreases the production of ROS/RNS during fasciolosis and correlates with an increase of splenic regulatory CD4$^+$ T cells in a process that might involve ICOSL in antigen-presenting cells or CTLA4 expression in Tregs.

To complement these results, we evaluated whether the inhibition of HO-1 by SnPP treatment affected the recruitment or the phenotypical characteristics of peritoneal F4/80$^+$ cells at the early stages of *F. hepatica* infection. To this end, we analyzed F4/80$^+$ cells in the peritoneal cavity of SnPP-treated mice at 1 dpi and compared them with both non-treated infected and control mice. We observed the presence of two different cell populations according to F4/80 expression (Figure 4A and Supplementary Figure S1). SnPP treatment increased both the frequency and number of F4/80^{int} cells, while it reduced F4/80^{hi} cells in the peritoneal cavity of infected mice at 1 dpi (Figure 4B). Nevertheless, F4/80^{int} cells expressed similar levels of HO-1 (Figure 4C) and ROS/RNS (Figure 4D) in F4/80^{int} cells regardless of SnPP treatment. Interestingly, peritoneal F4/80^{int} cells expressed higher levels of CCR2 (Figure 4E), while only those from SnPP-treated infected mice expressed

significantly increased levels of IL-33R (Figure 4F), which could be related to the initiation of an early immune response against the parasite. Thus, these results suggest that the presence of F4/80^{int} IL-33R^{+} cells in the peritoneum is induced by SnPP treatment, which in turn protects mice from infection.

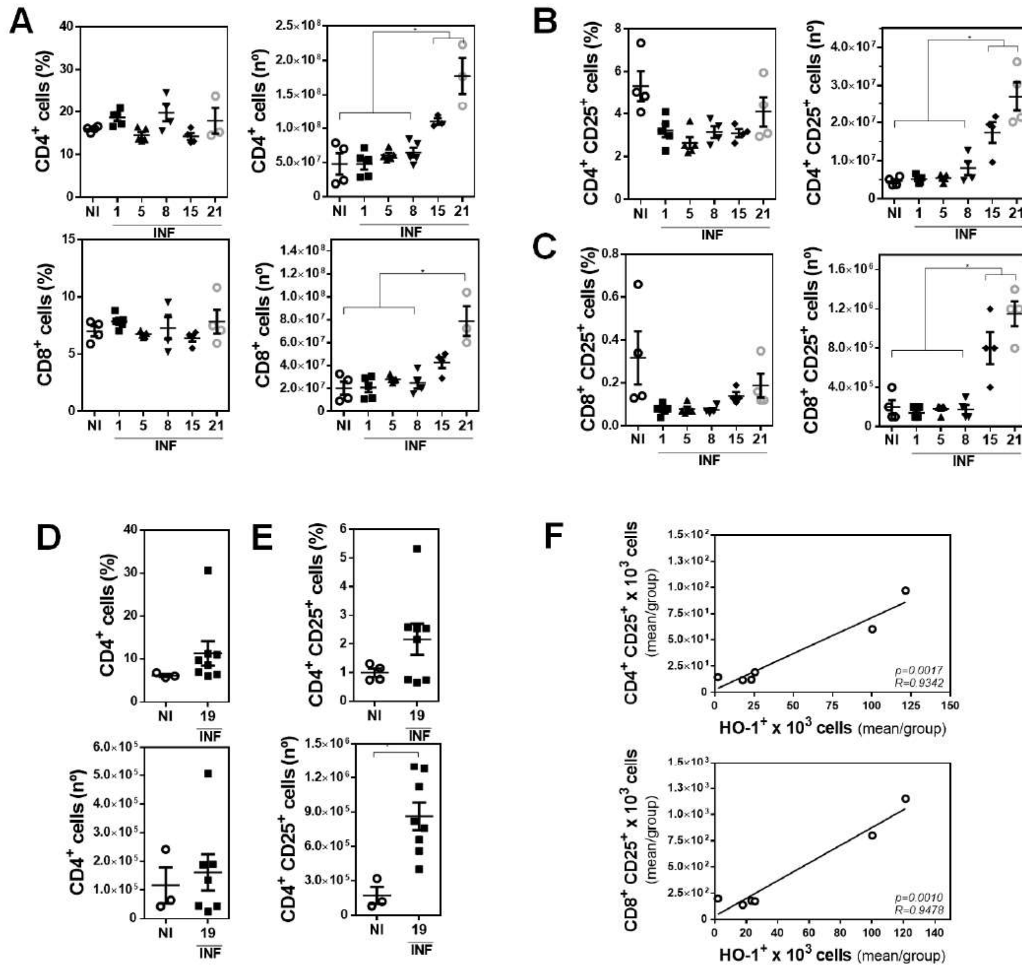

Figure 2. Peritoneal HO-1^{+} cells correlate with increased splenic CD4^{+} CD25^{+} and CD8^{+} CD25^{+} cells during *F. hepatica* infection. Mice (n = 4–8/group) were infected with 10 metacercariae and sacrificed at 1, 8, 15, and 21 dpi. Non-infected mice were used as control (NI). (**A**) Frequency and cell number of splenic CD4^{+} or CD8^{+} cells from infected and control (NI) mice. (**B**,**C**) Frequency and cell number of splenic CD4^{+} CD25^{+} (**B**) or CD8^{+} CD25^{+} (**C**) cells from infected and control (NI) mice. (**D**) Frequency and cell number of hepatic CD3^{+} CD4^{+} cells from infected and control (NI) mice. (**E**) Frequency and cell number of hepatic CD4^{+} CD25^{+} cells from infected and control (NI) mice (right). (**F**) Splenic CD4^{+} CD25^{+} or CD8^{+} CD25^{+} cell number in function of the number of peritoneal HO-1^{+} cells in NI and infected mice. The mean of CD4^{+} CD25^{+} (in Figure 2B), CD8 CD25^{+} (in Figure 2C), and HO-1^{+} cells (in Figure 1C) was plotted. Gate analyses by flow cytometry are shown in Supplementary Figures S2 and S3. The results shown represent one experiment. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

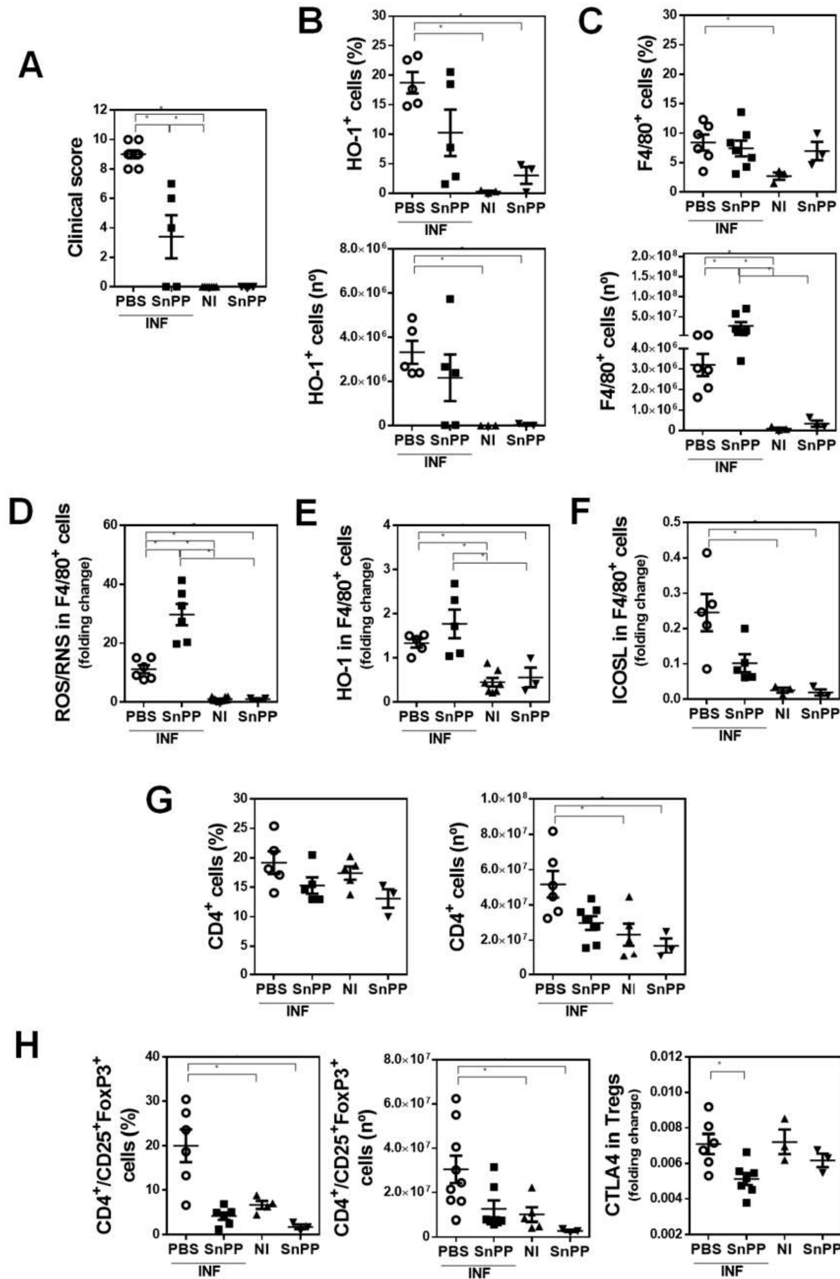

Figure 3. The HO-1 pharmacological inhibitor SnPP decreases the production of ROS/RNS by peritoneal F4/80⁺ cells and correlates with an increase of splenic FoxP3⁺CD25⁺/CD4⁺ T cells induced by *F. hepatica* infection. The HO-1 pharmacological

inhibitor SnPP decreases the production of ROS/RNS by peritoneal F4/80$^+$ cells and correlates with an increase of splenic FoxP3$^+$CD25$^+$/CD4$^+$ T cells induced by *F. hepatica* infection. Mice were injected with SnPP (40 mg/kg) or vehicle (PBS) one day before infection and every 4 days until the end of the experimental protocol. Mice (n = 5/group) were infected with 10 metacercariae (day 0) and sacrificed at 20 dpi. Non-infected (NI) mice (n = 3/group) both treated and untreated with SnPP were used as control. (**A**) Clinical signs of infected mice were analyzed to assess disease severity [17]. (**B**) Frequency and cell number of HO-1$^+$ cells in the peritoneal cavity of SnPP-treated or untreated infected and control (NI) animals by flow cytometry. (**C**) Frequency and cell number of F4/80$^+$ cells in the peritoneal cavity of SnPP-treated or untreated infected and control (NI) animals by flow cytometry. (**D**) ROS/RNS quantification in F4/80$^+$ cells of the peritoneal cavity of SnPP-treated or untreated infected and control (NI) mice using the DCFDA probe by flow cytometry. HO-1 (**E**) and ICOSL (**F**) expression in F4/80$^+$ cells in the peritoneal cavity of SnPP-treated or untreated infected and control (NI) mice. (**G**) Splenic CD4$^+$ T cell frequency and number from SnPP-treated or untreated infected and control (NI) mice. (**H**) Splenic CD4$^+$/CD25$^+$FoxpP3$^+$ cell frequency and number from SnPP-treated or untreated infected and control (NI) mice (left). CTLA4 expression in splenic CD4$^+$/CD25$^+$FoxpP3$^+$ cells from SnPP-treated or untreated infected and control (NI) mice. Gate analyses by flow cytometry are shown in Supplementary Figures S2 and S4. Results obtained for one representative experiment are shown. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

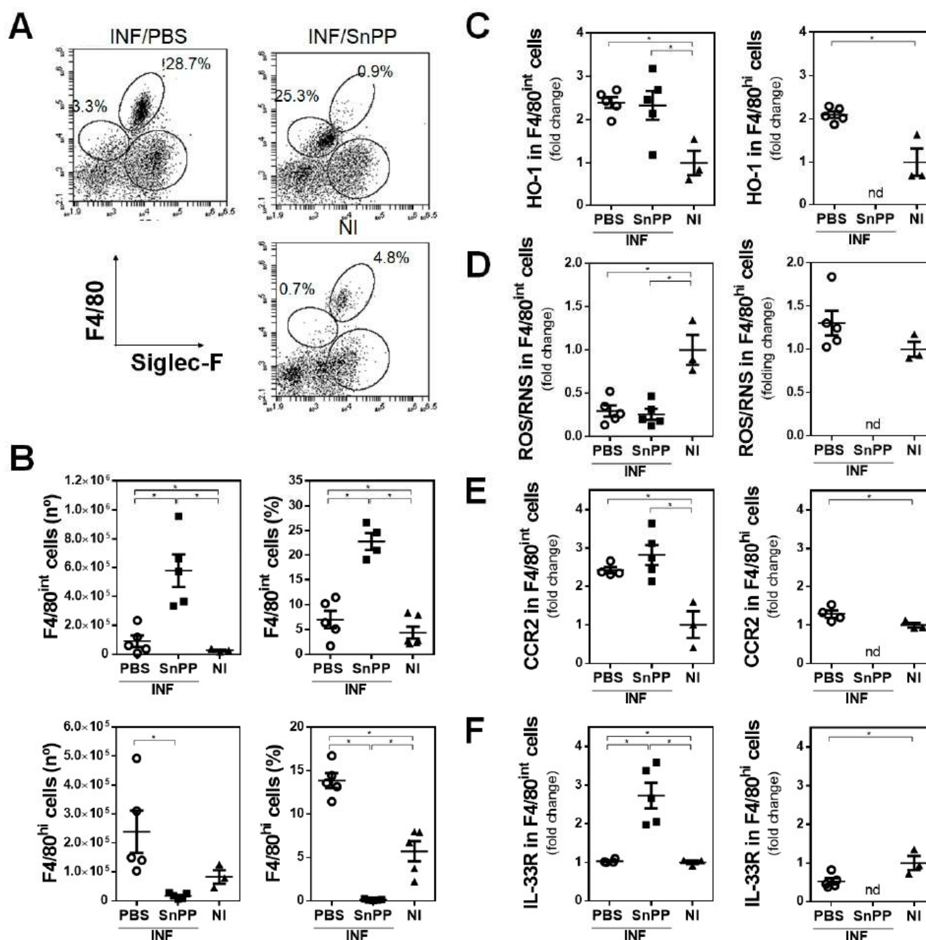

Figure 4. Analyses of peritoneal F4/80$^+$ cells at the early events of *F. hepatica* infection. Mice (n = 5/group) were injected with

SnPP (40 mg/kg) or vehicle (PBS) one day before infection, infected with 10 metacercariae (day 0), and sacrificed at 1 dpi. Non-infected (NI) mice (n = 3/group) both treated and untreated with SnPP were used as a control. (**A**) Analysis of F4/80^{int} and F4/80^{hi} cells in PEC from SnPP-treated or untreated infected and control (NI) mice by flow cytometry. (**B**) Frequency and cell number of F4/80^{int} (upper plots) or F4/80^{hi} (lower plots) cells in the peritoneal cavity of SnPP-treated or untreated infected and control (NI) animals. HO-1 (**C**), ROS/RNS (**D**), CCR2 (**E**), and IL-33R (**F**) expression in peritoneal F4/80^{int} and F4/80^{hi} cells by flow cytometry. "nd" means none detected, since barely any F4/80^{hi} cells in SnPP-treated infected mice were detected. Median fluorescence intensity is shown (MeFI) in the plot. Gate analyses by flow cytometry are shown in (**A**) and Supplementary Figure S1. Representative experiments are shown. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

3.4. The Inhibition of HO-1 Activity by SnPP Controls the Gene Expression of Antioxidant Molecules

To deeply analyze the relationship between HO-1 expression in peritoneal cells induced by *F. hepatica* infection with the production of ROS/RNS, the gene expression of different molecules involved in the oxidative and antioxidative responses was evaluated. PECs of SnPP-treated infected mice were characterized by a significant decrease in the mRNA levels of the transcription factor *nrf2* (Figure 5A). Moreover, the SnPP-induced decrease in *nrf2* gene expression levels was associated with decreased mRNA levels in the antioxidant enzymes *catalase,* glutathione peroxidase 2 (*gpx2*), and superoxide dismutase 2 (*sod2*) (Figure 5B). However, no differences were found in the gene expression of *gpx1*, while an increase in *sod1* expression was observed (Figure 5B). Finally, an unexpected decrease in the mRNA levels of the NADPH oxidase subunits *gp91^{phox}* and *p47^{phox}* was observed with SnPP treatment of infected mice (Figure 5C).

3.5. Deficiency of Functional NADPH Oxidase Partially Protects Mice from Liver Damage Induced by F. hepatica and Limits the Production of IL-10

Considering the fact that SnPP treatment protected mice from parasite infection and that reduced levels of *gp91^{phox}* mRNA were found in PECs from infected mice, we analyzed the infection in gp91^{phox} knockout mice. gp91^{phox} deficiency reduced the clinical signs and liver damage induced by *F. hepatica* infection (Figure 6A and Supplementary Figure S5). This partial protection was associated with a lower increase of HO-1$^+$ peritoneal cells, both in frequency and number (Figure 6B). Moreover, HO-1$^+$ cells from gp91^{phox} knockout infected mice expressed lower levels of MHCII (Figure 6C) and CD40 (Figure 6D), but not CD80 (Figure 6E), than those from wildtype mice, indicating that NADPH oxidase may play a role both in the immune response and the pathogenesis induced by *F. hepatica* infection. Further characterization of the peritoneal cells from these mice indicated that the increase of F4/80$^+$ cells was abrogated in the absence of gp91^{phox} (Figure 7A), and as expected, very low levels of ROS/RNS produced by these cells (Figure 7B). Additionally, these cells expressed lower levels of Sirpα (Figure 7C), ICOSL (Figure 7D), and IL-10 (Figure 7E). Finally, lower numbers of CD4$^+$ (Figure 7F and Supplementary Figure S6) and CD4$^+$/CD25$^+$FoxP3$^+$ Tregs (Figure 7G and Supplementary Figure S6) were found in gp91^{phox} knockout infected mice with respect to wildtype mice. However, no significant differences in the production of IFNγ by splenocytes stimulated with parasite components (FhTE) between gp91^{phox} knockout and wildtype infected mice were detected (Figure 7H).

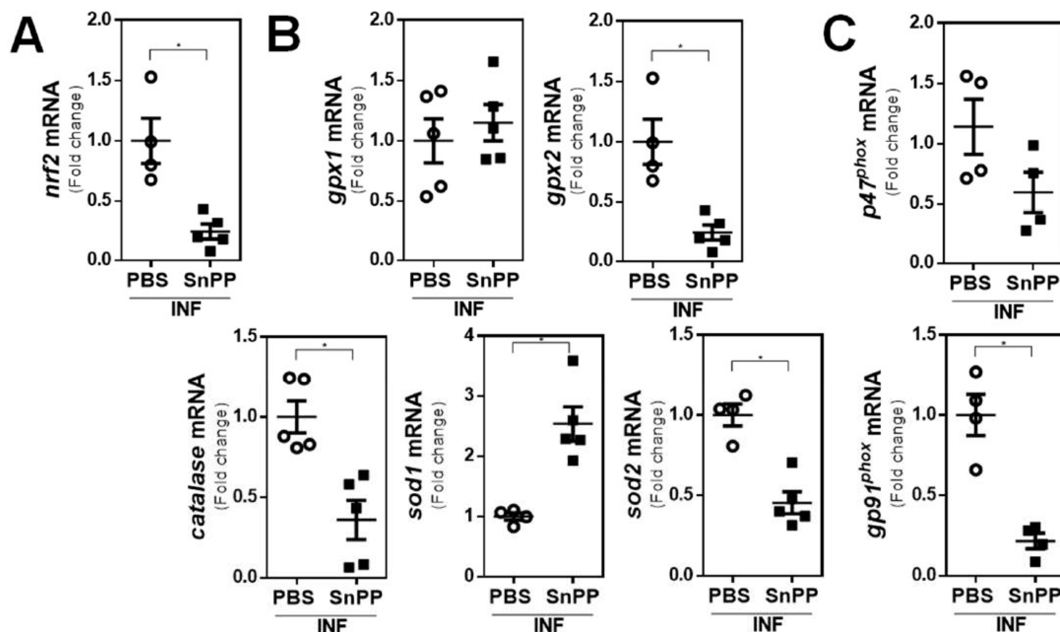

Figure 5. mRNA levels of antioxidative and oxidative genes in the infection by *F. hepatica*. Mice (n = 5/group) were injected with SnPP (40 mg/kg) or PBS 1 day before infection, infected with 10 metacercariae (day 0), and sacrificed at 20 dpi. *nrf2* (**A**), *catalase, gpx1, gpx2, sod1, sod2* (**B**), and *p47phox* and *gp91phox* (**C**) gene expression in PECs from SnPP-treated and control infected mice evaluated by qRT-PCR. mRNA levels were analyzed by qRT-PCR with respect to *gapdh* expression in PECs from SnPP-treated and control infected mice (PBS). Results were compared to the infected (control) group of mice and represented as the ratio between gene expression in SnPP-treated and control mice. Asterisks indicate significant differences with $p < 0.05$, performed by the Student's *t*-test.

3.6. IL-10 Signaling Is Crucial for HO-1 Expression in F. hepatica-Infected Mice

Considering the fact that IL-10 induces HO-1 expression that can favor the production of IL-10, and that IL-10 is crucial for Treg differentiation [36], we analyzed whether there was a relationship between IL-10 signaling and HO-1 expression during *F. hepatica* infection. To this end, we treated infected mice with a neutralizing antibody of IL-10 receptor (IL-10R). The results demonstrate that IL-10R blocking reduced the clinical signs associated with parasite infection (Figure 8A). Although the recruitment of F4/80$^+$ cells in the peritoneum of infected mice was not affected by IL-10R neutralization (Figure 8B), it abrogated the elevated expression of HO-1 induced by *F. hepatica* infection (Figure 8C). Interestingly, IL-10R neutralization reduced the frequency, but not the number, of CD4$^+$ (Figure 8D and Supplementary Figure S7) and CD4$^+$CD25$^+$ (Figure 8E and Supplementary Figure S7) T cells in the spleens of infected animals. Altogether, these results indicate that IL-10 signaling is essential for HO-1 expression of F4/80$^+$ cells during *F. hepatica* infection, likely affecting the differentiation of regulatory T cells.

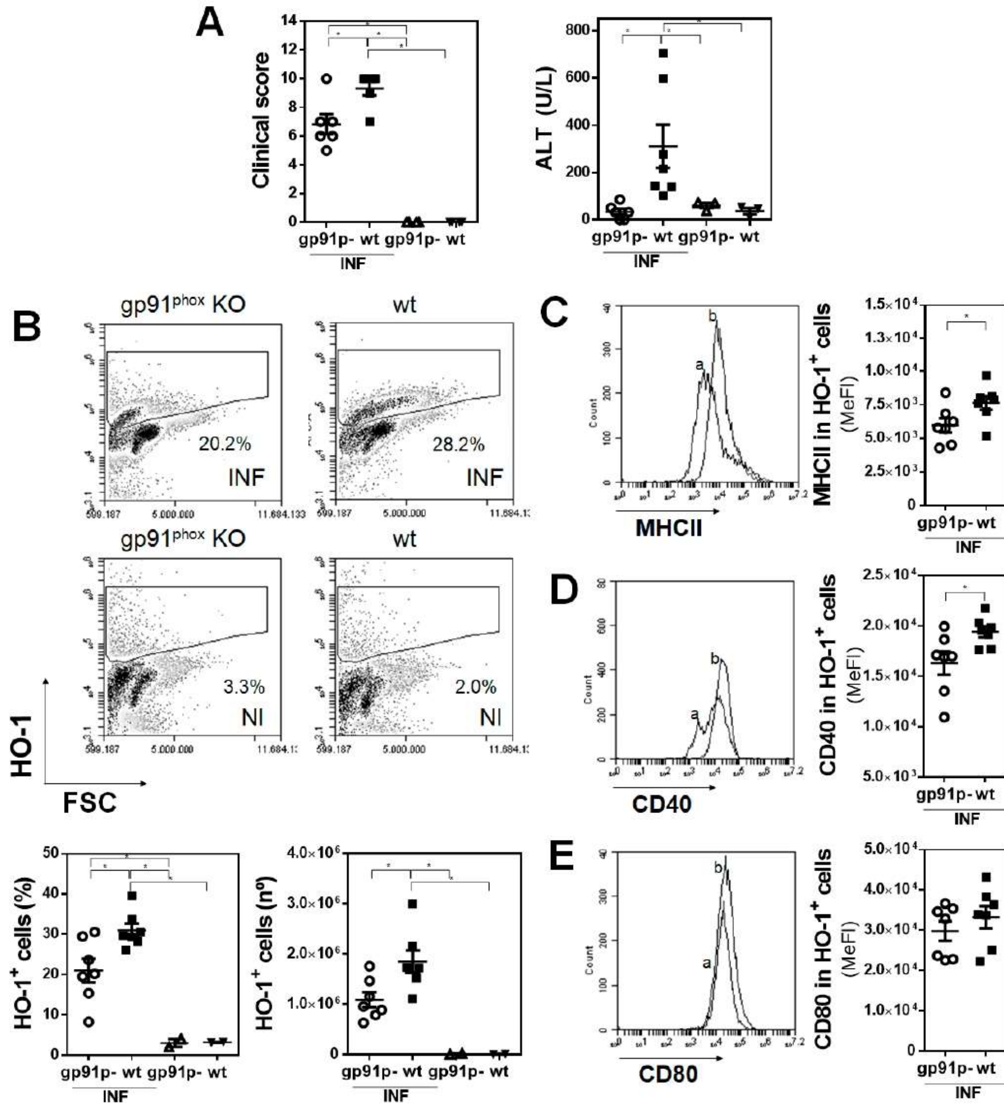

Figure 6. Gp91^{phox} (gp91p-) knockout (n = 6–8/group) and littermate control (n = 2/group) mice were infected with 10 metacercariae and sacrificed at 20 dpi. Non-infected mice were used as a control. (**A**) Clinical signs analyzed to assess disease severity [17]. Quantification of liver damage by ALT activity in sera. (**B**) Flow cytometry analysis of HO-1$^+$ in PECs from gp91^{phox} knockout mice and littermate controls (upper panel). (**C**) Frequency and cell number of HO-1$^+$ cells in the peritoneal cavity of infected and non-infected animals by flow cytometry (lower panel). MHCII (**C**), CD40 (**D**), and CD80 (**E**) expression in HO-1$^+$ cells in the peritoneal cavity of infected and control mice. Letters on histograms correspond as follows: a: infected gp91^{phox}, b: infected wildtype littermates. Median fluorescence intensity is shown (MeFI) in the plot. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

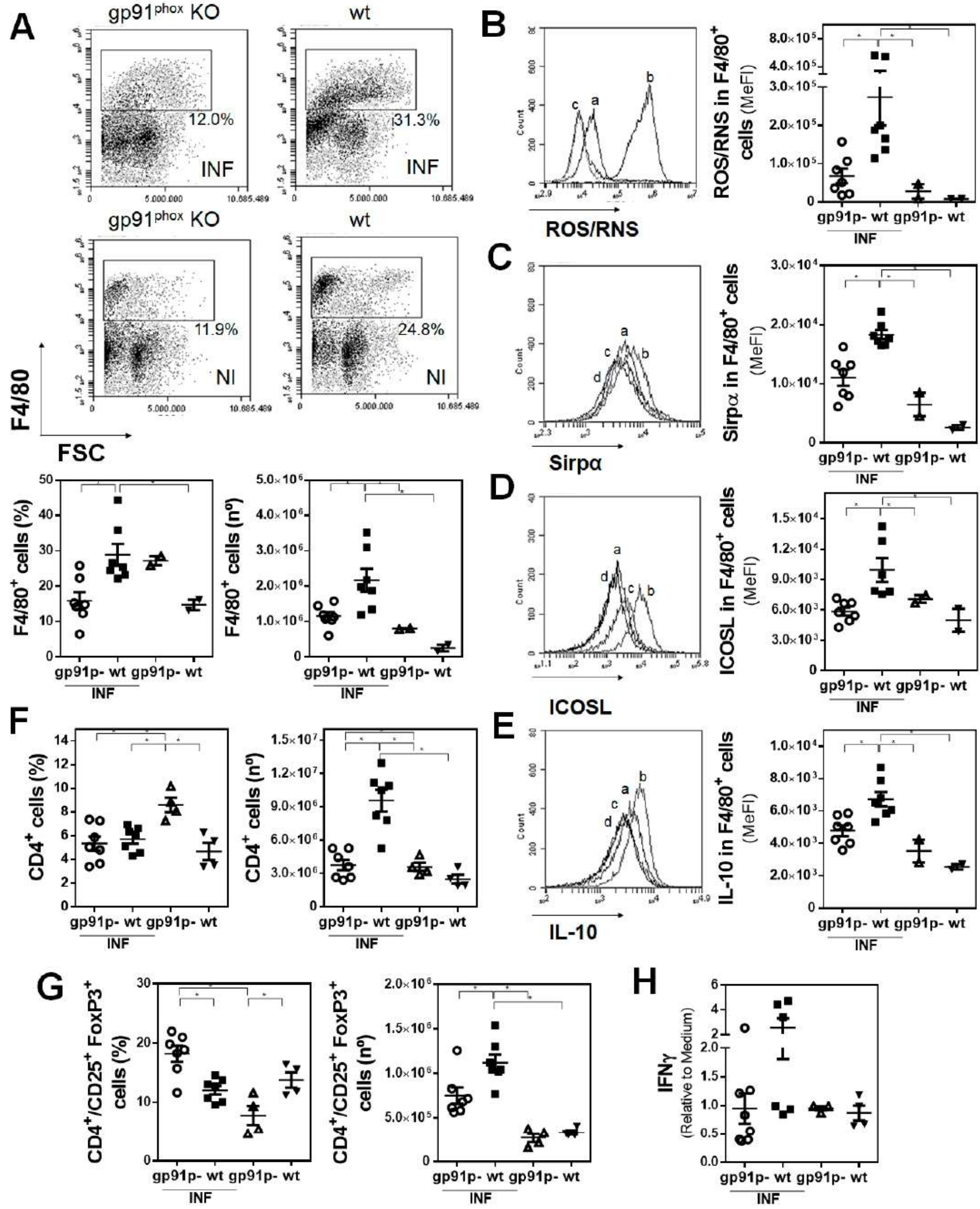

Figure 7. Peritoneal F4/80⁺ cells from wildtype infected mice present a regulatory-like phenotype. Gp91^{phox} (gp91p-) knockout

(n = 7–8/group) and littermate control (n = 2–4/group) mice were infected with 10 metacercariae and sacrificed at 20 dpi. Non-infected mice were used as a control. (**A**) Flow cytometry analysis of F4/80+ cells in PEC from gp91phox knockout mice and littermate controls (upper panel). Frequency and cell number of F4/80+ cells in the peritoneal cavity of infected and non-infected animals by flow cytometry (lower plots). ROS/RNS (**B**), Sirpα (**C**), ICOSL (**D**), and IL-10 (**E**) expression in F4/80+ cells in the peritoneal cavity of infected and uninfected mice. Letters on histograms correspond as follows: a: infected gp91phox, b: infected littermates, c: uninfected gp91phox, and d: uninfected littermates. Median fluorescence intensity is shown (MeFI) in the plot. (**F**) Frequency and cell number of splenic CD4+ cells from infected and non-infected mice. (**G**) Frequency and cell number of splenic CD4+/CD25+ Foxp3+ cells from infected and non-infected mice. Gate analyses by flow cytometry are shown in (**A**) and Supplementary Figure S1. (**H**) IFNγ levels in culture supernatants of splenocyte proliferation assay cultured with FhTE for 5 days at 37 °C. The results shown represent one experiment. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

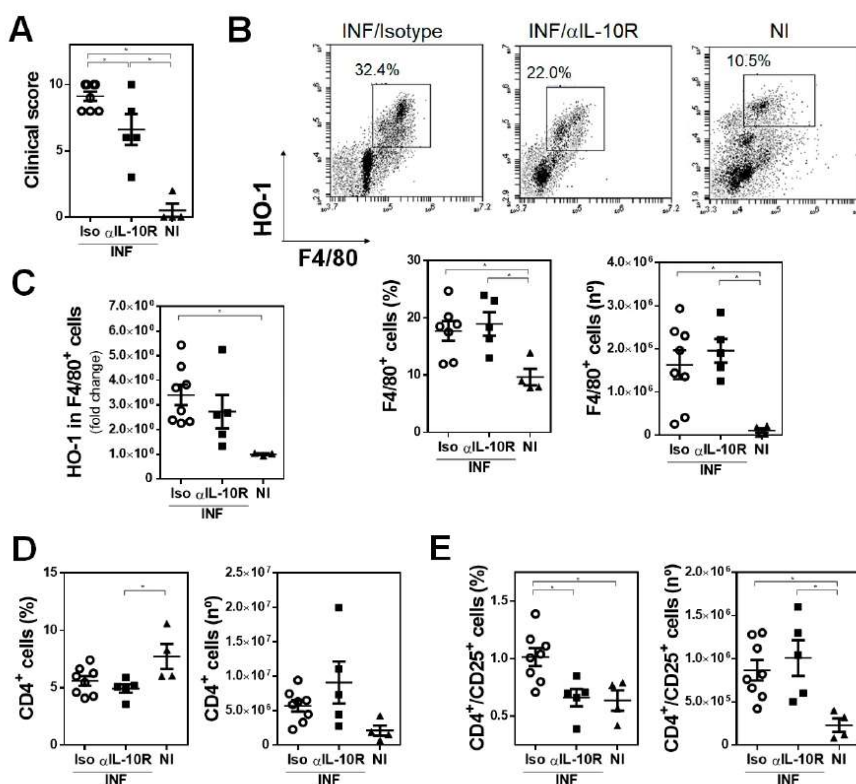

Figure 8. IL-10 signaling is essential for HO-1 expression in *F. hepatica*-infected mice. Fifteen μg of monoclonal rat IgG_{2a} anti-IL-10R antibody was administrated by intraperitoneal injection the day before and after infection with *F. hepatica* and every 3 days until sacrifice (n = 5–8/group). The control group (n = 4/group) received an isotype control antibody. At day 20 post-infection, animals were sacrificed and splenocytes were analyzed by flow cytometry. (**A**) Clinical signs were analyzed to assess disease severity [17]. (**B**) Analysis by flow cytometry of F4/80+ cells in PEC from infected and non-infected (NI) mice showing frequency and number of F4/80+ cells in PECs. (**C**) HO-1 expression in F4/80+ cells in the peritoneal cavity of infected and uninfected mice. (**D**) Frequency and cell number of CD4+ T cells in spleens from infected and non-infected (NI) mice. (**E**) Frequency and cell number of CD4+ CD25+ T cells in spleens from infected and non-infected (NI) mice. Gate analyses by flow cytometry are shown in (**B**) and Supplementary Figure S7. Representative results of one representative are shown. Asterisks indicate significant differences with $p < 0.05$, performed by one-way ANOVA followed by Tukey's test with multiple comparisons.

4. Discussion

In this work, we have examined the cellular and molecular mechanisms that govern the expansion or differentiation of Tregs induced by HO-1$^+$ cells in *F. hepatica* infection. We presented evidence showing that HO-1 activity results in decreased ROS/RNS production by F4/80$^+$ antigen-presenting cells, thereby enhancing the pathological effects caused by *F. hepatica* and promoting parasite infection. Furthermore, apart from its antioxidant capacity, HO-1 has other functions, such as its immunoregulatory properties and controlling gene expression as a transcription factor [14,21,26,32,38]. Indeed, HO-1 inhibition promotes IFNγ- and NOS2-mediated control of *M. tuberculosis* infection in mice [39]. Furthermore, it has been previously reported that HO-1 has a role in suppressing pro-inflammatory Th1 immune responses in experimental colitis, and sickle cell alloimmunization has been reported, and it protects from atherosclerosis [40,41]. Finally, HO-1 can impair the immunity against other pathogens, such as *Plasmodium yoelii* [42].

Indeed, we demonstrated that during *F. hepatica* experimental infection in mice, there is an increase in the expression of HO-1 in F4/80$^+$ cells in the peritoneal cavity and it inversely correlates with ROS/RNS production. Furthermore, we demonstrated an association between the expression of HO-1 and the presence of putative Tregs in the spleens of infected animals (Figure 9A). These results were also confirmed when using the HO-1 inhibitor SnPP, which inhibits its enzymatic activity. At first sight, the inhibition of HO-1 activity by SnPP would suggest that its effects are caused by the heme-catabolizing activity rather than by its expression and function as a transcription factor. Indeed, F4/80$^+$ peritoneal cells from SnPP-treated mice did not show a decrease in HO-1 expression, although a significant increase in ROS/RNS production was detected. SnPP is a metalloporphyrin that acts as a competitive inhibitor of HO-1 both in vitro and in vivo. Its efficiency can be explained by its higher binding affinity to HO-1/2 than to heme [43,44]. However, enzymatically inactive HO-1 can still mediate protection against hydrogen peroxide-induced toxicity, probably by promoting the gene expression of antioxidant proteins [14,45], although the mechanisms underlying these effects are still unclear. Thus, the possibility that HO-1 would act as transcription factor cannot be discarded, since the nuclear localization of HO-1 in F4/80$^+$ cells derived from *F. hepatica*-infected mice with or without SnPP treatment was not investigated. Furthermore, it is unlikely that the protective outcome of SnPP treatment represents a direct effect on *F. hepatica*, since the degree of infection and pathological effects induced by the parasite were also related to an increase in Tregs, evidencing that HO-1 activity influences the host adaptive immunity in vivo. Indeed, our results indicate that the increase of the mRNA levels of *nrf2*, a transcription factor responsible for the regulation of cellular redox balance and protecting antioxidant responses [46,47], is accompanied by an increase in some antioxidant enzyme genes, demonstrating that the infection, HO-1, Tregs, and the Nrf2 master regulator comprise a complex axis of antioxidant and immunoregulatory properties in *F. hepatica* infection. However, the function of these enzymes should be determined in order to confirm their antioxidant role during *F. hepatica* infection. On the other hand, heme-activated murine macrophages have functional anti-inflammatory features that are dependent on the enzymatic activity of HO-1 [38]. Thus, the immunoregulatory and immunosuppressive properties of HO-1 together with its antioxidant properties demonstrate that its function during *F. hepatica* infection goes far beyond heme degradation itself.

The role of ROS/RNS in helminth parasite killing is still controversial. Some reports showed that the infection by *Strongyloides papillosus* induced an oxidative/nitrosative stress in sheep [48], although its effect on the parasite itself has not been demonstrated. On the other hand, *Schistosome* infection relates to an immense oxidative stress by the host that is not sufficient to control infection [49]. Further data demonstrated that excretory/secretory factors from *Mesocestoides corti* inhibit ROS-induced neutrophil extracellular traps, showing that the parasite could use this mechanism to attenuate the effects induced by ROS [50]. It should be highlighted, however, that although oxidative mechanisms are induced by helminth parasite infections, their detrimental role in the parasite itself as well as in the host surroundings is not well-established yet [51–53]. A recent report has demonstrated

a high oxidative status in serum and liver in rabbits infected with *F. gigantica*, together with a decline in the SOD and catalase gene expression and enzyme activity in sera from infected animals [54], which is not in agreement with data from our work in *F. hepatica* experimentally infected mice. However, the authors came to the conclusion that the disruption of antioxidant and detoxification cascades by *F. gigantica* likely leads to the pathogenic response from the host [54].

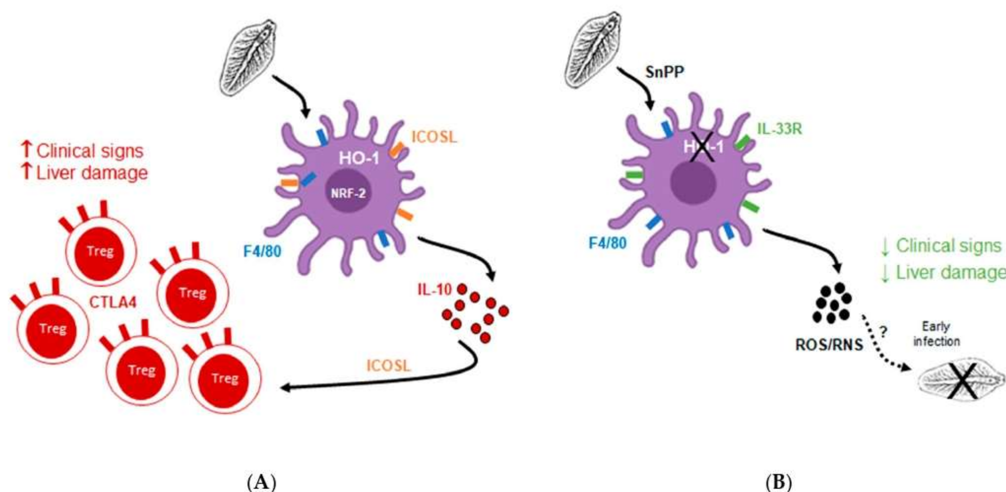

Figure 9. General hypothesis. (**A**) ICOSL$^+$ F4/80$^+$ APC express HO-1 induced by parasite infection that promotes IL-10 production and differentiation or expansion of CTLA4$^+$ Tregs. (**B**) SnPP treatment inhibiting HO-1 activity in IL-33R$^+$ F4/80$^+$ APC allows ROS/RNS production, that induce parasite damage in early stages of the infection.

It is worth noting that in our work, we used a DCFDA fluorescent probe that does not distinguish between ROS and RNS. Therefore, these studies should be complemented with others using ROS-specific probes such as DHE or specific inhibitors of nitric oxide production (such as L-Name). In order to analyze the ROS produced by NADPH-oxidase, we used, instead, gp91phox knockout mice. Interestingly, the fact that mice that are deficient in NADPH oxidase function, with a considerable decrease in ROS production, were partially protected against *F. hepatica* infection, suggests that the moment when ROS is produced by NADPH oxidase might be crucial to limit *F. hepatica*-induced damage (Figure 9B). Indeed, an exacerbated ROS production induced by a pro-inflammatory immune response can be detrimental to leukocyte cell function or viability and induced damage to the immune system [54]. Thus, a prolonged and not regulated production of ROS by F4/80$^+$ cells could benefit the parasite, and not the host. Of note, these cells expressed higher levels of ICOSL and IL-10 than those from gp91^{phox} knockout mice, which could be associated with the differentiation or expansion of a higher number of splenic Tregs, which in turn express higher levels of CTLA4. Indeed, both ICOSL [55,56] and CTLA4 [57] are key mediators of Treg differentiation. In the same line, macrophages can suppress T cell responses and favor the expansion of Tregs [58]. Furthermore, ROS levels on T cell activation seem to be important, since small quantities of ROS result in antigen hypo-responsiveness, while high doses lead to oxidative stress-induced apoptosis [59]. Further analysis of the role of IL-10 produced by antigen-presenting cells in the differentiation or expansion of Tregs showed that IL-10 signaling is essential to increase HO-1 expression in peritoneal F4/80$^+$ cells and likely the production of Tregs. Interestingly, it would seem that the parasite exploits the host defense mechanisms, on the one hand by recruiting HO-1$^+$ cells with less antioxidative functions that produce IL-10, and on the other hand by in

turn inducing the differentiation to Tregs. Nevertheless, the production of IL-10 by the host would also protect host cells in the acute pro-inflammatory immune response, caused either by damage induced by the parasite in the early state of the infection or by liver damage, at least in this experimental model. However, more experiments are needed in order to confirm these results, and to determine the role of ROS in the induction of Tregs and its relationship with IL-10.

One hypothesis that can explain these results might be the fact that ROS/RNS production is (partially) effective only during early stages of *F. hepatica* infection (Figure 9B). After ingestion of metacercariae by the mammalian host, juvenile flukes penetrate the host intestine wall and reach the liver through the peritoneal cavity between 4 and 6 days in livestock, although it is thought that it takes around 24 h in mice [3]. To further understand the early events that take place during *F. hepatica* infection in mice, we analyzed HO-1 expression and F4/80$^+$ cell recruitment at 1 dpi, finding that two different populations expressing different levels of F4/80 are present in the peritoneum, and those elicited in SnPP-treated mice expressed higher levels of IL33R (Figure 9B). IL33 is an alarmin that participates in the type 2 innate immune response, promoting innate lymphoid cells type 2. However, during *Schisotosoma* infection, IL-33 seems to contribute to the development of pathology via the induction of type 2 innate lymphoid cells and alternative activation of macrophages, thus favoring the infection [60–62]. Therefore, the functions of IL-33 during *F. hepatica* infection in mice, and in particular the overexpression of its receptor in antigen-presenting cells at the early events of the infection, remain to be elucidated.

In conclusion, our work showed that HO-1 is a key molecule that favors *F. hepatica* infection, by which HO-1 could control ROS/RNS production and Treg differentiation and how the parasite elicits/triggers these mechanisms. Altogether, these results contribute to the elucidation of the immunoregulatory and antioxidant roles of HO-1 induced by *F. hepatica* in the host, providing interesting checkpoints that might control fasciolosis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10121938/s1, Figure S1: Gates used for flow cytometry analyses of PECs from infected and control mice corresponding to Figures 1B,D and 4A; Figure S2: Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figures 2A–C and 3G,H; Figure S3: Gates used for flow cytometry analyses of hepatic leukocytes from infected and control mice corresponding to Figure 2D,E; Figure S4: Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figure 3B,C; Figure S5: Representative images of livers from gp91^{phox} KO and C57BL/6 liitermates infected and control mice; Figure S6: Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figure 7F,G; Figure S7: Gates used for flow cytometry analyses of splenocytes from IL 10R or isotype treated infected and control mice corresponding to Figure 8D,E.

Author Contributions: M.C. performed all the experiments, with the exception of the anti-IL-10R experiment, and wrote the original draft of the manuscript; V.d.C. and S.F. performed biological sample collection and flow cytometry analyses, and reviewing the manuscript; M.F.F., M.L., S.A.R.-Z. and P.L. helped with cell cultures, and performed flow cytometry analyses and interpretation; M.F.F. critically reviewed the manuscript; P.C. performed the IL-10R experiment, conceptualization, and reviewed the manuscript; T.F. performed conceptualization, experiment design and supervision, analyses of data, and writing, review, and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Programa de Desarrollo de Ciencias Básicas (PEDECIBA), Agencia Nacional de Investigación e Innovación (SNI-ANII, FCE_1_2017_1_136094 and FCE_1_2019_1_156295) to Teresa Freire.

Institutional Review Board Statement: Animal experimentation was carried out according to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences. Procedures involving animals were approved by the Universidad de la República's Committee on Animal Research (Comisión Honoraria de Experimentación Animal, CHEA Protocol Number 07153-000817-18).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article and supplementary materials.

Acknowledgments: We are grateful to Unidad de Reactivos Biológicos de experimentación at Facultad de Medicina, UdelaR, for animal housing and care and to Ignacio Anegón for his help.

Conflicts of Interest: The authors declare no conflict of interest.

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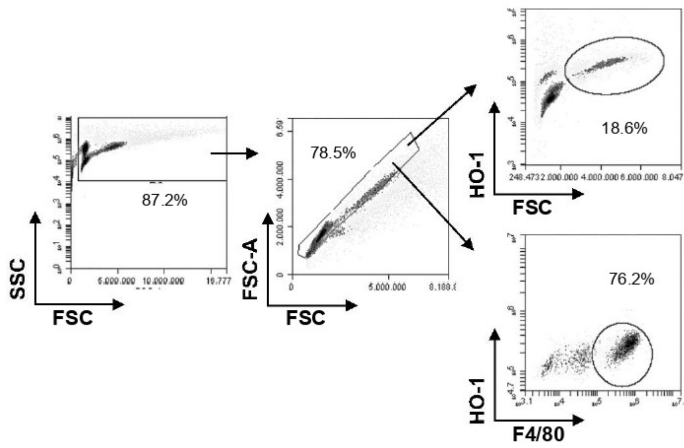
Supplementary Figure S1

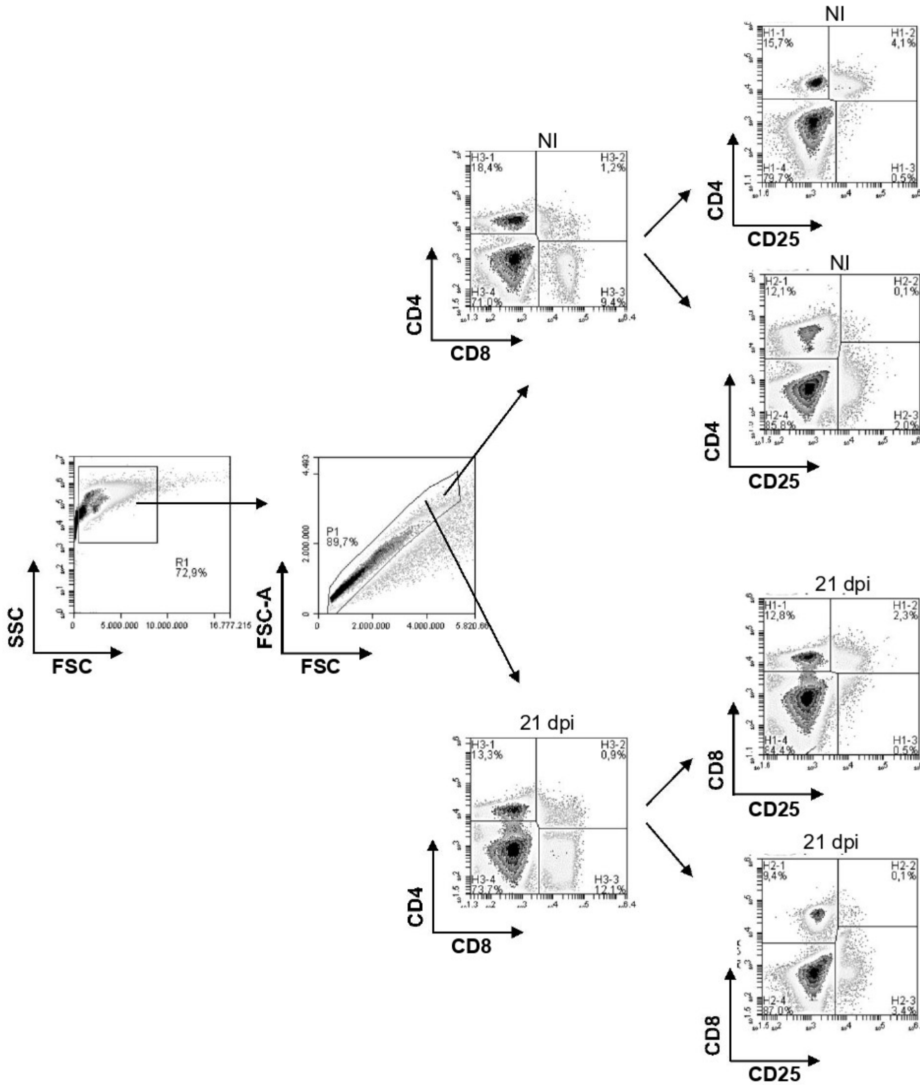
Costa *et al.*



Supplementary Figure S1.
Gates used for flow cytometry analyses of PECs from infected and control mice corresponding to Figure 1B, 1D and 4A.

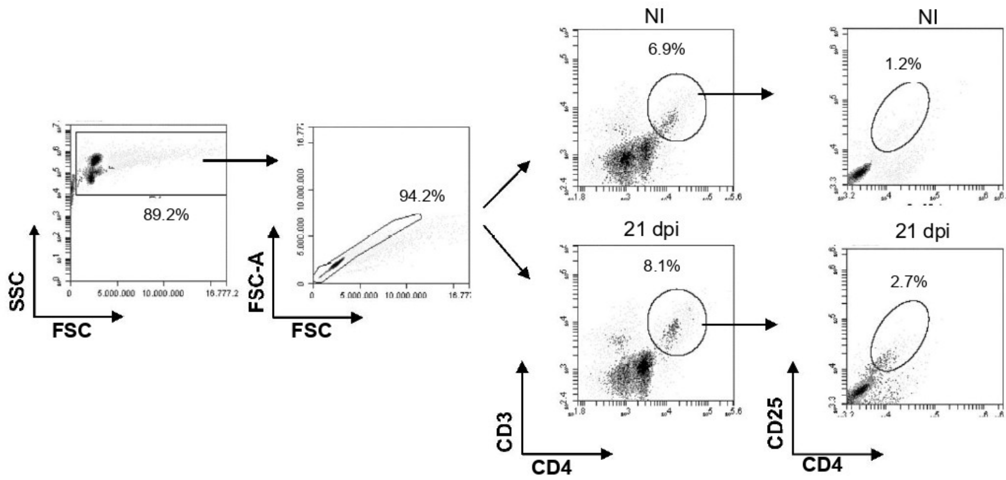
Supplementary Figure S2

Costa *et al.*



Supplementary Figure S2.
Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figure 2A, 2B, 2C, 3G and 3H.

Supplementary Figure S3 Costa *et al.*

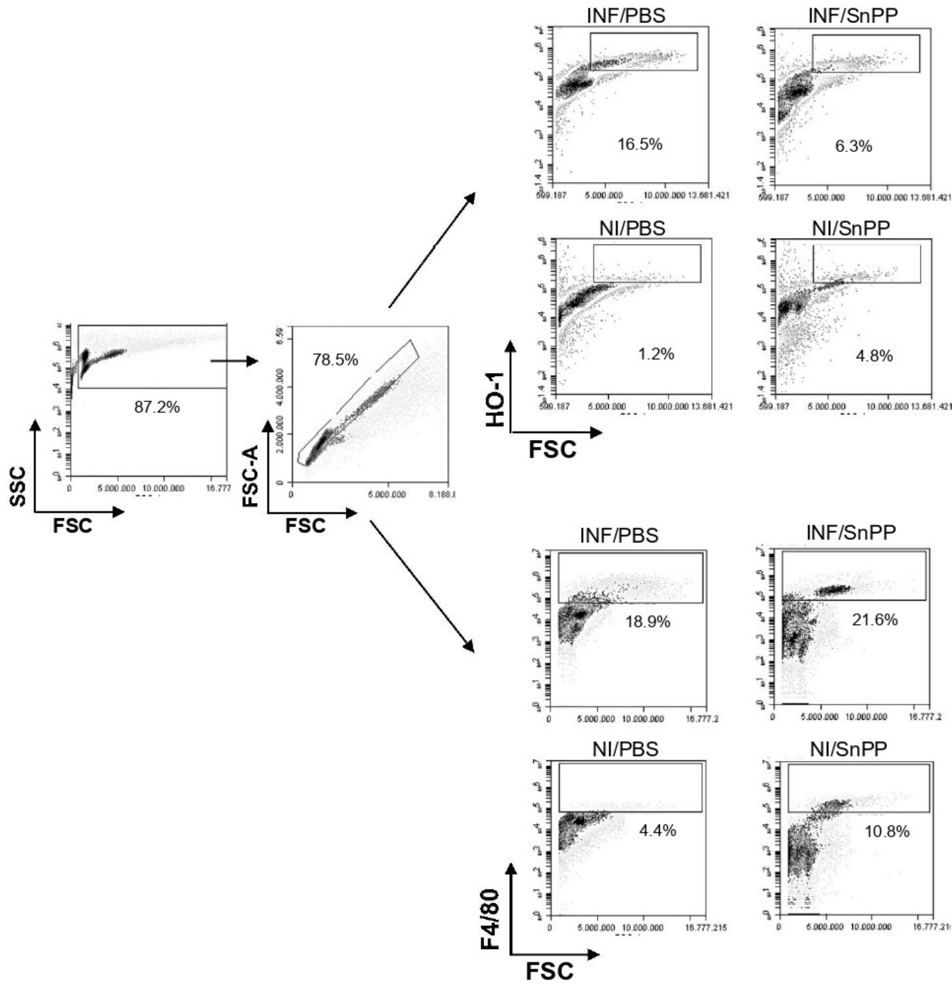


Supplementary Figure S3.
Gates used for flow cytometry analyses of hepatic leukocytes from infected and control mice corresponding to Figure 2D and 2E.

3

Supplementary Figure S4

Costa *et al.*

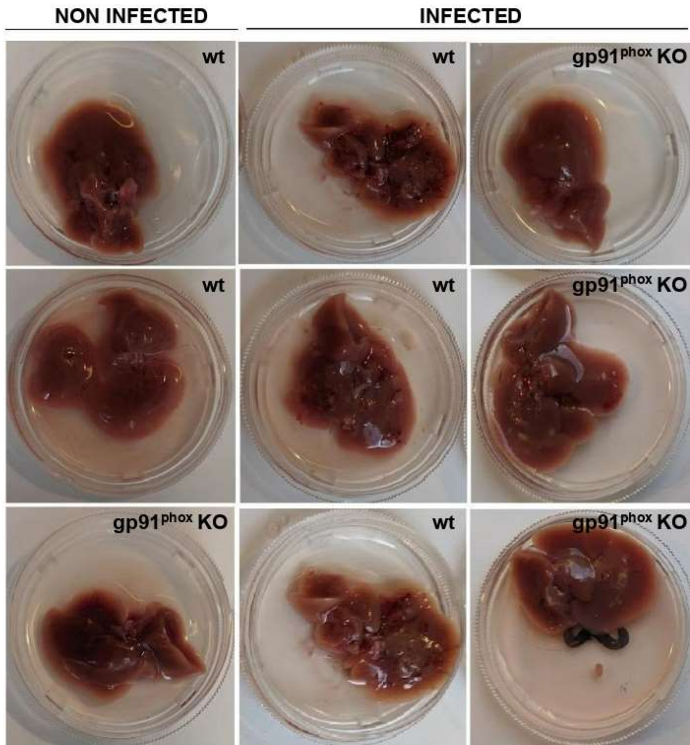


Supplementary Figure S4.
Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figure 3B and 3C.

4

Supplementary Figure S5

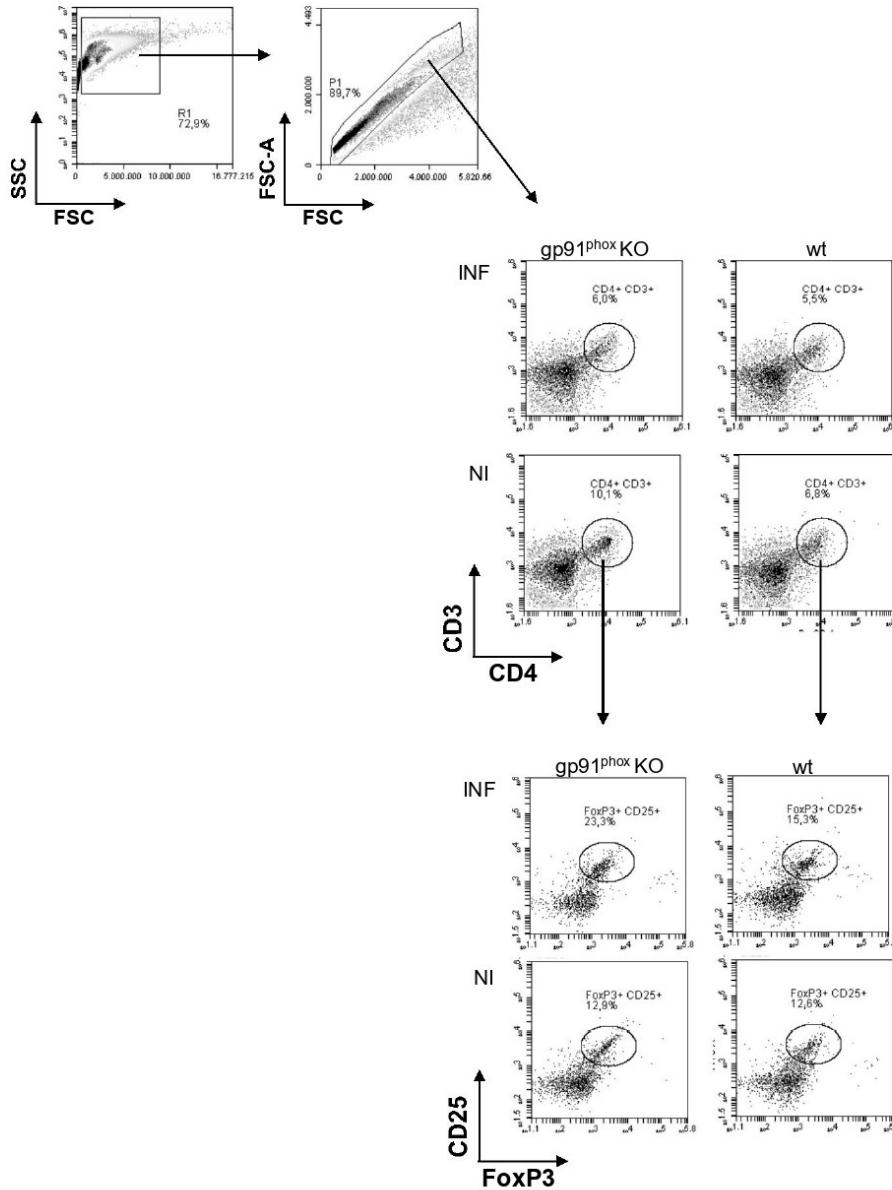
Costa *et al.*



Supplementary Figure S5.
Representative images of livers from gp91^{phox} KO and C57BL/6 liitermates infected and control mice.

Supplementary Figure S6

Costa *et al.*

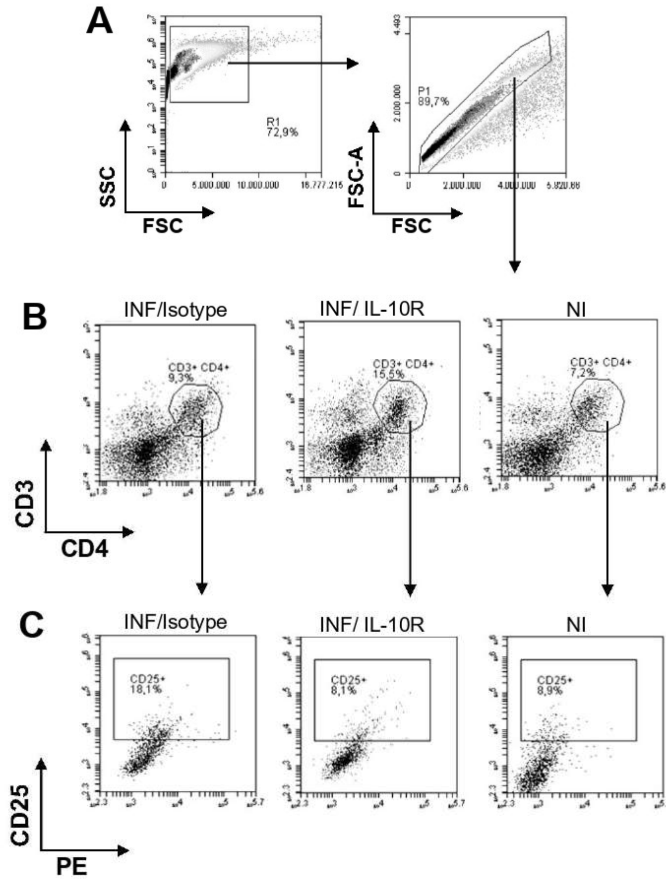


Supplementary Figure S6.
Gates used for flow cytometry analyses of splenocytes from infected and control mice corresponding to Figure 6F and 6G.

6

Supplementary Figure S7

Costa *et al.*



Supplementary Figure S7.
Gates used for flow cytometry analyses of splenocytes from IL-10R- or isotype-treated infected and control mice corresponding to Figure 8D and 8E.

5.2 Role of MGL2^{+} cells in the induction of Treg *F. hepatica*-infected mice

This section details the findings related to objective 2.

The main results of this part 2 of the chapter are contained in the attached publication (Art 2)

Costa, M., da Costa, V., Lores, P., Landeira, M., Rodríguez-Zraquia, S. A., Festari, M. F., & Freire, T. (2022). Macrophage Gal/GalNAc lectin 2 (MGL2^{+}) peritoneal antigen presenting cells during *Fasciola hepatica* infection are essential for regulatory T cell induction. *Scientific Reports*, *12*(1), 17661.

5.2.1 The importance of Macrophage Gal/GalNAc lectin 2 (MGL2 $^{+}$) in peritoneal APC in the induction of Treg cells during *F. hepatica* infection

In this second study, we focused on investigating the role of MGL2^{+} APCs in the induction of Tregs during *F. hepatica* infection. This paper was published in Scientific Reports in October of 2022.

As previously stated, *F. hepatica* can modulate the host immune system. Glycoconjugates can help the parasite escape from the host immune system (Van Die & Cummings, 2010). For instance, *F. hepatica* produces glycoconjugates that regulate the function and maturation of DCs (Rodríguez et al., 2015, 2017) and macrophages (Guasconi et al., 2011, 2018) in a process that can be mediated by C-type lectin receptors (CLRs). CLRs on APCs can decode the information carried by *F. hepatica* glycans and have a role in their immunomodulatory function (Aldridge & O'Neill, 2016b; Guasconi et al., 2011, 2015; D. Li & Wu, 2021; McGreal et al., 2005). MGL2 (CD301) is a surface receptor mainly expressed on immature, tolerogenic, or type-2 DCs (Higashi et al., 2002; Rodríguez, Kalay, et al., 2017; van Vliet et al., 2006b) and alternatively-activated macrophages (Ilarregui et al., 2019). It interacts with Gal/GalNAc carbohydrates, including the O-glycosylated Tn antigen (GalNAc-αThr/Ser) that can be expressed in parasite glycoconjugates and tumor cells (V. da Costa et al., 2021). The activation of MGL decreases immune responses by inducing IL-10 synthesis by DCs (Rodríguez, Kalay, et al., 2017; van Vliet et al., 2013), promoting Treg differentiation (V. da Costa et al., 2021), inducing T cell apoptosis, and suppressing T cell activation (van Vliet et al., 2006b).

Previous studies from our group demonstrated that human MGL can interact with *F. hepatica* components through the Tn antigen. This interaction modulates the TLR2-induced maturation of human monocyte-derived DCs by up-regulating the production of IL-10 and TNFα (Rodríguez, Kalay, et al., 2017). In an experimental mouse model of *F. hepatica* infection, we have found that MGL2$^+$ cells express various regulatory markers, such as IL-10, TNFα, and TGFβ. These cells expand modified Th2 immune responses while suppressing Th1 polarization (Rodríguez, Kalay, et al., 2017).

CCR2 mediates the cell migration from blood and the recruitment of monocytes, myeloid suppressor cells (Kurihara et al., 1997; Kuziel et al., 1997) or monocyte-derived DC-like cells (Heyde et al., 2018) that express MHC class II together with CD11c and F4/80 into damaged tissue or inflamed tissues. To confirm whether MGL2$^+$ cells expressing both CD11c and F4/80 are a population of DCs or SPM, the expression of MerTK, a macrophage-specific molecule expressing mostly by residents' macrophages (LPM) (Gautier et al., 2012), was evaluated.

Considering that MGL2 recognizes *F. hepatica* glycoconjugates and that MGL2$^+$ cells could have immunoregulatory properties, we aimed at studying their role in Treg induction *in vivo* in *F. hepatica* infected mice. Our findings demonstrate that MGL2$^+$ peritoneal APCs are essential for the induction of splenic Tregs during *F. hepatica* infection.

5.2.2 Results

5.2.2.1 F. hepatica infection in MGL2-DTR transgenic mice increases immunoregulatory F4/80$^+$ cells in the peritoneal cavity and splenic Treg

To investigate the role of MGL2$^+$ F4/80$^+$ cells during parasite infection, we employed a transgenic animal model that enables the depletion of MGL2$^+$ cells by administering diphtheria toxin (DT), as these cells express the receptor for DTR (V. da Costa et al., 2021). We injected DT (0.5 µg/mouse) intraperitoneally into the MGL2-DTR and PBS into the control group on day -1 and subsequently every 2-3 days until the end of the experiment.

As expected, after 3 wpi, infected animals showed high clinical scores (Figure 1A) based on their overall health condition (Carasi et al., 2017) and the levels of Alanine transaminase (ALT) activity

in serum (Figure 1A), a standard marker to detect hepatic dysfunction, liver damage, and fibrosis. The infection was associated with an increase of F4/80$^+$ cells in the peritoneal cavity of infected mice (Figure 1B). Peritoneal F4/80$^+$ cells from infected mice showed higher levels of CCR2 and CD11c (Figures 2A, and 2B respectively). Still, it did not exhibit an increase in the expression of the macrophage-associated marker MerTK (Figure 2C).

As mentioned in the previous chapter, CCR2 is responsible for recruiting monocytes to sites of inflammation (Kurihara et al., 1997). CD11c is a complement receptor frequently used as a marker of murine DC, but it can be expressed by many macrophages, including some of those present in the serous cavities (Bain & Jenkins, 2018; Bou Ghosn et al., 2010; D. L. Costa et al., 2016), and the MerTK is an efferocytosis receptor on macrophages. It is an important phagocytic receptor in the immune system (Gautier et al., 2012).

Flow cytometry analysis revealed significantly increased expression of Sirpα (Figure 2D) and MGL2 (Figure 2E) in infected mice. SIRPα is a transmembrane protein predominantly expressed in monocytes, granulocytes, DC and hematopoietic stem cells (S. Takahashi, 2018). Furthermore, F4/80$^+$ MGL2$^+$ cells upregulated MHC class II expression with the infection (Figure 2F). Peritoneal APCs F4/80$^+$ MGL2$^+$ upregulated the immunoregulatory molecule PD-L1 (Figure 3A) but not ICOS-L (Figure 3B). Additionally, they expressed higher levels of IL-10 (Figure 3C), TNFα (Figure 3D), and HO-1 (Figure 3E). As shown in the previous chapter (5.1), the HO-1$^+$ F4/80$^+$ cells present in the peritoneal cavity during *F. hepatica* infection are associated with the presence of T regs in infected mice. In this study, we aimed to investigate whether MGL2-DTR mice exhibited similar behavior to the C57/BL6 mice that were used previously. Additionally, we sought to thoroughly characterize these cell populations with a focus on MGL2 expression that also expressed HO-1 (MGL2$^+$ HO-1$^+$ cells).

Figure 4A shows the analyses of the presence of Tregs in spleens of MGL2-DTR mice at 3 wpi. A decreased frequency of CD4$^+$ T cells was observed in the spleens of infected mice, despite their number being higher than that in non-infected mice. This could be attributed to significant splenomegaly resulting from the infection, as depicted in Supplementary Figure 2. In CD4$^+$ T cells,

the frequency and number of FoxP3^{+} CD25^{+} increased with infection compared to non-infected mice (Figure 4B).

5.2.2.2 Effect of MGL2^{+} cell depletion in F. hepatica infection

Mice that had MGL2^{+} cells depleted showed significantly lower clinical signs compared to control (PBS) mice at 3 wpi (Figure 5A). This was also correlated with a significant reduction in serum ALT activity (Figure 5B), which was associated with lower liver damage and fibrosis than that observed in control (PBS) infected mice (Figure 5C). In addition, the decrease in clinical symptoms was linked to a reduction in the frequency and number of MGL2-expressing F4/80^{+} cells from 1 dpi, as shown in Figures 5D and 5E. Interestingly, the depletion of MGL2^{+} cells in infected mice prevented the decrease of CD4^{+} T cell percentage in the spleen from 1 wpi (Figure 6A and Supplementary Figure 3). However, no significant increase in the CD4^{+} T cell counts was detected in comparison with infected control mice (Figure 6A).

In addition, the depletion of MGL2^{+} cells in infected mice shows a tendency to decrease CD4^{+}/FoxP3^{+} CD25^{+} cells, indicating that MGL2^{+} F4/80^{+} cells could play a crucial role in inducing a regulatory immune response (Figure 6B). Upon restimulation with molecules derived from the parasite (FhTE), splenocytes from infected mice depleted of MGL2^{+} cells produced higher levels of IFN-γ than those from infected non-depleted mice (Figure 6C). However, no differences were found in IL-4 and IL-10 cytokine secretion between infected groups (data not shown). No difference in the secretion of IL-4, IFN-γ, and IL-10 by splenic CD4^{+} T cells between infected mice with or without MGL2^{+} cell depletion by flow cytometry (Supplementary Figure 3).

Finally, we performed an adoptive transfer experiment to determine if the F4/80^{+} cells in the peritoneal cavity of infected animals could induce regulatory T lymphocytes. To this end, we infected MGL2-DTR animals and treated them with PBS (control) or DT (to deplete MGL2^{+} F4/80^{+} cells) and obtained cells from the peritoneal cavity present at 3 dpi (Figure 7A). These cells were inoculated into the peritoneal cavity of C57BL/6 ^{wt} animals (1x10^{6} cells/mouse) (Figure 7B). After one week of adoptive transfer, the spleen of recipient mice was analyzed by flow cytometry to detect the presence of CD4^{+} CD3^{+} T cells (Figure 7C) and Foxp3^{+} CD25^{+}/CD4^{+} T

cells (Figure 7D). The analysis showed that the group that was transferred with peritoneal cells had an increase in splenic Tregs, while the depletion of MGL2$^+$ F4/80$^+$ cells in infected mice did not increase splenic Tregs.

5.2.3 Discussion

Several studies have shown that CLRs play a significant role in recognizing, internalizing, and signaling upon stimulation with helminth glycoconjugates (Drickamer & Taylor, 2015; Guasconi et al., 2015). The uptake and internalization of parasite molecules are crucial for allowing antigen processing and presentation, which may affect the immune response and promote parasite survival (Dambuza & Brown, 2015; Higashi et al., 2002; Vázquez-Mendoza et al., 2013).

This study focused on the role of peritoneal MGL2$^+$ myeloid APC (F4/80$^+$ HO-1$^+$) in inducing splenic Tregs during *F. hepatica* infection. In the previous chapter of this thesis (5.1), we demonstrated that F4/80$^+$ myeloid cells in the peritoneal cavity of infected animals express HO-1, and this expression induces the production of IL-10, which is necessary for parasite establishment in the host (M. Costa et al., 2021). Based on our previous unpublished data, we have found that the cells expressing F4/80$^+$ and HO-1$^+$ are the same cells that express MGL2. The experimental model of MGL2- DTR mice was used in parallel as an alternative strategy to evaluate the role of F4/80$^+$ peritoneal cells in *F. hepatica* infection, particularly in the induction of T lymphocytes.

Here, we show that MGL2$^+$ F4/80$^+$ cells are induced into the peritoneal cavity, likely through an increase in CCR2, a chemokine receptor associated with the recruitment of inflammatory monocytes in parasite infections (Dunay et al., 2010; C. Terrazas et al., 2017). CCR2 mediates the cell migration from the blood and the recruitment of monocytes, myeloid suppressor cells (Kurihara et al., 1997; Kuziel et al., 1997) or monocyte-derived DC-like cells (Heyde et al., 2018) that express MHCII together with CD11c and F4/80 into damaged tissue or inflamed tissues. To confirm whether the MGL2$^+$ cells expressing both CD11c and F4/80 are a population of DCs or SPM, the expression of MerTK, a macrophage-specific molecule (Gautier et al., 2012), was evaluated. The results showed a low MerTK expression and high Sirpα levels (Figure 2D). Furthermore, these cells also expressed PD-L1, IL-10, HO-1, and TNFα (Figure 3). This suggests

that these cells recruited to the peritoneal cavity of *F. hepatica* infected-mice are CD11c$^+$ F4/80$^+$ CCR2$^+$ MGL2$^+$ and could consist of a population of monocyte-derived DCs or SPM with regulatory properties.

It has been previously reported that in *F. hepatica* infection, MGL2$^+$ regulatory DCs can suppress Th1 differentiation and induce the production of IL-10 by CD4$^+$ T cell lymphocytes in vitro (Rodríguez, Kalay, et al., 2017) . These regulatory functions could be mediated by PD-L1, the ligand of PD-1, expressed on regulatory DCs. PD-L1, along with PD-L2, is an immune receptor that inhibits T cell proliferation, induces Treg differentiation, and helps maintain immune homeostasis (Daneshmandi et al., 2015; Gerdes & Zirlik, 2011). PD-L2 negatively regulates the Th1-mediated immunopathology during *F. hepatica* infection (Guasconi et al., 2015). Thus, PD-L1 on DCs could control the induction of parasite-specific immunity that allows its survival. In this line, previous work showed that CCR2$^+$ cells recruited in the early stage of infection with *T. crassiceps* express PD-L1 and suppress T cell proliferation *in vitro* (Becerra-Díaz et al., 2021). It is worth noting that the hepatocyte growth factor receptor can induce the upregulation of both HO-1 and PD-L1 in cancer through the activation of Ras (Balan et al., 2015; Mukae et al., 2020). Additionally, myeloid cells that express both HO-1 and PD-L1 in breast tumors are known to suppress the activity of T cells (Muliaditan et al., 2018). Further experiments are needed to determine if MGL2 signaling correlates with HO-1 or PD-L1 expression in peritoneal DC-like cells during *F. hepatica* infection in mice.

MGL2$^+$ cell depletion showed partial resistance to the infection and did not experience an increase in CD4$^+$/CD25$^+$FoxP3$^+$ Tregs induced by the infection. This suggests that MGL2$^+$ cells play a crucial role in determining the infection's extent and could be used as immune checkpoints to control *F. hepatica* infection. Furthermore, the transfer of peritoneal cells from infected mice with or without MGL2$^+$ cell depletion showed that MGL2$^+$ cells from infected mice can acquire an immunoregulatory program that licenses them to induce Treg differentiation. Nevertheless, the parasite molecules that trigger this immunoregulatory pathway on peritoneal MGL2$^+$ myeloid cells during *F. hepatica* infection have not yet been identified.

In conclusion, we demonstrate that MGL2$^+$ HO-1$^+$ cells are recruited to the peritoneal cavity during experimental *F. hepatica* infection in mice. They express immunoregulatory molecules associated with increased clinical signs and expansion of Tregs, thus favoring infection. Altogether, these results suggest that strategies based on MGL2 targeting could be helpful in the control of fasciolosis.

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Macrophage Gal/GalNAc lectin 2 (MGL2)+ peritoneal antigen presenting cells during *Fasciola hepatica* infection are essential for regulatory T cell induction

Monique Costa, Valeria da Costa, Pablo Lores, Mercedes Landeira, Santiago A. Rodríguez-Zraquia, María Florencia Festari & Teresa Freire✉

***Fasciola hepatica*, one of the agents that causes fasciolosis, modulates the host immune system to allow parasite survival in the host. *F. hepatica* expresses carbohydrate-containing glycoconjugates that are decoded by C-type lectin receptors, such as Dectin-1, mannose receptor, DC-SIGN and MGL, that are mainly present on myeloid antigen presenting cells (APCs) and can mediate immunoregulatory properties on T cells. In particular, Macrophage Gal/GalNAc lectin 2 (MGL2) expands modified Th2 immune responses, while suppressing Th1 polarization, upon recognition of GalNAc-glycosylated parasite components. In this study, by using MGL2-DTR transgenic mice that encode human diphtheria toxin receptor in MGL2$^+$ cells, we demonstrate the role of peritoneal APCs during *F. hepatica* infection in favoring parasite survival. This process might be mediated by the induction of splenic Tregs in vivo, since the depletion of MGL2$^+$ cells conferred mice with partial resistance to the infection and abrogated the increase of CD4$^+$/CD25$^+$ FoxP3$^+$ Tregs induced by the parasite. Therefore, MGL2$^+$ cells are critical determinants of *F. hepatica* infection and could constitute immune checkpoints to control parasite infection.**

Fasciola hepatica is a trematode parasite that causes fasciolosis, a zoonotic disease that affects humans[1,2]. It also infects livestock, causing significant economic losses worldwide[1,2]. To survive in its mammalian hosts, *F. hepatica* is capable of modulating the host immune system by inducing a modified type-2 responses characterized by potent immune regulatory processes such as differentiation of regulatory T cells (Tregs), alternative activation of macrophages, involvement of regulatory dendritic cells (DCs), upregulation of IL-10 and TGFβ and down-regulation of Th1 cytokines[3–7].

Helminths express carbohydrate-containing glycoconjugates that are extremely important in their life cycles and pathogeny since they can participate in immune escape[8]. Indeed, glycoconjugates produced by *F. hepatica* are able to modulate the maturation and function of DCs[9,10] and macrophages[11–13]. Furthermore, *F. hepatica* glycans participate in parasite migration through the intestine in early stages of the infection[14,15].

The immunomodulatory role of parasite glycans relies on the ability of lectins to decode their information, such as C-type lectin receptors (CLRs), that are mainly present on myeloid antigen presenting cells (APC)[16,17]. Previously data have demonstrated that CLRs are key mediators of the immunoregulatory properties induced by *F. hepatica*. In fact, Dectin-1 on macrophages interacts with *F. hepatica* excretory-secretory products inducing an alternative activated macrophage phenotype[11,12] and likely exerting T cell anergy via selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way[18]. However, in-depth studies on the function of these cells are still necessary[12]. Injection of *F. hepatica* tegumental antigens induces anergic-like T cells via DCs in a mannose receptor (MR)-dependent manner, although the role of MR during *F. hepatica* infection has not been determined so far[19]. In contrast, no implication of the MR was found in the suppression of LPS-induced cytokines by bone marrow derived DCs treated with *F. hepatica* derived-molecules[13]. On the other hand, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) interacts with *F. hepatica* glycoconjugates

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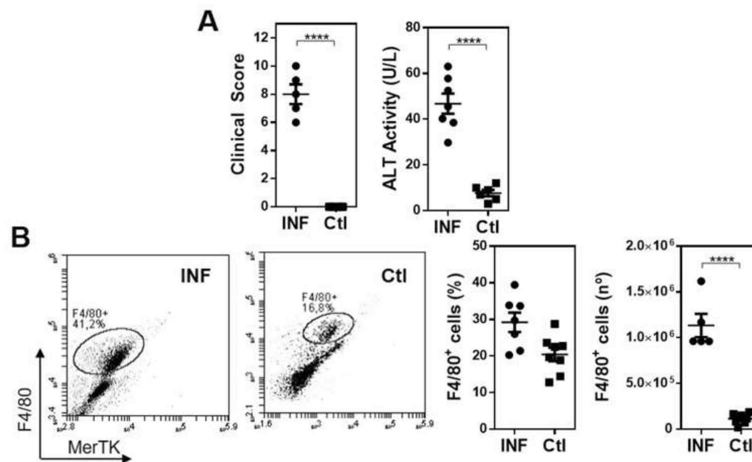

Figure 1. *F. hepatica* infected MGL2-DTR mice present an increase of F4/80⁺ cells in the peritoneal cavity. (**A**) Clinical score and ALT in serum after 3 wpi. MGL2-DTR mice were infected with 10 *F. hepatica* metacercariae (INF). Non infected mice injected with PBS were used as controls (Ctl). (**B**) Frequency and number of F4/80⁺ cells in the peritoneal cavity determined by flow cytometry. A representative figure of three independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (****$p < 0.0001$).

through mannose and fucose residues on regulatory DCs and decreases allogeneic T cell proliferation, via the induction of anergic T cells[10].

Macrophage Gal/GalNAc lectin 2 (MGL2, CD301) binds to terminal GalNAc residues, including the Tn antigen (GalNAc-αThr/Ser) and is mainly expressed on immature, tolerogenic or type-2 DCs[6,20,21] and alternatively-activated macrophages[22]. Moreover, MGL activation dampens immune responses, by inducing synthesis of IL-10 by DCs[6,23], promoting the differentiation of Tregs[24], inducing T cell apoptosis and suppressing T cell activation[25]. Previous studies from our group demonstrated that human MGL can interact with *F. hepatica* components through the Tn antigen and modulate the TLR2-induced maturation of human monocyte derived DCs by up-regulating the production of IL-10 and TNFα[6]. In addition, we have shown that MGL2⁺ cells in *F. hepatica* infected mice express a variety of regulatory markers, including IL-10, TNFα and TGFβ, expand modified Th2 immune responses and suppress Th1 polarization[6]. However, the role of MGL2⁺ cells during *F. hepatica* infection in inducing Tregs in vivo has not yet been investigated.

In this study, we demonstrate that MGL2-expressing APCs recruited to the peritoneal cavity during *F. hepatica* experimental infection in mice are essential for parasite survival as well as for the induction of splenic Tregs in vivo. We used MGL2-DTR transgenic mice that encode human diphtheria toxin receptor (DTR) in MGL2⁺ cells. Thus, these cells can be depleted with diphtheria toxin (DT) injection[24]. The depletion of MGL2⁺ cells conferred mice with partial resistance to the infection and abrogated the increase of CD4⁺/CD25⁺ FoxP3⁺ Tregs induced by the infection. Therefore, MGL2⁺ cells could constitute immune checkpoints to control *F. hepatica* infection.

Results

F. hepatica infection in MGL2-DTR transgenic mice is characterized by the recruitment of immunoregulatory F4/80⁺ cells in the peritoneum and higher levels of splenic Treg. First, we infected MGL2-DTR transgenic mice and analyzed the clinical signs and immunological response induced by *F. hepatica* infection. After 3 weeks post-infection (wpi), infected animals presented a high clinical score, determined by the general state of the animal (Supplementary Table 1) and alanine transaminase (ALT) activity in serum, a common marker to detect hepatic dysfunction, and liver damage and fibrosis (Fig. 1A). Furthermore, infection was associated with an increase of F4/80⁺ cells in the peritoneal cavity of infected mice. However, the frequency of these cells was not significantly different from those determined in non-infected mice (Fig. 1B and Supplementary Fig. 1A). In addition, peritoneal F4/80⁺ cells from infected mice expressed higher CCR2 (Fig. 2A and Supplementary Fig. 1B) and CD11c (Fig. 2B) levels. However, they did not upregulate MerTK expression (Fig. 2C). Additional phenotyping by flow cytometry showed a significantly enhanced expression of Sirpα (Fig. 2D) and MGL2 (Fig. 2E) in infected mice. Furthermore, F4/80⁺ MGL2⁺ cells upregulated MHC class II expression with the infection (Fig. 2F). On the other hand, F4/80⁺ MGL2⁺ peritoneal APCs upregulated different immunoregulatory molecules, such as PD-L1 (Fig. 3A), but not ICOS-L (Fig. 3B). Last, they also expressed

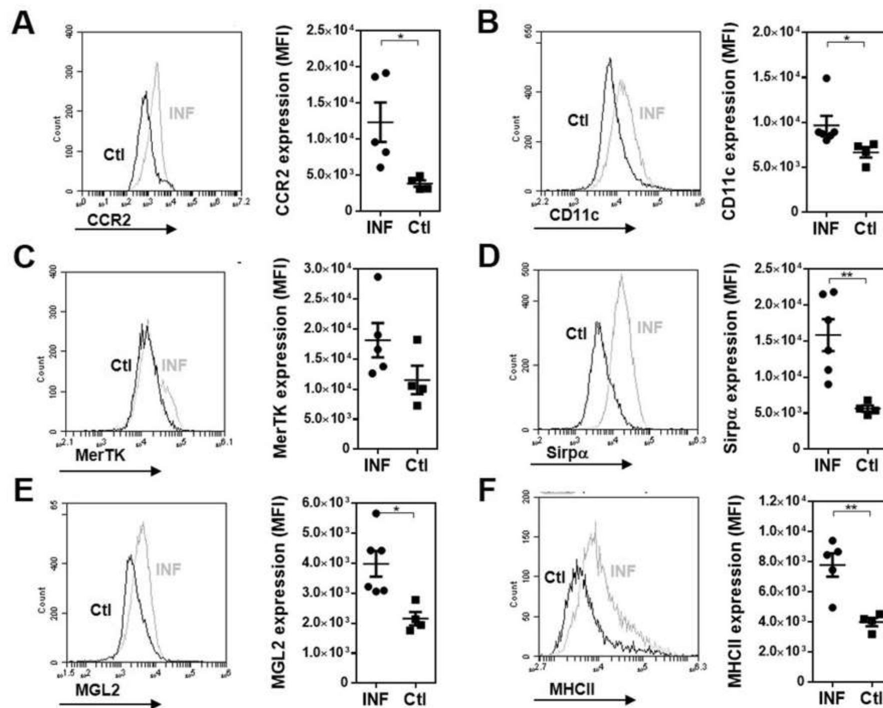

Figure 2. Peritoneal F4/80⁺ cell phenotype in *F. hepatica* MGL2-DTR mice. Expression of CCR2 (**A**), CD11c (**B**), MerTK (**C**), Sirpα (**D**), MGL2 (**E**) and MHC class II (**F**) is shown in F4/80⁺ peritoneal cells from *F. hepatica* infected mice (INF) and non-infected animals (Ctl). Grey lines in histograms represent infected mice while black lines indicated control mice. A representative figure of three independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (*$p<0.05$, **$p<0.01$).

higher levels of immunomodulatory molecules such as IL-10 (Fig. 3C), TNFα (Fig. 3D) and hemoxygenase-1 (HO-1) (Fig. 3E).

Then, we analyzed the presence of Tregs in spleens of 3 wpi MGL2-DTR mice. As depicted in Fig. 4A, a lower frequency of CD4⁺ T cells in spleens of infected mice was detected, although their number was higher than in non-infected mice, likely due to prominent splenomegaly in response to the infection (Supplementary Fig. 2). In addition, FoxP3⁺ CD25⁺ in CD4⁺ T cells increased with infection, both in frequency and number, in relation to non-infected mice (Fig. 4B).

MGL2⁺ cell depletion is associated with resistance to *F. hepatica* infection. In order to determine the role of MGL2⁺ F4/80⁺ cells during *F. hepatica* infection, we injected MGL2-DTR transgenic mice with DT one day before and after the infection and every 3 days. Mice depleted of MGL2⁺ cells showed significantly lower clinical signs than control (PBS) mice at 3 wpi (Fig. 5A), which was also related to a significant decrease in serum ALT activity (Fig. 5B), associated with a lower liver damage and fibrosis than the one observed in control (PBS) infected-mice (Fig. 5C). Accordingly, the decrease in clinical signs was associated with a reduction, from 1 day post-infection (dpi), in the frequency and number of MGL2-expressing F4/80⁺ cells (Figs. 5D,E).

MGL2⁺ cell depletion abrogates splenic Treg increase induced by the infection. The depletion of MGL2⁺ cells in MGL2-DTR infected mice prevented the decrease of CD4⁺ T cell percentage in the spleen from 1 wpi (Fig. 6A and Supplementary Fig. 3). In contrast, no significant increase in the CD4⁺ T cell counts was detected in comparison with DT-non treated infected control mice (Fig. 6A). Moreover, the depletion of MGL2⁺ cells in infected mice abrogated the increase in frequency and number of CD4⁺/FoxP3⁺ CD25⁺ cells (Fig. 6B), suggesting that MGL2⁺ F4/80⁺ cells are critical players in the induction of an adaptive regulatory immune response.

Of note, when splenocytes from infected mice depleted of MGL2⁺ cells were restimulated with molecules derived from the parasite (FhTE), they produced higher levels of IFNγ than those from infected non-depleted mice (Fig. 6C). However, no differences were observed in IL-4 and IL-10 cytokine secretion between infected

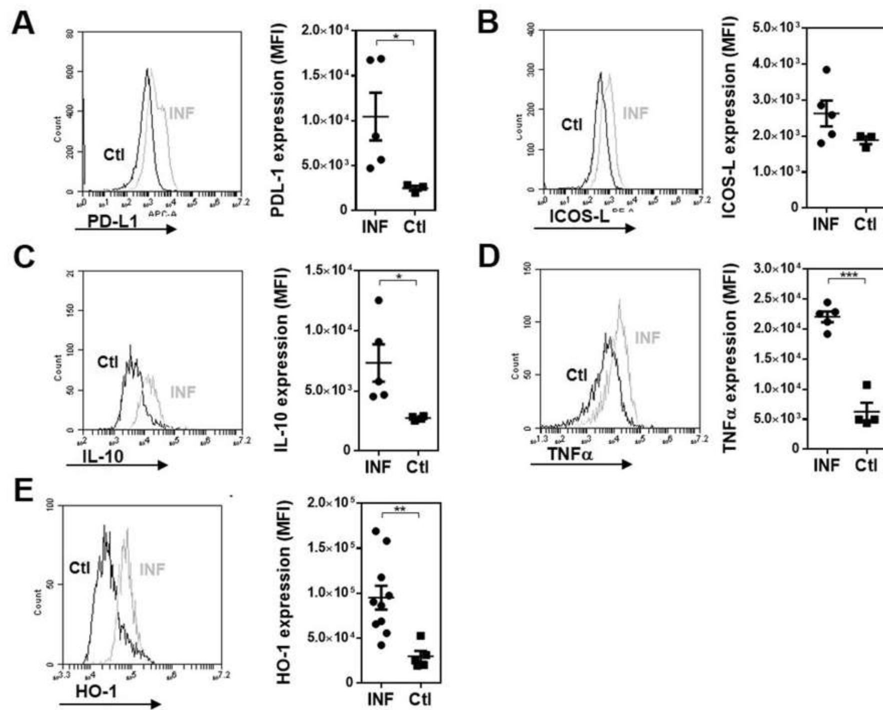

Figure 3. Peritoneal F4/80+ cells in *F. hepatica* MGL2-DTR mice express immunoregulatory molecules. Expression of PD-L1 (**A**), ICOS-L (**B**), IL-10 (**C**), TNFα (**D**) and HO-1 (**E**) is shown in F4/80+ peritoneal cells from *F. hepatica* infected mice (INF) and non-infected animals (Ctl). Grey lines in histograms represent infected mice while black lines indicated control mice. A representative figure of three independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (* $p<0.05$, ** $p<0.01$).

groups (not shown). We were also unable to detect a difference in IL-4, IFNγ and IL-10 secretion by splenic CD4+ T cells between infected mice with or without MGL2+ cell depletion by flow cytometry (Supplementary Fig. 3).

Peritoneal MGL2+ F4/80+APCs from infected mice are essential for the induction of splenic Tregs. To confirm the role of MGL2+ F4/80+ peritoneal APCs induced by *F. hepatica* infection in the generation of Tregs in the spleen, we infected MGL2-DTR mice and treated them either with DT to deplete MGL2+ cells or with PBS as control. After 3 dpi, peritoneal cells from both groups of mice were adoptively transferred to the recipient non-infected mice (Fig. 7A,B). The presence of CD4+ CD3+ T cells (Fig. 7C) and Foxp3+ CD24+/CD4+ T cells (Fig. 7D) in the spleen of recipient mice was analyzed by flow cytometry revealing that the group transferred with F4/80+ cells presented an increase in splenic Tregs, while depletion of F4/80+ cells in infected mice did not induce and increase in splenic Tregs.

Discussion

Various studies have independently demonstrated the role of CLRs in recognizing, internalizing and signaling upon the stimulation with helminth glycoconjugates[18,26]. The uptake and the internalization of parasite molecules are crucial to allow the antigen processing and presentation that may influence the immune response and promote parasite survival in the host[20,27,28]. In this work, we focused on the role of peritoneal MGL2+ myeloid APC during *F. hepatica* infection in inducing splenic Tregs in vivo. Macrophages and DCs represent heterogeneous myeloid cell populations specialized in antigen presentation. However, DCs are unique in their capacity to orchestrate the adaptive immune response by activating naïve T cells and inducing their differentiation into different effector T cells depending on the pathogen[29,30]. Both macrophages and DCs can secrete pro-inflammatory or anti-inflammatory cytokines since they exhibit functional plasticity that enables them to adapt to various local conditions and to restore homeostasis after inflammation[29–31]. Here, we show that MGL2+ F4/80+ cells are recruited to the peritoneal cavity likely through an increase in CCR2, the receptor of the monocyte chemoattractant proteins 1 and 3[32,33]. Indeed, CCR2 is a chemokine receptor associated with the recruitment of inflammatory monocytes in parasite infections[34,35] and it mediates the cell migration from the bone marrow and the

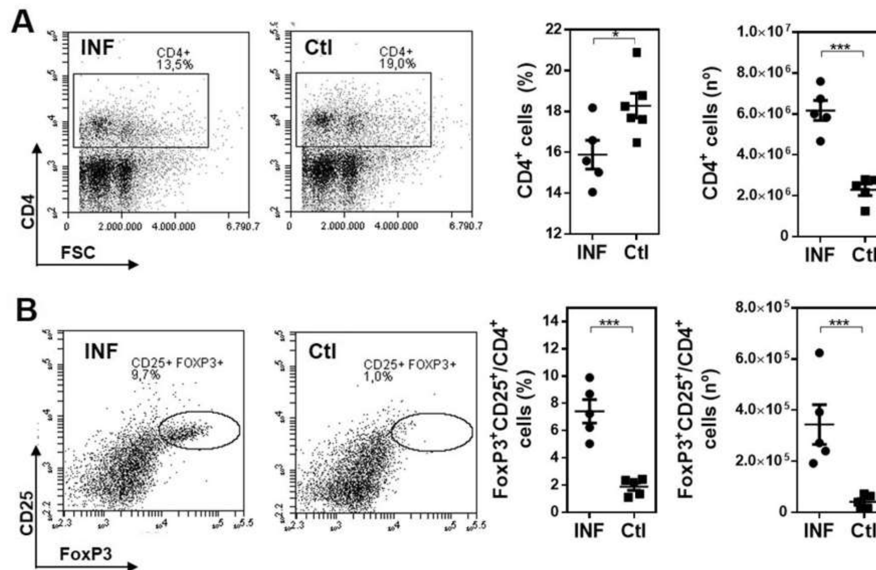

Figure 4. *F. hepatica* infection is associated with an increase in splenic Tregs. Frequency and number of $CD4^+$ cells (**A**) and $FoxP3^+$ $CD25^+/CD4^+$ cells. (**B**) in spleens were determined by flow cytometry. Infected MGL2-DTR mice were infected with 10 *F. hepatica* metacercariae (INF). Non infected mice were used as controls (Ctl). A representative figure of three independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$, ***$p < 0.001$).

recruitment of monocytes, myeloid suppressor cells[32,33] or monocyte-derived DC-like cells[36] that express MHC II together with CD11c and F4/80 into damaged tissue or inflamed tissues.

MGL is a type II transmembrane protein expressed on professional APC. Even though there is only one MGL in humans (hMGL), two orthologues are present in mice that possess different glycan specificity (mMGL1 and mMGL2)[37,38]. Both mMGL2 and hMGL display similar specificity for terminal GalNAc moieties, including the Tn antigen (αGalNAc-O-Ser/Thr) and LacDiNAc (GalNAcβ1-4GlcNAc)[39] and can recognize glycoconjugates from helminth parasites, such as *Schistosoma mansoni*[40], *Trichuris suis*[41] and *Taenia crassiceps*[42]. Furthermore, it has been proposed that $MGL2^+$ dermal DCs are specialized in inducing Th2 responses both in allergy and helminth-infection models[42].

In order to confirm that $MGL2^+$ cells that express both CD11c and F4/80 may constitute a population of DCs, we evaluated the expression of MerTK, a macrophage specific molecule[43]. Due to the low expression of MerTK and high levels of Sirpα, PD-L1, IL-10 and TNFα, we suggest that the $CD11c^+$ $F4/80^+$ $CCR2^+$ cells, that also express MGL2 during *F. hepatica* infection, constitute a population of monocyte-derived DCs with regulatory properties. Moreover, as previously described by our team, and in accordance with our studies performed in MGL2-DTR transgenic mice, regulatory DCs from *F. hepatica* express high levels of Sirpα and IRF4[6]. Both IRF4 and Sirpα participate in immunoregulation and can promote Treg differentiation[44–46]. In *F. hepatica* infection in mice, $MGL2^+$ regulatory DCs can suppress Th1 differentiation and induce the production of IL-10 by $CD4^+$ T cell lymphocytes in vitro[6]. These regulatory functions could be mediated by PD-L1, the ligand of PD-1, expressed on regulatory DCs. This molecule, together with PD-L2, is an immune inhibitor receptor expressed on T cells that limits cell proliferation, induces Treg differentiation and serves to maintain immune homeostasis[47,48]. Indeed, PD-L2 negatively regulates Th1-mediated immunopathology during *F. hepatica* infection[18]. Therefore, PD-L1 on DCs could play a role in controlling the induction of parasite-specific immunity that allows its survival. In addition, a previous work demonstrated that $CCR2^+$ cells recruited in the early stage of infection with *T. crssiceps* express PDL-1, and suppress T cell proliferation in vitro[49]. Furthermore, molecules from the tapeworm *Hymenolepis diminuta* induce a CCR2-dependent recruitment of myeloid monocyte-like cells that express high levels of PD-L1 to the peritoneum. These cells enhance both IL-10 and IL-4 secretion by activating T cells in vitro and have an immunosuppressive role in vivo[50].

In a recent report we have shown that $F4/80^+$ myeloid cells in the peritoneum of infected animals express HO-1[51], the inducible rate-limiting enzyme involved in the catabolism of free heme[52] that promotes anti-inflammatory cytokine secretion[53,54]. In fact, HO-1 expression by DCs induces the production of IL-10 and inhibits T cell proliferation[55]. In *F. hepatica* infection it induces production of IL-10 which is necessary for parasite establishment in the host[51]. Of note, hepatocyte growth factor receptor induces Ras-dependent upregulation of both HO-1 and PD-L1 in cancer[56,57]. In addition, myeloid cells expressing both HO-1 and PD-L1 in breast tumors

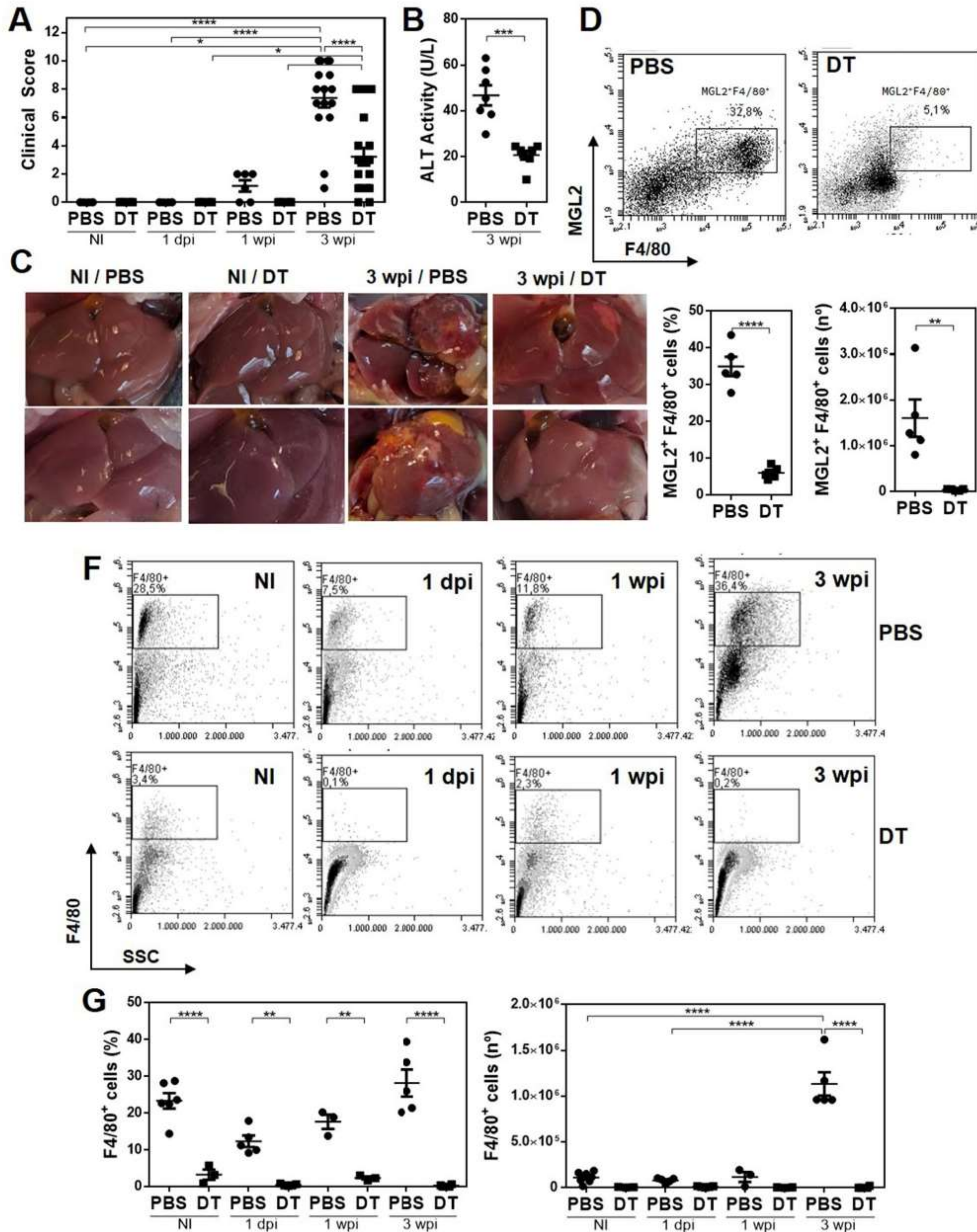

Figure 5. Depletion of F4/80⁺ MGL2⁺ cells during *F. hepatica* infection attenuates the clinical signs induced by the parasite. MGL2-DTR mice were infected with 10 *F. hepatica* metacercariae and sacrificed at 1 dpi, 1 wpi and 3 wpi. MGL2⁺ cells were depleted with DT-treatment. Control mice were injected with PBS. Non infected mice were used as controls (NI). Clinical score (**A**) and ALT (**B**) in serum of infected and control DT- or PBS-treated mice. (**C**) Representative images of livers from DT- or PBS-treated 3 wpi mice. (**D**) Peritoneal F4/80⁺ MGL2⁺ cells gating in *F. hepatica* infected mice treated with DT. (**E**) Frequency and number of MGL2⁺ F4/80⁺ peritoneal cells in infected DT- or PBS-treated mice. (**F**) Peritoneal F4/80⁺ cells gating during *F. hepatica* infection in DT-treated mice. (**G**) Frequency and number of F4/80⁺ peritoneal cells in infected DT- or PBS-treated mice at 1 dpi, 1 wpi and 3 wpi. Data from three independent experiments is shown (±SEM, indicated by error bars). Asterisks indicate statistically significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

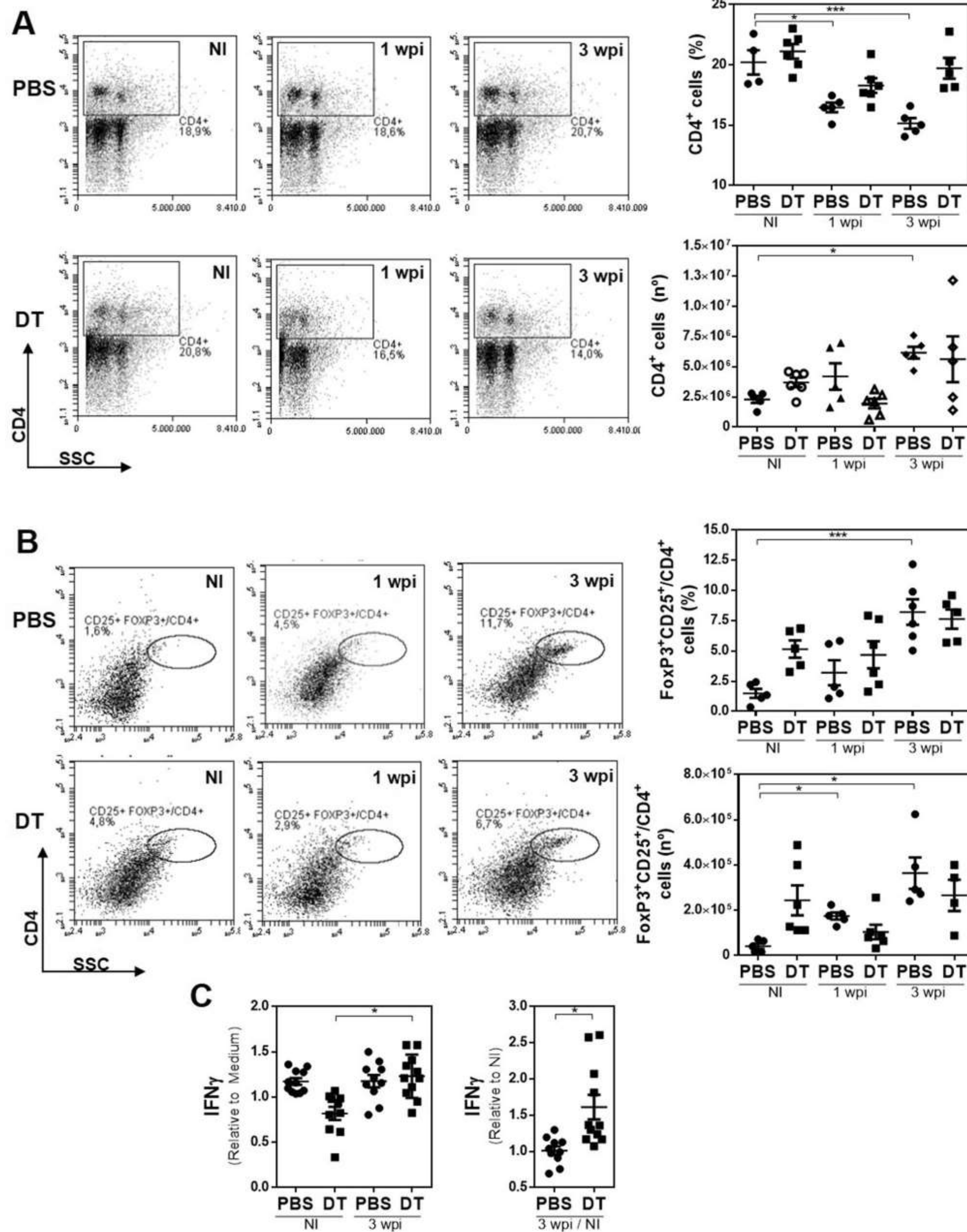

Figure 6. Depletion of F4/80⁺ MGL2⁺ cells during *F. hepatica* infection abrogates splenic Treg expansion. Frequency and number of CD4⁺ cells (**A**) and FoxP3⁺ CD25⁺/CD4⁺ cells. (**B**) in spleens were determined by flow cytometry. MGL2-DTR mice were infected with 10 *F. hepatica* metacercariae and sacrificed at 1 dpi, 1 or 3 wpi. MGL2⁺ cells were depleted with DT-treatment. Control mice were injected with PBS. Non infected mice were used as controls (NI). (**C**) Production of IFNγ evaluated by specific ELISA on supernatants of splenocyte culture stimulated with PMA/Ionomicin. A representative figure of three independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$, ***$p < 0.001$).

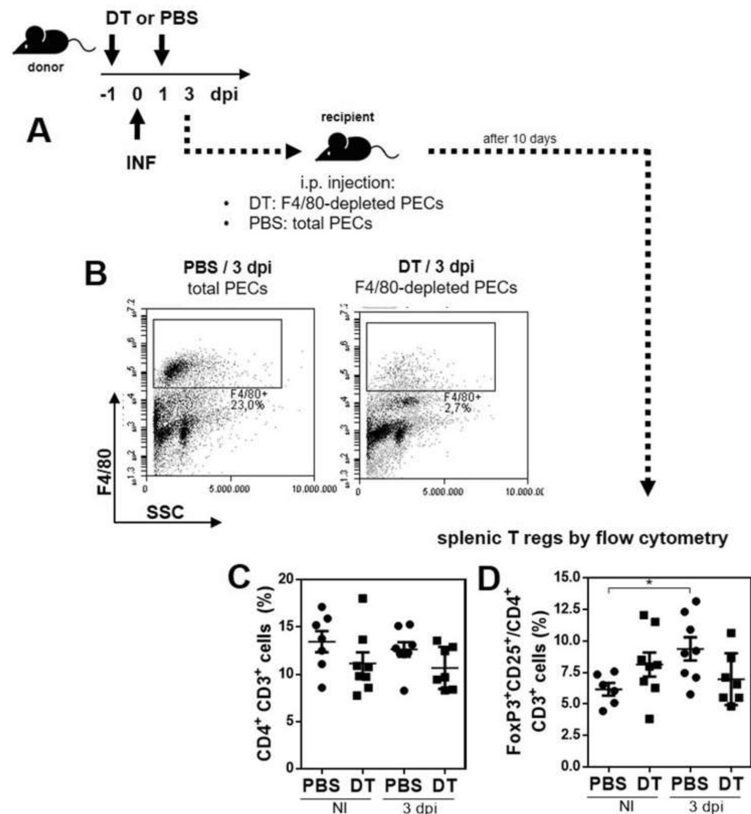

Figure 7. MGL2⁺ F4/80⁺ peritoneal cells induced by *F. hepatica* infection are necessary for Treg induction. (**A**) Adoptive transfer from infected MGL2-DTR mice to recipient non-infected mice. (**B**) F4/80⁺ peritoneal cell depletion with DT treatment after 3 dpi. Frequency and number of CD4⁺ CD3⁺ cells (**C**) and FoxP3⁺ CD25⁺/CD4⁺ (**D**) cells in spleens from recipient mice determined by flow cytometry. A representative figure of two independent experiments is shown (± SEM, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$).

suppress T cell activity[58]. However, further experiments are necessary to explore whether MGL2 signaling is related with HO-1 or PD-L1 expression in peritoneal DC-like cells in *F. hepatica* infection in mice.

We have previously demonstrated that MGL2⁺ cells induce the production of IL-10 by CD4⁺ T cells[6]. To explore the involvement of these cells in the differentiation of Tregs, we depleted MGL2⁺ cells from *F. hepatica* infected mice. We found that they are crucial for infection and differentiation of Tregs, since an increased number of CD4⁺/FoxP3⁺CD25⁺ cells was found in the spleens of infected mice and their depletion abrogated the Treg expansion induced by the infection. Furthermore, adoptive transfer of peritoneal cells from infected mice with or without MGL2⁺ cell depletion revealed that MGL2⁺ DCs from infected mice can acquire an immunoregulatory program that licenses them to induce Treg differentiation. Nevertheless, the parasite molecules that trigger this immunoregulatory pathway on peritoneal MGL2⁺ myeloid cells during *F. hepatica* infection have not yet been identified. Indeed, although the identification of differentially-expressed genes involved in the glycosylation process of *F. hepatica* proteins has been performed[59] the immunomodulatory role of glycans in certain proteins is still unknown. On the contrary, several studies have identified the glycan motifs from *F. hepatica* that can interact with CLRs on these cells, such as the Tn antigen that interacts with MGL2[6,10]. Other glycans such as mannosylated glycoconjugates favor anergic T cells or Tregs that silence the immune system of the host through CD209 (or DC-SIGN)[10]. In addition, MR was described to interact with *F. hepatica* molecules and to mediate the partial inhibition of TLR-induced maturation of bone marrow-derived DCs[13,19]. Last, both MR and Dectin-1 immunomodulate Arginase-1 and PD-L2 expression and TGFβ production by macrophages in response to *F. hepatica* excretory–secretory products[12,18]. These data suggest that the parasite targets more than one CLR in order to evade immunity. Further experiments are needed to determine whether different factors participate

in this process, such as glycans in parasite microvesicles[60,61]. In addition, other works have reported the anti-inflammatory role of *F. hepatica* molecules in training the innate immunity of macrophages[62]. However, the role of glycans in the induction of epigenetic imprinting of cells has not yet been determined.

In conclusion, we demonstrate that MGL2$^+$ DC-like cells are recruited to the peritoneum during experimental *F. hepatica* infection in mice. They express immunoregulatory molecules that associate with an increase in clinical signs and expansion of Tregs, thus, favoring infection. Altogether, these results suggest that strategies based on MGL2 targeting could be helpful in the control of fasciolosis.

Methods

Ethics statement. Adult worms were collected during the routine work of a local abattoir (Frigorífico Carrasco) in Montevideo (Uruguay). Protocols were approved by the Uruguayan Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, Uruguay).

Mice. MGL2-DTR six- to eight-week-old mice were purchased from Jackson Laboratory (USA). They were used to analyze the role of MGL2$^+$ cells during *F. hepatica* infection. MGL2$^+$ cells can be depleted with DT injection[24]. Animals were kept in the animal house (URBE, School of Medicine, UdelaR, Uruguay) with water and food supplied ad libitum. Mouse handling, care and experiments were carried out in compliance with institutional guidelines and regulations from the National Committee on Animal Research (CNEA, http://www.cnea.org.uy/, National Law 18.611, Uruguay) and in accordance with ARRIVE guidelines. Procedures involving animals were approved by the Universidad de la República's Committee on Animal Research (Comisión Honoraria de Experimentación Animal, CHEA Protocol Number 070153–000,811-19).

Parasite infection, animal treatment and sample obtention. Ten *F. hepatica* metacercariae (Montevideo, Uruguay) were orally administered per mouse. Viability of metacercariae was analyzed by in vitro excystment (> 70%) as previously evaluated[63]. Mice were bled at 3 wpi and peritoneal exudate cells (PECs), spleens, and livers were removed after either 1 dpi, 1 or 3 wpi, depending on the experiment. Non-infected animals were used as controls (0 dpi). To evaluate parasite infection while depleting MGL2$^+$ cells, metacercariae were administered on day 0 into MGL2-DTR mice that were previously intraperitoneally (i.p.) injected with diphtheria toxin (DT, 0.5 μg/mouse) (The Native Antigen Company, USA) or PBS on day − 1 and every 2 or 3 days until the end of the experiment. Each experimental group contained at least six mice. PECs and spleens were processed as already described[64]. Red cells were lysed with ammonium chloride potassium buffer. The alanine aminotransferase (ALT) activity in sera was used to quantify liver damage and was determined with a colorimetric commercial kit (Spinreact, Spain) according to the manufacturer's instructions. The infection severity was assessed with a defined clinical score according to the following parameters: presence or absence of peritoneal hemorrhage, presence of macroscopic liver damage and splenomegaly, and the amount of cell content in the peritoneal cavity[51,64,65], where the minimum score was 0 and the maximum was 10 (described in detail in Supplementary Table 1).

Flow cytometry. Cell suspensions from PECs and spleens were washed twice with PBS containing 2% FBS and 0.1% sodium azide (FACS buffer) and stained with specific antibodies for 30 min at 4 °C as previously published[51]. The following antibodies (Biolegend, USA) were used: anti-Sirpα (P-84), -CD11c (N418), -CCR2 (SA203G11), -PD-L1 (10F9G2) and ICOS-L (HK5.3). Expression of FoxP3, HO-1 and IL-10 was analyzed by intracellular staining by permeabilizing with Cytofix and Perm wash buffers (Biolegend, USA), incubated with anti-IL-10 (JES5-1E3), -FoxP3 (MF14), TNFα (MP6-XT22) and HO-1 (clone ab13248 from Abcam, USA) specific antibodies. Analyses were performed using a BD Accuri C6 Plus cytometer and software (BD-Biosciences, USA).

Proliferation assay and cell culture. Parasite protein extract (FhTE) was prepared from live adult flukes obtained from infected bovines as previously published[51]. Splenocytes (0.5×10^6/well) from infected mice or uninfected naïve mice (control group) were cultured for 5 days at 37 °C and 5% CO_2, in RPMI-1640 with 400 μg/ml glutamine (Capricorn Scientific, Germany) complete medium containing 10% heat-inactivated fetal bovine serum (FBS, Capricorn Scientific, Germany), 50 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Merk, Sigma-Aldrich, USA) in presence or absence of FhTE (75 μg/ml) as previously described[9]. An IFNγ-specific sandwich ELISA assay (Biolegend, USA) was used to quantify IFNγ levels in culture supernatants.

Adoptive transfer of peritoneal cells from infected mice. To evaluate the capacity of MGL2$^+$ cells to induce Tregs, 6 MGL2-DTR mice that were i.p. injected with DT (0.5 μg/mouse) or PBS on days − 1, + 1 and + 3, were infected with 10 *F. hepatica* metacercariae on day 0. As control groups non-infected MGL2-DTR mice injected either with DT or PBS were used (n = 6). At 4 dpi, PECs from the four groups were collected, red cells were lysed and remaining cells were counted. Depletion of peritoneal MGL2$^+$ cells with DT treatment was verified by flow cytometry. 1×10^6 cells were i.p. injected in recipient non-infected MGL2-DTR mice that did not receive DT treatment. After 10 days, spleens were removed and CD3$^+$ CD4$^+$/FoxP3$^+$ CD25$^+$ cells were analyzed by flow cytometry as described above.

Statistical analysis. The obtained results were expressed as mean ± SEM. Statistical analyses were performed with GraphPad Prism version 6.04 for Windows (GraphPad Software, USA) was used to perform statistical analyses. Results were analyzed using one-way ANOVA followed by Tukey's test, or two-tailed student's

t-test, depending on the experiment. Significant differences shown by asterisks were considered when $^{*}p < 0.05$, $^{**}\,p < 0.01$, $^{***}\,p < 0.001$, $^{****}\,p < 0.0001$.

Data availability

All data generated or analyzed during this study are included in this published article or available upon request (and its Supplementary Information files).

Received: 26 May 2022; Accepted: 28 September 2022
Published online: 21 October 2022

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Acknowledgements
We are particularly grateful to abattoirs 'Frigorífico Carrasco' for their help with worm collection. The authors wish to thank to Agencia Nacional de Investigación e Innovación (ANII) for funding (FCE_1_2019_1_1_156295) to TF.

Author contributions
M.C. performed the experiments, analyzed data and reviewed the manuscript. V.C. assisted with MGL2-DTR mice model to deplete MGL2^{+} cells and contributed with manuscript revision. S.R-Z, M.L. P.L. and M.F.F. assisted with in vivo animal and organ treatment. M.L. P.L. and M.F.F revised the final manuscript. TF contributed to supervision and design of all experiments shown in this paper, analyzed data and finally prepared and revised the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-21520-w.

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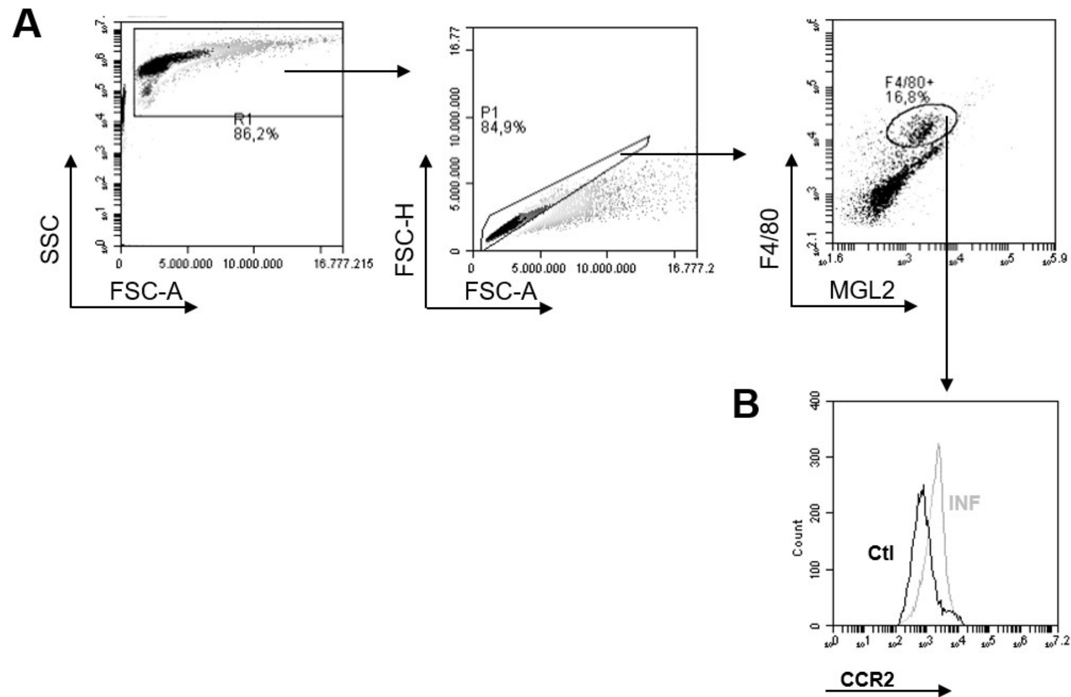
Supplementary Table 1. Clinical score of *F. hepatica* infected mice used in this study.

Ascites		Spleen		Number of lesions / hepatic lobe		Liver lobes	
Score	**Description**	**Score**	**Size**	**Score**	**Description**	**Score**	**Description**
0	None (normal cell content)	0	Normal	0	None	0	Healthy
1	Mild (medium cell content)	1	Splenomegaly (< x2)	1	< 3 lesions	1	1 hepatic lobe affected
2	Moderate (high cell content)	2	Splenomegaly (> x2)	2	> 3 lesions	2	> 2 hepatic lobes affected
3	Severe (high cell and blood content)			3	Complete affection of lobes		

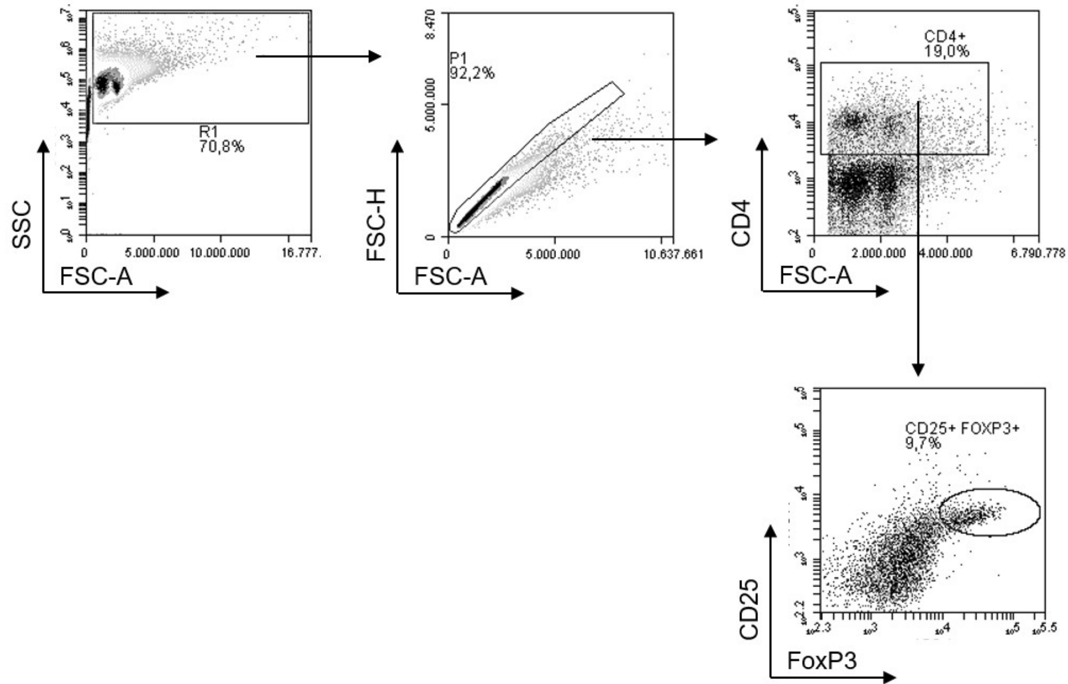
Maximal score is 10

Supp. Fig.1 **Costa *et al.***



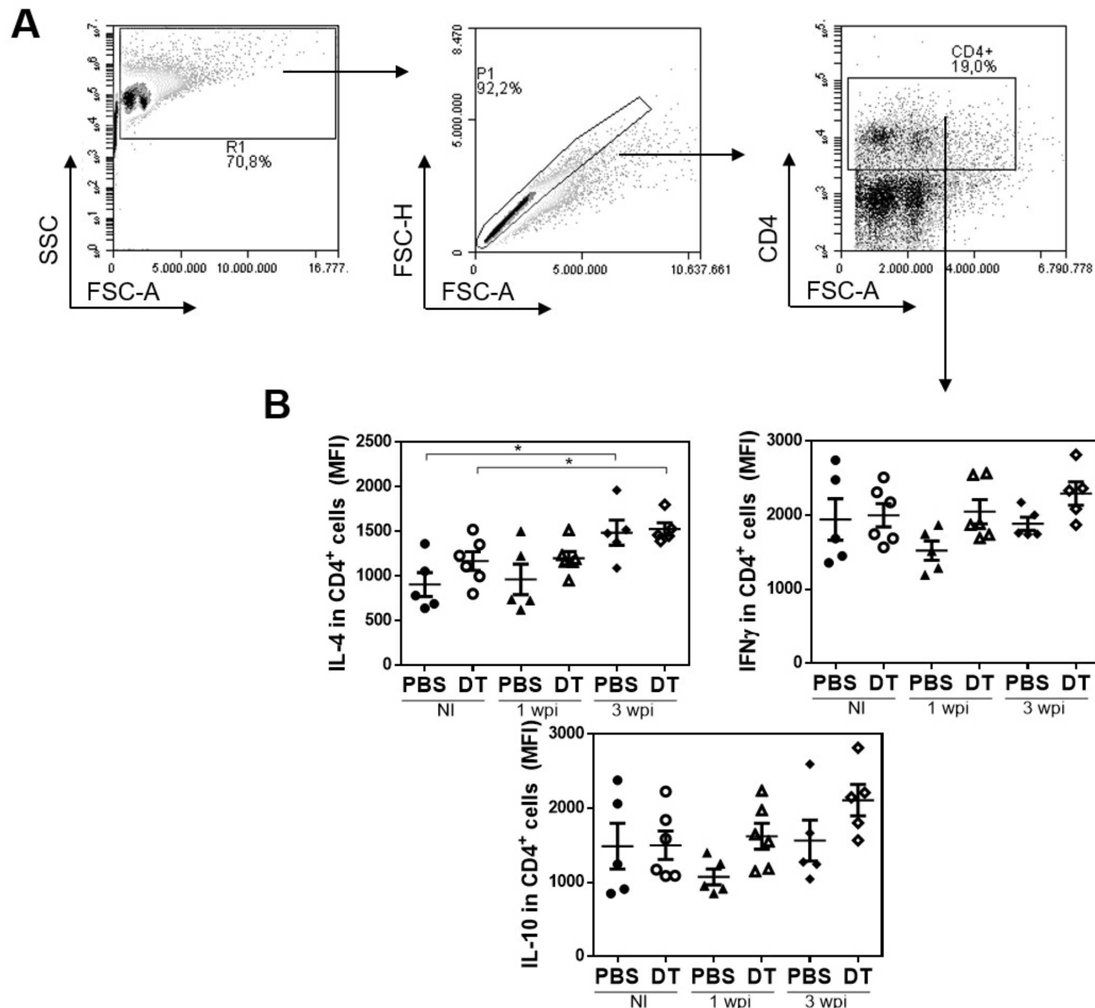
Supplementary Figure 1. Gates used for flow cytometry analyses of PECs from infected and non-infected mice. PEC suspensions were gated according to side and forward scatter and singlets were selected. Cells were stained with anti-F4/80 and -MGL2 antibodies **(A).** CCR2 expression (shown as an example) was determined according to Median Fluorescence intensity on F4/80^{+} cells after staining with a specific antibody in non-infected (Ctl) and infected mice (INF) **(B)**.

Supp. Fig.2 **Costa *et al.***



Supplementary Figure 2. Gates used for flow cytometry analyses of spleens from infected and non-infected mice. Splenocyte suspensions were gated according to side and forward scatter and singlets were selected . Cells were stained with anti-CD4, -CD25 and -FoxP3 antibodies.

Supp. Fig. 3 **Costa *et al.***



Supplementary Figure 3. Cytokine expression in CD4$^+$ splenocytes from infected and non-infected mice. Splenocyte suspensions were gated according to side and forward scatter and singlets were selected. Cells were stained with anti-CD4, -IL-4, -IFNγ and -IL-10 antibodies **(A)**. Cytokine expression was represented as Median Fluorescence Intensity (MFI) in CD4$^+$ cells from infected and non-infected mice in the presence or absence of DT-treatment to deplete MGL2$^+$ cells **(B)**.

6 Relationship between liver damage, haematological and circulating leukocyte parameters caused by *F. hepatica* infection in cattle

This section details the findings related to objective 3.

The main results of this part 1 of the chapter are contained in the attached publication (Art 3).

Costa, M., Saravia, A., Ubios, D., Lores, P., da Costa, V., Festari, M. F., Landeira, M., Rodriguez -Zraquia, S., Banchero, G. & Freire, T. (2022). Liver function markers and haematological dynamics during acute and chronic phases of experimental *Fasciola hepatica* infection in cattle treated with triclabendazole. *Experimental Parasitology*, *238*, 108285.

6.1 Liver function markers and haematological dynamics during acute and chronic phases of experimental *F. hepatica* infection in cattle treated with triclabendazole

This study investigates the relationship between hepatic dysfunction, hematological and circulating leukocyte parameters, and liver damage caused by *F. hepatica* in cattle in different stages of infection. This paper was published in the Experimental Parasitology Journal in May of 2022.

In the experimental model of infection in cattle, fasciolosis can be divided into two phases: acute and chronic. Acute fasciolosis is generally characterized by the presence of immature flukes in the liver that destroy hepatic parenchyma and cause hemorrhage and extensive liver damage (Mazeri et al., 2016). The chronic phase of fasciolosis occurs 12 weeks post-infection (wpi). Once in the bile ducts, flukes become sexually mature (allowing egg production and shedding), extensively ingest blood, damage the mucosa, and cause cirrhosis, anemia, and hypoproteinaemia(Mazeri et al., 2016; M. A. Taylor et al., 2015).

Fasciolosis can be treated with anthelmintic drugs. However, this type of treatment does not prevent liver damage caused by the parasite or reinfection (Kelley et al., 2016). One of the most used fasciolicide drugs is triclabendazole (TCZ), a benzimidazole derivative whose mechanism of action involves disruption of the parasite tegument and causes severe damage to the reproductive system of *Fasciola* spp. (Coyne et al., 2020; Fairweather et al., 2020). It is the only chemical drug

that kills juvenile, early immature, and adult *F. hepatica.* Unfortunately, reports of drug resistance are increasing nowadays (Fairweather et al., 2020).

Several studies have evaluated the effectiveness of TCZ against *F. hepatica*. The efficacy was determined by analyzing the fecal egg count reduction after treatment but did not include any correlation with liver damage (Brockwell et al., 2013; Mooney et al., 2009; Romero et al., 2019). However, it is important to note that FEC is not necessarily correlated with the liver fluke burden (Braun et al., 1995; Hutchinson et al., 2009). The measurement of liver protein, AST, ALT, and GGT levels in the sera serves as a means for the indirect assessment of the condition of the liver. Still, it is not normally used in routine clinical practice in rural settings.

The pathological characterization of *F. hepatica* in naturally infected cattle is challenging because parasites constantly infect animals, and the effects of the disease are deduced from the clinical signs of the animals or the liver damage at slaughter. Thus, more research is needed regarding the correlation between hepatic function, treatment with TCZ, and the long-term effects of infection.

This part of our work mainly aimed to investigate the relationship between haematological parameters and liver function markers in acute and chronic phases of *F. hepatica* infection and evaluate TCZ treatment's effects on chronically infected cattle. As described below, our findings showed elevated levels of serum AST and GGT in the early stages of the experimental infection.

Additionally, we found that high levels of circulating eosinophil count and plateletcrit (PCT) were directly correlated with the number of flukes present in the livers of infected cattle. Despite reducing parasite burden and liver damage in cattle during the chronic phase of infection, the TCZ treatment was not completely effective.

6.1.1 Results

6.1.1.1 Characterization of acute and chronic stages of F. hepatica infection

To study and characterize the local and physiopathological effects during both acute and chronic *F. hepatica* infection in cattle, we experimentally infected steers with 500 metacercariaee per animal. The steers were divided into three groups of 12 animals: i) infected, ii) infected and TCZ-treated, and iii) control. After 115 dpi, the second group of animals was treated with TCZ (12 mg/kg, Fasimec®, Novartis) (Figure 1).

The infected animals showed increased egg counts per gram (EPG) at day 87, although this increase was significantly higher after only 115 dpi (Figure 2A). As expected, the non-infected animals did not display detectable EPG in feces during the whole experiment (Figure 2A). Furthermore, the rise in EPG in animals infected with *F. hepatica* fluctuated during the infection. Between days 115 and 193, the EPG levels in fecal samples were consistently high. However, at day 213, there was a decrease in EPG levels, although they remained significantly higher than those of control steers (as shown in Figure 2A).

The livers of infected animals analyzed at slaughter showed a varying number of recovered flukes, with an average of 89 flukes per liver in infected steers (Figure 2B). However, only 5 to 40% of flukes were recovered in infected animals to the initial number of *metacercariaee* that were inoculated in them (Figure 2C). Non-infected cattle did not show any flukes in their livers (as seen in Figure 2B).

Last, the number of recovered flukes was significantly associated with EPG on the day of slaughter (at 213 dpi) (Figure 2D) and with liver damage (Figure 2E). The livers from infected animals were characterized (Figure 2F and G) by a high degree of fibrosis, right lobe hypertrophy, hyperplasia of bile ducts and a pale color compared to livers from control animals (Figure 2H and I and Table 1).

6.1.1.2 Liver function during the infection

To evaluate the liver function of infected animals, we analyzed albumin and total protein production ratio, bilirubin, and transaminase activity in the sera of infected and control steers. The infection decreased albumin/total protein production from 71 to 115 dpi (Fig. 3A). The differences were more apparent when the liver's albumin/total protein production ratio was compared to control animals (Figure 3B).

In both the acute and early chronic stages of the infection, direct bilirubin levels significantly increased compared to non-infected steers, as shown in Figure 3C. It's worth noting that both groups had a statistical difference in bilirubin levels throughout the infection, which could be attributed to seasonal or nutritional changes related to animal maintenance.

To expand the study of the pathological effect induced by *F. hepatica* in bovine livers, we also analyzed AST, ALT, and GGT activities in sera from infected and controlled animals. Figures 3D-F show that infected animals had higher transaminase activity in their serum than control steers. However, the increase was different for the three analyzed enzyme activities. AST activity in sera was increased in infected steers during both acute and chronic phases of infection (between 28 and 157 dpi) compared to control animals (Figure 3D).

On the other hand, ALT activity significantly increased only between 71 and 87 dpi (Figure 3E) Last, GGT activity in sera of infected animals increased at day 71 after infection. However, it increased significantly until animal slaughter (Figure 3F). AST enzymatic activity in serum significantly correlated with fecal EPG and liver fluke recovery (Figure 3G).

6.1.1.3 Characterization of circulating red and white cells from infected cattle

Considering the hematophagous characteristic of the parasite, we examined RBC and hemoglobin levels in groups of infected and non-infected animals. Overall, the number of RBC, total hemoglobin levels (Hb), and hematocrit levels (HCT) remained stable during the infection in steers. However, a significant increase in HCT was observed at 87 dpi (Figure 4A-C), indicating no signs of anemia associated with the parasite infection.

During infection, platelet number (PLT) and plateletcrit (PCT) increased in infected animals, as shown in Figure 4D and E, respectively. However, there was no significant difference in mean platelet volume (MPV) and platelet distribution width (PDW) between infected and control steers (Figure 4F and G). Of note, only the fluke recovery number, was significantly associated with PCT (Figure 4H). These results indicate that platelet increase is related to *F. hepatica* infection, which could mediate liver fibrosis and regeneration (Kurokawa & Ohkohchi, 2017).

The number of circulating leukocytes in steers remained similar during the experimental infection. However, interestingly, control animals increased their number in the acute and early chronic stages of the infection. In contrast, those from infected steers did not change over time (Figure 5A). A similar result was found for blood lymphocytes, which decreased at 49 dpi in infected animals (Figure 5B). After day 87, monocytes and neutrophils increased in the non-infected group of animals while these cells from infected steers remained stable throughout the infection period (Figures 5C and D). Last, granulocyte frequency, such as basophils or eosinophils in circulation, significantly increased upon infection (Figures 5E and F).

Infected animals had two peaks of basophils: one during the acute and one during the chronic phase of infection, while non-infected animals remained stable throughout the study period (Figure 5E). As expected, eosinophils largely increased from 48 dpi, remaining high during both the acute and early chronic phases of the infection (Figure 5F). On the day of slaughter, we found no significant difference in the frequency of eosinophils between infected and control animals (Figure 5F). At 43 dpi, no significant correlation was found between the frequency of circulating eosinophils and fecal EPG. However, a significant positive correlation was observed between eosinophils'

frequency during the infection's acute phase and the number of fluke recoveries in the livers (Figure 5G).

6.1.1.4 Treatment with TCZ does not eliminate hepatic flukes or abrogate liver damage

To assess the impact of TCZ treatment on parasite infection, liver damage, and dysfunction, 12 out of 24 infected steers received treatment during the chronic phase (115 dpi) of *F. hepatica* infection (Table 1). The TCZ treatment induced, as expected, a considerable decrease in fecal EPG from 157 dpi concerning infected animals (Figure 6A), which remained unchanged as the control group until the end of the study.

After being treated with TCZ, the animals infected with *F. hepatica* showed a reduction in fecal EPG by 10-15% compared to their initial EPG count (as shown in Figure 6B). Although TCZ treatment was administered, it did not completely eradicate all flukes in the biliary tracts of the livers. A significant increase in fluke recovery (6-35 flukes) was observed in comparison to control animals, although the number of flukes was significantly reduced compared to non-treated *F. hepatica* steers (32–192 flukes) (Figure 6C and Table 2).

Fluke recovery in infected steers varied from 3 to 38% to the initial inoculation of 500 metacercariae (100%), while TCZ treatment reduced this number to 1–7% (Figure 6D and Table 2). However, the efficiency of TCZ treatment was 5 to 39%, considering the mean of fluke recovery from infected animals (Figure 6E and Table 2).

We created a scoring system to assess the extent and severity of liver damage in steers infected with *F. hepatica* and treated with TCZ. Our scoring system quantifies damage, fibrosis, and lobe hypertrophy. The results showed that infected animals had significantly higher levels of hepatic damage (as shown in Figure 7A) and fibrosis (as shown in Figure 7B) compared to the control group. TCZ-treated infected animals also showed higher liver damage and fibrosis levels than control steers, although they were lower than non-treated animals (Figure 7A and B).

It is worth noting that the treatment with TCZ prevented the increase in liver mass (as shown in Figure 7C) and right hepatic lobe hypertrophy (as shown in Figure 7D) that was caused by the

infection (as shown in Figure 7E and Table 1). Notably, there was a significant correlation between liver damage (Figure 7F) and fibrosis (Figure 7G) with the number of recovered flukes.

We also analyzed the effect of TCZ treatment on hepatic function and circulating leukocytes. Figure 7H shows no significant difference in the albumin/total protein ratio between TCZ-treated and non-treated infected animals. However, only infected animals showed significantly decreased albumin/total protein levels compared with the control group (Figure 7H).

In addition, both GGT and AST activity levels in serum from infected animals remained significantly higher than in TCZ-treated infected and control groups (Figure 7I). Last, no significant changes in circulating neutrophils, monocytes, eosinophils, or basophils were associated with TCZ-treatment of infected animals (Figure 7J). Although TCZ treatment reduced parasite burden and liver damage, it could not eliminate them.

6.1.1.5 Discussion

In our experiment, we found high heterogeneity in EPG and in the recovered flukes from livers in the infected group. However, infections were carefully handled and administration of metacercariaee in capsules was successful. This suggests that the individual genetic background of selected animals might affect parasite survival in the infected animals.

The EPG during the infection process only started to increase significantly after 16 wpi, while it decreased at the time of slaughter (30 wpi). It has been reported that EPG can be detected in cattle from 10 to 11 weeks post-infection (Valero et al., 2011). It is already known that EPG, although used as the standard assay, has a low sensitivity in cattle since it can only detect chronic infection and also because egg shedding is discontinuous (Braun et al., 1995; Castro-Hermida et al., 2021), highlighting the need for alternative diagnostic methods (Mirzadeh et al., 2021). However, it is a cost-effective and simple method that does not require specialized equipment (Mirzadeh et al., 2021; Peters et al., 2021).

Both protein and bilirubin determination in sera allowed the assessment of hepatic dysfunction in protein synthesis and possible cholestasis in the livers of infected animals, especially in the acute (until 10 wpi) and early chronic phases of the infection (from 10 to 16 wpi). However, after 16 wpi, these differences were no longer detected, indicating that liver regeneration can supply adequate protein levels.

At 22 wpi, infected steers increased platelet count and plateletcrit, which may promote liver fibrosis and regeneration during the chronic phase of *F. hepatica* infection. It has been shown that platelets directly promote liver regeneration in hepatocytes, although their role in liver diseases is still discussed (Kurokawa & Ohkohchi, 2017).

Our study also demonstrates that the analysis of different transaminase activities in sera from *F. hepatica*-infected animals reveals different increased kinetic profiles, as already suggested in previous studies (Bulgin et al., 1984; Lotfollahzadeh et al., 2008; Wyckoff 3rd & Bradley, 1985). The different obtained profiles may be related to their organ-specific expression or function according to the hepatic dysfunction or biliary tract obstruction.

According to our study, *F. hepatica* infection was associated with an early increase (at 4 wpi) of AST in sera, suggesting hepatic dysfunction likely due to liver damage in the parenchyma induced by the juvenile flukes, which can be observed up to 6 wpi (Beesley et al., 2018). These results suggest that AST could detect acute stages of infection in experimentally infected cattle. On the other hand, GGT increased levels were detected after 10 wpi and lasted the whole period of infection (up to 30 wpi), indicating cholestasis associated with the chronic phase of infection. A recent report using a transcriptomic approach analyzing immune responses in peripheral blood mononuclear cells of experimentally infected cattle demonstrated that gene pathways for hepatic fibrosis and cholestasis were enriched at chronic stages (Garcia-Campos et al., 2019).

In addition, fasciolosis acquired by natural infection in different mammalian hosts was also associated with an increase in hepatic transaminases, although an association with the stage of the infection was difficult to determine (Jarujareet et al., 2018; Kitila & Megersa, 2014; Purnama et al., 2021). Thus, the use of ALT, AST, and GGT activity levels in plasma to detect natural infection

may be limited, likely due to the coexistence of immature and mature flukes in the livers of subclinical infected cattle.

Eosinophils play an important role in defending against multicellular parasites and in various immune disorders driven by Th2 (Long et al., 2016). The fact that an increase in the frequency of circulating eosinophils was detected in the acute phase of infection (at 4 wpi) and that they were significantly correlated with fluke recovery number at 43 dpi indicates that they would be more useful and reliable for detecting early stages of the experimental infection, together with other hepatic markers.

It is worth noting that the red or white blood counts from steers presented some variations during the analyzed period. However, they remained within reference values regardless of *F. hepatica* infection (Roland et al., 2014). This indicates that animals might be sensitive to other factors independently of parasite infection. Our animals were free grass-fed up to day 110 after the infection and then transported to a feedlot facility where they were intensively fed with high-energy diets. After transport, animals lost some weight. Therefore, both the transportation-induced stress and the nutritional changes during the experiment might have influenced the synthetic liver function and circulating blood and white cells, regardless of parasite infection.

The effectiveness of TCZ treatment has been demonstrated, particularly in the acute phase of fasciolosis, since it would prevent liver damage. Indeed, in recent studies, the efficacy of TCZ was confirmed in cattle (Kouadio et al., 2021). However, the histopathological effects of the parasite on the liver during the chronic stage of the infection were not thoroughly studied. A previous work analyzed the serological and coproantigen ELISA and EPG in cattle experimentally infected with *F. hepatica* until 126 dpi with TCZ treatment. The authors found that steers infected with 500 metacercariaee and treated at 84 dpi did not have detectable EPG or flukes in the liver (Brockwell et al., 2013), although no hepatic lesions were described.

Our findings demonstrate that using TCZ at 115 dpi in steers infected with the same parasitic load significantly reduced parasite burden in the liver. However, it did not eliminate all the flukes in the liver, which continue to cause significant hepatic damage. Thus, the TCZ treatment does not resolve the economic losses due to the confiscation of livers. In fact, condemnation of *Fasciola*-infected bovine livers at slaughter represents a significant loss of income for livestock worldwide (Mas-Coma et al., 2019), including Uruguay (da Costa et al., 2019).

In conclusion, the obtained results provide case-control groups and establishes a stronger association between liver pathological changes and serum biochemical alterations in *F. hepatica* experimental infection in cattle over a long time (30 weeks post-infection) (Zhang et al., 2005; Raadsma et al., 2008).

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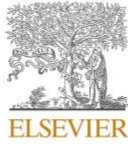

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Liver function markers and haematological dynamics during acute and chronic phases of experimental *Fasciola hepatica* infection in cattle treated with triclabendazole

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ARTICLE INFO

Keywords:
Fasciola hepatica
Fasciolosis
Liver
Fluke
Triclabendazole

ABSTRACT

Fasciola hepatica, a worldwide-distributed liver fluke, is one of the causative agents of fasciolosis, a zoonotic disease that affects livestock and humans. In livestock, fasciolosis causes huge economic losses worldwide, reducing animal fertility, milk production, weight gain and condemnation of livers. In spite of the availability of drugs, such as triclabendazole (TCZ), for the treatment of fasciolosis, they do not necessarily prevent liver damage or parasite reinfection and can eventually increase parasite resistance. The aim of this research was to relate the hepatic function, haematological parameters, leukocyte counts in circulation and parasite egg shedding during *F. hepatica* acute and chronic phases of infection in cattle as well as to determine how these parameters change with TCZ-treatment of chronically infected cattle. Our results show that increased levels of serum aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT) were detected in early stages of the experimental infection. Moreover, high circulating eosinophil count and plateletcrit levels were correlated with fluke number in livers from infected cattle. On the other hand, although TCZ-treatment in the chronic phase of infection reduced parasite burden and damage in the liver, it was not able to completely avoid them. In conclusion, our work sheds light into the physiopathological mechanisms induced during fluke infection in cattle, revealing the complexity of the host response to the infection, together with the effects of TCZ-treatment in chronically infected animals.

1. Introduction

Fasciola hepatica, a worldwide-distributed liver fluke, is one of the causative agents of fasciolosis, a zoonotic disease that affects livestock and humans (Mas-Coma et al., 2019). Indeed, the World Health Organization estimates that around 17 million people are infected in more than 70 countries worldwide (Cwiklinski et al., 2016; Howell and Williams, 2020). Furthermore, fasciolosis in livestock causes huge economical losses of approximately 3 billion dollars per year (Mas-Coma et al., 2019; Borgsteede et al., 2005; Beesley et al., 2018) due to reduced fertility and milk production, prevalence of secondary infections, reduced weight gain and condemnation of livers caused by chronic infections (Nonga et al., 2009; Howell et al., 2015; Ezatpour et al., 2015). In cattle, *F. hepatica* causes liver damage due to immature fluke migration and obstruction of the bile ducts by adult parasites (Mas-Coma et al., 2019; Beesley et al., 2018; Olaechea et al., 2013). A recent review on fasciolosis epidemiology has reported its prevalence in ruminant species, with up to 91% in cattle some regions of Africa and South America (Mehmood et al., 2017). In Uruguay, the overall prevalence of bovine fasciolosis in abattoirs was recently reported to be about 34%, while in some territories of the country it increases up to 55%, implicating considerable economic losses considering only liver confiscation

Abbreviations: TCZ, Triclabendazole; dpi: days post-infection, wpi: weeks post-infection.

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https://doi.org/10.1016/j.exppara.2022.108285
Received 13 September 2021; Received in revised form 24 May 2022; Accepted 27 May 2022
Available online 30 May 2022
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(da Costa et al., 2019). Furthermore, this report also concluded that *F. hepatica* infection in Uruguayan cattle is associated with poorer carcass quality parameters and lower weights at slaughter, and the effect on weight differs across age ranges (da Costa et al., 2019). The life cycle of *F. hepatica* is complex as the parasite goes through multiple stages before reaching its adult form and includes an intermediate host, a mud snail of the *Lymnaea* genus, and a definitive host, for instance livestock or humans (Howell and Williams, 2020; Beesley et al., 2018; Moazeni and Ahmadi, 2016). After ingestion of metacercariae, the infective form of *F. hepatica*, by the mammalian host, excystation occurs and the newly excysted juvenile flukes penetrate the host's intestine wall and reach the liver between 4 and 6 days. Juvenile flukes then burrow through the liver parenchyma for up to 6 weeks and damage considerable amount of tissue (Beesley et al., 2018). Eventually, the flukes reach the bile ducts, where they become sexually mature and start to shed eggs from 12 weeks post-infection (wpi) (Howell and Williams, 2020), although these characteristics can change depending on the mammalian host taking up to 30 weeks for the parasite to complete its life cycle (Moazeni and Ahmadi, 2016; Mazeri et al., 2016). Acute fasciolosis is generally characterized by the presence of immature flukes in the liver that destroy hepatic parenchyma and cause haemorrhage, extensive liver damage with fibrinous deposits on the capsule (Mazeri et al., 2016). Then flukes enter the bile ducts. Chronic phase of fasciolosis occurs when flukes, once inside the bile ducts, extensively ingest blood, damage the mucosa and cause cirrhosis, anaemia and hypoproteinaemia (Mazeri et al., 2016; Taylor et al., 2007). The damaged bile ducts become enlarged, or even cystic, and have thickened, fibrosed walls and, in cattle, they are usually calcified (Mazeri et al., 2016; Taylor et al., 2007). Chronic infection in cattle is usually analysed after 12 and up to 14 wpi (Niedziela et al., 2021; Garcia-Campos et al., 2019).

Triclabendazole (TCZ), a benzimidazole derivative, is usually used for the treatment of fasciolosis. However, this drug does not necessarily prevent liver damage induced by the parasite or parasite reinfection (Kelley et al., 2016). Furthermore, although several studies have assessed the efficacy of TCZ against *F. hepatica*, it was determined by assessing post-treatment reduction in the faecal egg count (Mooney et al., 2009; Romero et al., 2019; Brockwell et al., 2013), a parameter that is not necessarily correlated with the liver fluke burden (Braun et al., 1995; Hutchinson et al., 2009). Indeed, the detection of parasite egg shedding in faeces does not reflect parasite infection, since parasite eggs cannot be detected during the long prepatent period of 11–12 weeks post-infection and their shedding is discontinuous (Braun et al., 1995). Interestingly, a coproantigen ELISA test was demonstrated to be more sensitive than faecal egg count in experimental-infected cattle (Brockwell et al., 2013). Also, this study analysed the TCZ treatment on experimentally-infected cattle although no correlation with liver damage was included (Brockwell et al., 2013). Furthermore, while the detection of specific antibodies is more sensitive and sooner than coproantigen ELISA test, they persist after treatment with TCZ, while both coproantigen ELISA and faecal egg counts returned to negative status (Brockwell et al., 2013). In fact, the antibody response to infection is frequently associated with exposure to the infection and may not be useful for the diagnosis of natural *F. hepatica* active infection (Walsh et al., 2021; Jayraw et al., 2009). Last, reports of resistance to TCZ are increasing (Fairweather et al., 2020), evidencing one of the major drawbacks nowadays in the treatment of this disease.

The characterization of the pathological events induced by *F. hepatica* infections turns out to be difficult when working in naturally infected cattle, because animals are permanently challenged with parasite ingestion and the effects of the infection are deduced from the clinical signs of the animals or the liver damage at slaughter. Different works have been carried out to characterize damage caused by the parasite in the livers both in naturally and experimentally infected cattle (Garcia-Campos et al., 2019; Brockwell et al., 2013; Walsh et al., 2021; Jayraw et al., 2009; Lotfollahzadeh et al., 2008; Bulgin et al., 1984; Wyckoff and Bradley, 1985; Clery et al., 1996; DAS Baldissera et al., 2017; Kitila and Megersa, 2014), although correlations with hepatic function and TCZ-treatment for long periods after infection are scarce. The present work relates hepatic dysfunction, together with haematological and circulating leukocyte parameters with liver damage caused by *F. hepatica* in cattle through both the acute and chronic phases of the infection during 30 wpi and to determine how these parameters change with TCZ-treatment of chronically infected cattle.

2. Materials and methods

2.1. Parasite experimental infection

Animal handling and experiments were carried out in accordance with strict guidelines and regulations from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, http://www.cnea.gub.uy/, National Law 18.611, Uruguay), according with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All procedures involving animals were approved by INIA's Committee on Animal Research (CNEA Protocol Number: 0009/11). Eighteen-to twenty-four-month-old male Aberdeen Angus steers (mean body weight 421.88 ± 17,7 kg) were used in this experiment. Animals were negative to *F. hepatica* exposure determined by the sedimentation technique (Giovanoli Evack et al., 2020) in three previous consecutive tests (every 4–5 weeks) during 4 months before experimental infections. The animals were drenched on arrival with Dectomax® 1% (Doramectin 1 g/100 ml) with the recommended dose (1 ml/50 Kg body weight) and remained free of gastrointestinal parasites (as determined by the modified McMaster technique (Escribano et al., 2019) until *F. hepatica* experimental infection. Steers were kept outdoors and fed with high-quality pasture and water *ad libitum* at the experimental station of INIA La Estanzuela, Colonia, Uruguay. Animals were divided into three groups of 12 animals according to matched age and weight: i) infected, ii) infected and TCZ-treated, and iii) control groups (Fig. 1). Experimental infections (n = 24) were carried out with 500 TCZ-sensitive metacercariae (Ridgeway Laboratories, England) per animal, spread in saline solution, inserted into gelatine capsules (Torpac®) and delivered orally using a dosing gun. As control, non-infected steers (n = 12) were maintained under the same conditions of infected animals during the experiment in a separate space. After 115 days post-infection (dpi), the second group of animals was treated with TCZ (12 mg/kg, Fasimec®, Novartis) according to the recommendations of the drug supplier laboratory. One week after, animals were transported and fed with high-energy diets at the intensive animal farming facility (feedlot) of Marfrig Group in Fray Bentos, Río Negro, Uruguay. Establishments where animals were kept were free of snails. At day 213 post-infection animals were transported to an abattoir (Marfrig S. A. Tacuarembó) and sample collection, liver examination and fluke recovery were carried out (Fig. 1).

2.2. Collection of samples

Animals were bled for complete hemogram analysis before the infection (day 0) and after 43, 87, 157 and 213 dpi. Faecal and blood samples were collected before (day 0) and at 15, 28, 43, 59, 71 and 87 dpi (every approximately 15 days) and at 115, 157, 193 and 213 dpi (every approximately 30 days) during the acute and chronic phases of infection, respectively (Fig. 1). *F. hepatica* egg counts per gram (EPG) in faeces were determined individually using the sedimentation technique (Giovanoli Evack et al., 2020) before the infection (day 0) and at 15, 28, 43, 59, 71, 87, 115, 157, 193 and 213 dpi. Chronic infection was determined after 15 weeks of parasite infection.

2.3. Fluke recovery and liver damage

Livers, bile ducts and gallbladders were examined for the presence of

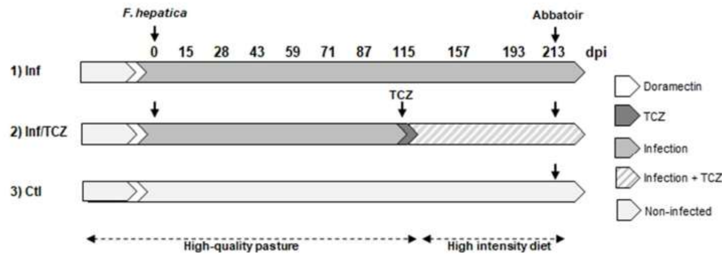

Fig. 1. *F. hepatica* experimental infection timeline. Groups of 12 Aberdeen Angus steers were used per group. Animals from all groups were drenched with Doramectin to treat gastrointestinal parasite infection. Group 1 and 2 were infected with 500 *F. hepatica* metacercariae, while group 3 (control) remained non-infected. Animals were fed with high-quality pasture until day 122. At day 115 post-infection animals from group 2 were treated with TCZ. At 122 dpi animals were transported and fed with high-energy diet at the intensive animal farming facility until slaughter at the abattoir.

F. hepatica parasites as previously described (Roberts et al., 1997). Fluke recovery was determined by the number of adult flukes from each processed liver. Livers were weighed and given a score according to the macroscopic liver damage (ranging from 0 to 3) at the time of dissection by a veterinary pathologist, where 0 represented absence of tissue necrosis and liver damage, 1 represented less than 30% (slight), 2 between 30 and 70% (moderate), and 3, more than 70% (severe) of liver necrosis and damage at the tissue surface. Fibrosis, capsule, consistency, calcification in the biliary ducts and abscesses, as well as atrophy of hepatic lobes were also determined by a similar score: 0 (absence), 1 (slight), 2 (moderate) and 3 (severe) (Marcos et al., 2007).

2.4. Hemogram and circulating leukocyte counting

Blood samples were processed for assessment of haematocrit mean corpuscular volume (MCV) and mean platelet volume (MPV) using the Counter 19 from Weiner lab. Leukocyte total counts were determined in a microscope using a Neubauer Haemocytometer. Thin smears were prepared on individually labelled microscope slides using one or two drops of blood. Smears were air-dried, fixed with absolute methanol, and stained with Giemsa to analyse leukocyte and lymphocyte counts. Sera were collected for the quantification of transaminase activity levels.

2.5. Hepatic synthetic functions and transaminase determination

Serum levels of albumin, total protein and hepatic enzyme activity were determined using an automatized spectrophotometer (Dimension RxL Max integrated chemistry system; Siemens). Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Gamma glutamyltransferase (GGT) activities were expressed as international units per litre (IU/l), Total Bilirubin (TBil) and Direct Bilirubin (DBil) determinations were expressed in mg/dl and Albumin (ALB) and Total Protein (TP) determinations were expressed in g/dl.

2.6. Statistical analysis

Results were analysed using GraphPad Prism software 6.0 (GraphPad Software, San Diego, CA) by non-parametric (with Krustal-Wallis test) or parametric one- or two-way ANOVA followed by the Tukey test for multiple comparisons, according to the experiment. Asterisks represent statistically significant differences as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

3. Results

3.1. Characterization of acute and chronic stages of F. hepatica experimental infection in steers

To study and characterise the local and physiopathological effects during both acute and chronic *F. hepatica* infection in cattle, we experimentally infected steers with 500 *F. hepatica* metacercariae per animal. After experimental infection, animals showed an increase in faecal EPG from day 87, although this increase was significantly higher only after 115 dpi (Fig. 2A). As expected, non-infected animals did not display detectable EPG in faeces during the whole experiment (Fig. 2A). Interestingly, the increase in EPG in *F. hepatica*-infected animals varied during the infection period: sustained faecal EPG were constant between days 115 and 193, while they decreased at day 213, although they remained significantly increased with respect to control steers (Fig. 2A).

The number of recovered flukes in the livers from infected animals analysed at slaughter was heterogeneous, with a mean of 89 flukes per liver from infected steers, while non-infected cattle did not show any flukes in livers (Fig. 2B). Moreover, only between 5 and 40% of flukes were recovered in infected animals with respect to the initial number of metacercariae inoculated in these animals (Fig. 2C). Last, the number of recovered flukes was significantly associated with faecal EPG at day of slaughter (Fig. 2D). Fluke recovery number was also significantly correlated with liver damage (Fig. 2E).

Last, livers from infected animals were characterized (Fig. 2F and G) by a high degree of fibrosis, right lobe hypertrophy, hyperplasia of bile ducts and a pale colour comparing to livers from control animals (Fig. 2H and I, and Table 1).

3.2. Increased levels of serum AST and GGT are detected in early stages of infection

To analyse the hepatic synthetic function of infected animals, we studied the production ratio between albumin and total protein, bilirubin, and different transaminase activities in sera from infected and control steers. Infection was associated with a decrease of albumin/total protein production from 71 to 115 dpi (Fig. 3A). These differences became clearer when the albumin/total protein production ratio by the liver was normalized to control animals (Fig. 3B). Direct bilirubin levels were significantly increased both in the acute and early chronic stage of the infection in relation with non-infected steers (Fig. 3C). Of note, a statistical difference was detected on bilirubin levels in both groups along the infection, probably due to seasonal or nutritional changes related with the maintenance of animals.

In order to expand the study of the pathological effect induced by *F. hepatica* in bovine livers, we also analysed AST, ALT and GGT activities in sera from infected and control animals. As indicated in Fig. 3D–F, an increase of the three studied enzyme activities in sera of infected animals was detected in relation to sera from control steers. However, the increase was different for the three analysed enzyme activities. AST activity in sera was increased in infected steers both in the acute and chronic phases of infection (between 28 and 157 dpi) with regard to control animals (Fig. 3D). On the other hand, ALT activity significantly increased only between 71 and 87 dpi (Fig. 3E). Last, GGT activity in sera of infected animals increased at day 71 after infection, as ALT, although it continued significantly increased until animal slaughter (Fig. 3F). AST enzymatic activities in serum significantly correlated with both faecal EPG and fluke recovery in livers (Fig. 3G).

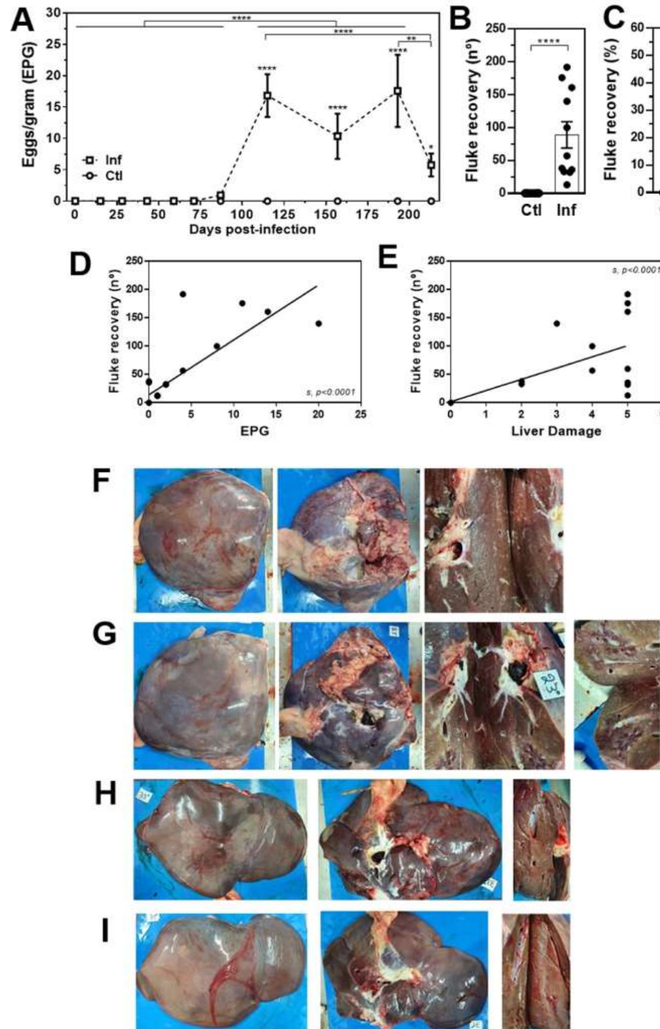

Fig. 2. Fluke recovery from *F. hepatica* -infected animals significantly correlates with faecal parasite eggs/gram (EPG). A) EPG in faeces from infected (squares) and non-infected animals (circles). Animals were orally infected with 500 metacercariae and EPG were determined by the sedimentation technique. Only the infected group and control groups are shown (n = 12). B–C) Fluke recovery from infected cattle at time of sacrifice both in number of flukes (B) or percentage in relation to the initial number of metacercariae used to infect animals (C). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test (A) or non-parametric one-way Anova with Krustal-Wallis test (B–C): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. D-E) Significant linear regression correlation between fluke recovery and EPG (D) and liver damage (E). F-G) Representative livers from infected that had 13 (F) and 57 (G) flukes in the liver. H–I) Representative livers from control animals.

Table 1
Macroscopic characteristics of livers from experimentally infected steers.

Groups	Score	LD	F	RLH	LLA	HBD	RB	CT	Cal	Con
INF	**0**	0 (0)	0 (0)	2 (17)	0 (0)	0 (0)	2 (17)	2 (17)	1 (8)	1 (8)
	1	1 (8)	1 (8)	0 (0)	3 (25)	2 (17)	0 (0)	4 (33)	1 (8)	0 (0)
	2	2 (17)	3 (25)	6 (50)	4 (33)	1 (8)	8 (67)	6 (50)	4 (33)	3 (25)
	3	9 (75)	8 (67)	4 (33)	5 (42)	9 (75)	2 (17)	0 (0)	6 (50)	8 (67)
INF /TCZ	**0**	0 (0)	2 (17)	8 (67)	0 (0)	1 (8)	7 (58)	7 (58)	5 (42)	5 (42)
	1	9 (75)	1 (8)	1 (8)	1 (8)	5 (42)	2 (17)	3 (25)	2 (17)	1 (8)
	2	2 (17)	6 (50)	3 (25)	7 (58)	4 (33)	2 (17)	2 (17)	3 (25)	3 (25)
	3	1 (8)	3 (25)	0 (0)	4 (33)	2 (17)	1 (8)	0 (0)	2 (17)	3 (25)
Control	**0**	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The number of animals together with the % (between brackets) is indicated for each score: n (%). LD: liver damage; F: fibrosis; RLH: right lobe hypertrophy; LLA: left lobe atrophy; HBD: Hyperplastic bile ducts; RB: round borders; CT: Capsule thickening; Cal: calcification; Con: consistency.

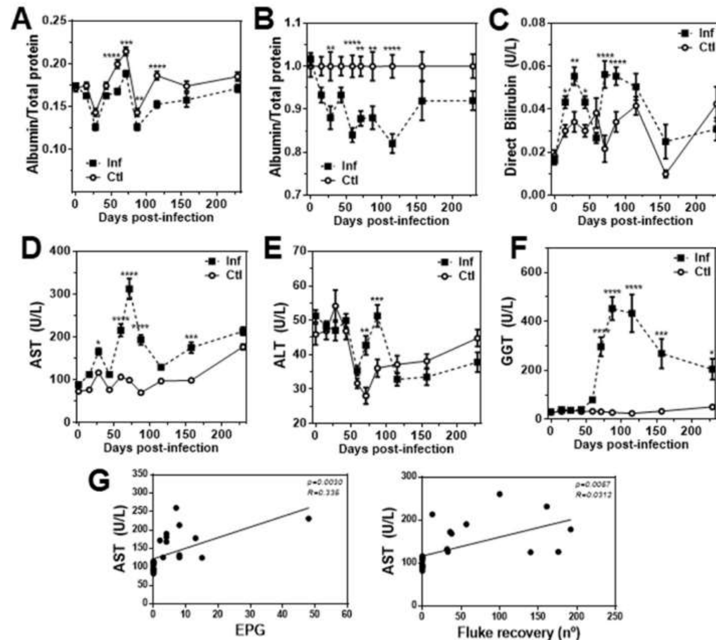

Fig. 3. Infected animals present a compromised liver function and hepatic damage. A) Albumin and total protein ratio determined in U/L in sera of infected and control cattle. B) Normalised albumin and total protein ratio in regard to the control group of infected and control cattle. C) Direct Bilirubin detection in U/L of infected and control cattle. D) aspartate transaminase (AST) serum levels. E) Alanine transaminase. (ALT) serum levels. F) Gamma-glutamyl transferase (GGT) serum levels. Only the infected group and control groups are shown (n = 12). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. G) Significant linear regression correlation between AST levels and EPG (left) at day 157 post-infection or fluke recovery (right).

3.3. High circulating eosinophil counts are correlated with fluke number in livers from infected cattle

Considering the hematophagous characteristic of the parasite, we analysed red blood cells and haemoglobin from infected and control groups of animals. From a general point of view red blood cell number (RBC), total haemoglobin (Hb) and haematocrit (HCT) remained constant during the infection in steers, although a significant increase in HCT was detected at 87 dpi (Fig. 4A–C), revealing no signs of parasite associated anaemia. Other related parameters such as mean platelet volume (MPV) or mean corpuscular volume (MCV) of red blood cells did not change during the course of the infection (not shown). On the other hand, platelet number (PLT) and plateletcrit (PCT) increased in infected animals during the course of infection (Fig. 4D and E, respectively), while mean platelet volume (MPV) and platelet distribution width (PDW) did not show any significant difference between infected and control steers (Fig. 4F and G, respectively). Of note, only fluke recovery number, and not faecal EPG, was significantly associated with PCT (Fig. 4H). These results indicate that an increase in platelets is related with *F. hepatica* infection, which could trigger liver fibrosis and regeneration (Kurokawa and Ohkohchi, 2017).

Steers presented similar levels of circulating leukocytes during experimental *F. hepatica* infection, although, interestingly, in the acute and early chronic stages of the infection control animals increased their number while those from infected steers did not change over time (Fig. 5A). A similar result was found for blood lymphocytes, which decreased at 49 dpi in infected steers (Fig. 5B). On the other hand, monocytes and neutrophils augmented after day 87 in the non-infected group of animals, while infected steers remained stable throughout the whole infection period (Fig. 5C and D, respectively). Last, granulocyte frequency, such as basophils or eosinophils in circulation, significantly increased upon infection. With regard to basophils, infected animals presented two picks: one at the acute and the other at the chronic phases of infection, while non infected animals remained unchanged during the period of study (Fig. 5E). As expected, eosinophils largely increased from 48 dpi remaining high during both the acute and early chronic phases of the infection (Fig. 5F). Nevertheless, we could not detect any difference between the frequency of eosinophils from infected and control animals at day of slaughter (Fig. 5F). On the other hand, although no significant correlation between the frequency of circulating eosinophils and faecal EPG was detected at 43 dpi, there was a significant positive correlation between the frequency of eosinophils at the acute phase of the infection and fluke recovery number in livers (Fig. 5G).

3.4. Treatment with TCZ does not completely eliminate hepatic flukes or abrogates liver damage

In order to evaluate the effect of a commonly used anti-helminth drug on parasite infection as well as on liver damage and dysfunction, 12 out of the 24 infected steers were treated with TCZ in the chronic phase of *F. hepatica* infection (at 115 dpi) (see Table 1). The TCZ-treatment induced, as expected, a considerable decrease in faecal EPG from 157 dpi with regard to infected animals (Fig. 6A), which remained unchanged as the control group until the end of the study. TCZ-treated *F. hepatica* infected animals showed a reduction between 10 and 15% of faecal EPG in comparison with the initial number of EPG before TCZ treatment (Fig. 6B). However, TCZ-treatment of infected steers did not completely eliminate all flukes in the biliary tracts of livers since a significant increase in fluke recovery (6–35 flukes) was determined in comparison to control animals, although their number was significantly reduced compared to the non-treated *F. hepatica* steers (32–192 flukes) (Fig. 6C and Table 2). Fluke recovery in infected steers varied from 3 to 38% in relation to the initial inoculation of 500 metacercariae (100%), while TCZ-treatment of infected animals reduced this number to 1–7% (Fig. 6D and Table 2). However, the efficiency of TCZ-treatment was from 5 to 39% taking into account the mean of fluke recovery from infected animals (Fig. 6E and Table 2).

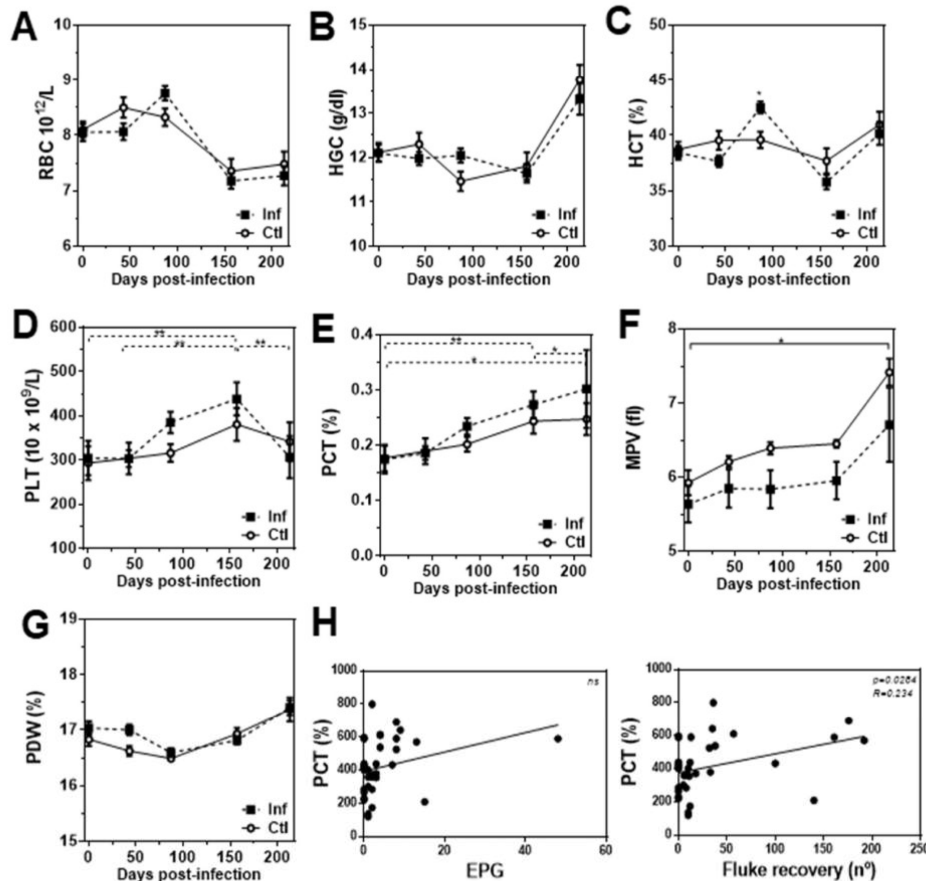

Fig. 4. Infected animals do not present any signs of anaemia associated with *F. hepatica* infection while platelet number and plateletcrit increase during *F. hepatica* infection. A-C) Red blood cells (RBC, A), total haemoglobin (Hb, B) and haematocrit (HCT, C) were determined using an automated counter in infected and control groups of steers. E-G) Platelet (PLT, D), Plateletcrit (PCT, E), Mean Platelet Volume (MPV, F) and Platelet distribution width (PDW, G) were determined using an automated counter in infected (black squares) and control groups (open circles) of animals. Dotted and continuous lines indicate significant differences between different time points in the infected and control groups, respectively. Only the infected group and control groups are shown (n = 12). Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. H) Significant linear regression correlation between PCT (%) and EPG (left) at day 157 post-infection or fluke recovery (right).

In order to analyse the type and degree of liver lesions associated with TCZ-treatment of *F. hepatica*-infected steers, we developed a score to quantify damage, fibrosis and lobe hypertrophy, as explained above. Infected animals showed significantly higher hepatic damage (Fig. 7A) and fibrosis (Fig. 7B) than controls. TCZ-treated infected animals also showed higher levels of liver damage and fibrosis than control steers, although they were lower than non-treated animals (Fig. 7A and B). Of note, TCZ-treatment completely abrogated the increase of liver mass (Fig. 7C) and right hepatic lobe hypertrophy (Fig. 7D) induced by the infection (Fig. 7E and Table 1). Interestingly, both liver damage (Fig. 7F) and fibrosis (Fig. 7G) were significantly correlated with the number of recovered flukes.

Last, we analysed how the hepatic function and circulating leukocytes were affected by TCZ-treatment of infected animals. As shown in Fig. 7H, TCZ-treated animals did not show any significant differences in the albumin/total protein ratio in comparison with non-treated infected animals. However, only infected animals showed a significant decrease in albumin/total protein levels compared with the control group (Fig. 7H). In addition, both GGT and AST activity levels in serum from infected animals remained significantly higher than TCZ-treated infected and control groups (Fig. 7I). Finally, no significant changes in circulating neutrophils, monocytes, eosinophils or basophils were associated with TCZ-treatment of infected animals (Fig. 7J). Altogether these results indicate that, although TCZ-treatment reduces parasite burden and damage in the liver while it is not able, however, to completely avoid them.

4. Discussion

This work deeply characterized liver function markers. haematological and circulating leukocyte dynamics in 30 weeks of an experimental *F. hepatica* infection in steers, together with the impact of TCZ-

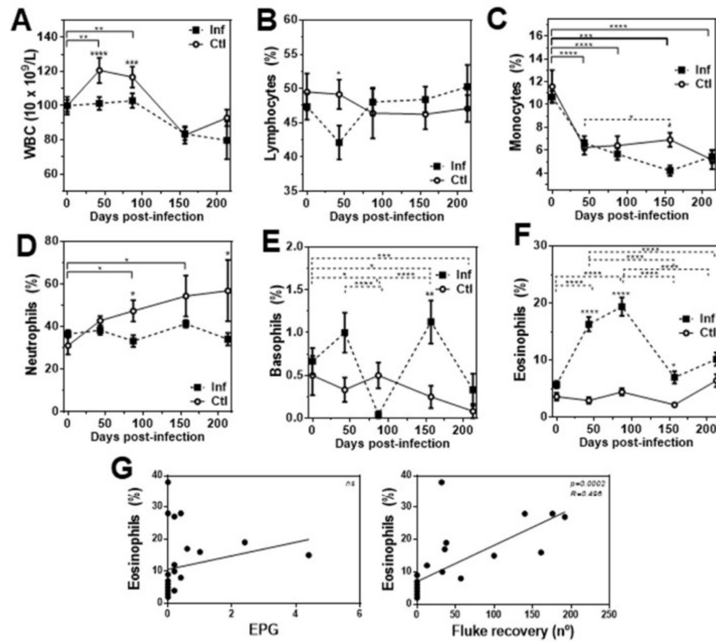

Fig. 5. ***F. hepatica*** **infection is associated with a decrease in white blood cells but an increment in eosinophils.** White blood cells (WBC, A), lymphocytes (B), monocytes (C), neutrophils (D), basophils (E) and eosinophils (F) in blood were detected in blood smears prepared on individually labelled microscope slides and stained with Giemsa. Dotted and continuous lines indicate significant differences between different time points in the infected and control groups, respectively. Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. G) Linear regression correlation between eosinophils (%) and EPG (left) at 43 dpi or fluke recovery (right).

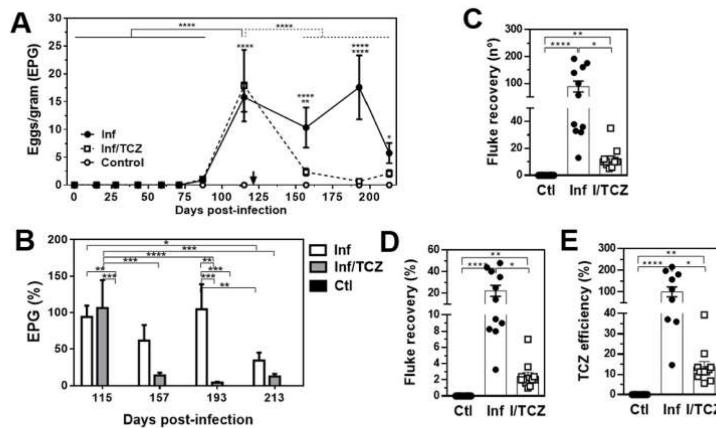

Fig. 6. Treatment with TCZ decreases fluke recovery although does not completely eliminate hepatic damage. A) EPG from infected (close squares), TCZ-treated infected (open squares) and non-infected animals (open circles). Animals were orally infected with 500 metacercariae and EPG were determined by the sedimentation technique. The arrow indicates the moment of TCZ administration (115 dpi). B) EPG percentage from infected cattle with or without treatment with TCZ. Number of recovered flukes from livers of infected cattle with or without TCZ-treatment at time of sacrifice (213 dpi). D) Percentage of recovered flukes considering the initial number of metacercariae administrated as 100% (500). E) TCZ efficiency calculated with respect to non-treated infected animals. The mean of recovered flukes from infected animals was considered as 100% (88.1 flukes). Infected group, TCZ-treated/infected animals and control groups are shown (n = 12/group). Asterisks indicate statistically significant differences between infected and control animals calculated with non-parametric two-way Anova followed by a Tukey multiple comparison test (A and B) or non-parametric one-way Anova with Krustal-Wallis test (C–E): *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

treatment in liver damage during the course of chronic experimental parasite infection.

Faecal EPG from infected animals varied during the infection process and only started to significantly increase after 16 wpi, while it decreased at time of slaughter (30 wpi). It is already known that faecal EPG, although used as the gold standard assay, has a low sensitivity in cattle since it can only detect patent infection and also because egg shedding is discontinuous (Braun et al., 1995; Castro-Hermida et al., 2021). However, it is cost effective, simple and does not require specialized instrumentation (Mirzadeh et al., 2021; Peters et al., 2021). Surprisingly, previous reports in cattle indicate that faecal EPG can be detected from 10 to 11 wpi (Valero et al., 2011), much earlier than what it was found in our experimental infection (16 wpi). We also found great heterogeneity in EPG and recovered flukes from livers in the infected group, although infections were carefully handled and administration of metacercariae in capsules was successful. Thus, altogether, these facts

Table 2
Fluke recovery from *F. hepatica*-infected steers with or without TCZ-treatment.

	Fluke recovery (n°)	Fluke recovery (%)	Fluke recovery (%) for TCZ efficiency[a]
F. hepatica	88.1 (32-192)	17.8 (3-38)	100 (15-198)
F. hepatica + TCZ	12.3 (6-35)	2.5 (1-7)	13.8 (5-39)

[a] To calculate TCZ efficiency the n° of flukes (88.1, column 1) was considered as 100% (column 3).

highlight the drawbacks of coprological methods for fasciolosis diagnosis (Mirzadeh et al., 2021) and the need of alternative diagnostic methods. Indeed, an integral diagnostic can be more reliable, especially those that combine both coprological and immunological methods including antigen detection and serological assays (Mirzadeh et al., 2021; Mas-Coma et al., 2005; Kuerpick et al., 2013).

The heterogeneity found in faecal EPG was also correlated with fluke recovery from livers from infected animals demonstrating that individual genetic background of selected animals and/or the experimental infection procedure might affect parasite survival in the infected animals. This fact is relevant, considering that we analysed an experimental infection with minimal variability, as compared to natural infections, in which cattle might be permanently challenged with metacercariae present in the pastures, and where acute or chronic phase of the disease is difficult to determine since immature versus mature fluke coexist in cattle (Kitila and Megersa, 2014).

Both protein and bilirubin determination in sera allowed the assessment of hepatic dysfunction in protein synthesis and possible cholestasis in the livers of infected animals, especially in the acute (until 10 wpi) and early chronic phases of the infection (from 10 to 16 wpi). However, these differences were not detected after 16 wpi, indicating that liver regeneration can take place to supply adequate protein levels. Interestingly, infected-steers presented an increase in both platelet number and plateletcrit at 22 wpi which could be promoting liver fibrosis and regeneration in the chronic phase of *F. hepatica* infection. Indeed, it has already been demonstrated that platelets improve liver regeneration directly on hepatocytes, although their role in liver diseases is still controversial (Kurokawa and Ohkohchi, 2017).

This study also demonstrates that the analysis of different transaminase activities in sera from *F. hepatica*-infected animals reveal

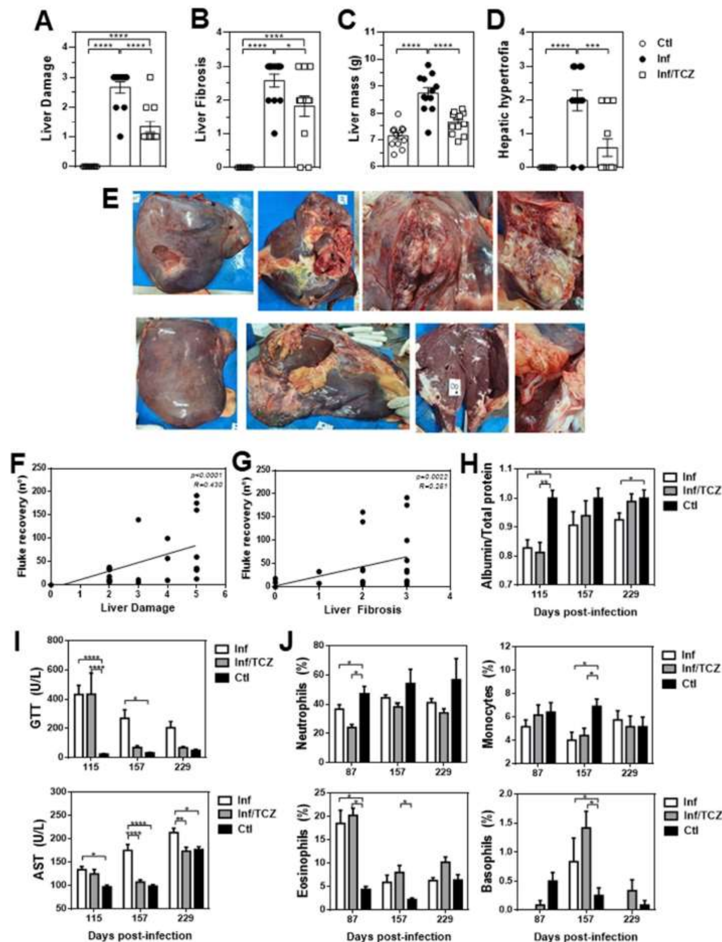

Fig. 7. TCZ-treated cattle present liver damage and liver fibrosis, which correlates with fluke recovery. Liver damage (A), fibrosis (B) and right lobe hypertrophy (C) was determined by a veterinary anatomopathologist anatomic. Livers were also weighted (D). Significant linear regression correlation between fluke recovery and liver damage and EPG (E) or fibrosis (F) of both infected and infected/TCZ treated groups. G) Albumin and total protein ratio determined in U/L in sera from *F. hepatica* infected animals with or without TCZ-treatment and control steers at 115, 157 and 213 dpi. H) Gamma-glutamyl transferase (GGT) and aspartate transaminase (AST) plasmatic levels. Percentage of neutrophils, monocytes, basophils and eosinophils (I) in blood at 115, 157 and 213 dpi were detected in blood smears prepared on individually labelled microscope slides and stained with Giemsa. Infected group, TCZ-treated/infected animals and control groups are shown (n = 12/group). Asterisks indicate statistically significant differences between infected and control animals calculated with one-way Anova (A–D) or two-way Anova followed by a Tukey multiple comparison tests (G–I): *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. J-K) Representative livers from TCZ-treated infected animals that had 10 (J) and 11 (K) flukes in the liver.

different increased kinetic profiles, as already suggested in previous studies (Lotfollahzadeh et al., 2008; Bulgin et al., 1984; Wyckoff and Bradley, 1985). The different profile obtained for the studied transaminases may be related with their organ-specific expression or function according to the hepatic dysfunction or biliary tract obstruction. In fact, ALT is the only enzyme produced by hepatocytes, being more specific than AST and GGT and results to be a very specific marker of hepatocellular injury (Lala et al., 2021). However, its expression can fluctuate during the day and according to the level of strenuous exercise (Lala et al., 2021). This latter fact might explain the differences obtained for both infected- and control groups of animals during the studied period of time. On the other hand, AST and GGT can be produced by other organs, such as the kidney, apart from the liver. Nevertheless, AST usually rises in conjunction with ALT to indicate hepatocellular injury, while high serum GGT activity suggests biliary tract obstruction (cholestasis) (Lala et al., 2021). Furthermore, this study indicates that *F. hepatica* infection was associated with an early increase (at 4 wpi) of AST in sera, while both GGT and ALT increased levels were detected after 10 wpi suggesting hepatic dysfunction likely due to liver damage in the parenchyma induced by the juvenile flukes, which can be observed up to 6 wpi (Beesley et al., 2018). On the other hand, increased levels of GGT were detected after 10 wpi and lasted the whole period of infection (up to 30 wpi), indicating cholestasis associated with the chronic phase of infection. Importantly, a recent report using a transcriptomic approach analysing immune responses in peripheral blood mononuclear cells of experimentally infected cattle demonstrated that gene pathways for hepatic fibrosis and cholestasis were enriched at chronic stages (Garcia-Campos et al., 2019). Moreover, the hepatic damage of experimental infected cattle was associated with excessive reactive oxygen species production (Das et al., 2017). Altogether, these results suggest that AST could be used to detect acute stages of infection in experimental infected-cattle, which turns out to be at least as earlier as antigen detection or serological tests. These last methods are capable of diagnosing fasciolosis between 2 and 4 wpi (Walsh et al., 2021; Mirzadeh et al., 2021; Mas-Coma et al., 2014; Calvani et al., 2017) or 2 wpi, respectively (Mirzadeh et al., 2021; Mas-Coma et al., 2014; Sarkari and Khabisi, 2017). In addition, fasciolosis acquired by natural infection in different mammalian hosts was also associated with increase of hepatic transaminases, although an association with the stage of the infection was difficult to determine (Kitila and Megersa, 2014; Purnama et al., 2021; Afshan et al., 2020; Jarujareet et al., 2018). Thus, the use of ALT, AST and GGT activity levels in plasma to detect natural infection may be limited likely due to the coexistence of immature and mature flukes in the livers of subclinical infected cattle.

Eosinophils participate mainly in the defence against multicellular parasites and in several Th2-driven immune disorders (Long et al., 2016). The classical functions of eosinophils include mainly degranulation triggered by antibodies in a mechanism known as antibody-dependent cell cytotoxicity (Long et al., 2016; Makepeace et al., 2012). Their functions have been well characterized in helminth infections, and in particular, our group has recently demonstrated that they play a protective role during experimental *F. hepatica* infection in mice (Frigerio et al., 2020). The fact that an increase in the frequency of circulating eosinophils was detected in the acute phase of infection (at 4 wpi) and that they were significantly correlated with fluke recovery number, but not with EPG, at 43 dpi, indicates that they would be more useful and reliable to detect early stages of the experimental infection, together with other hepatic markers. It also confirms the fact that EPG is a late and likely a non-confident technique to follow infection, as it has been previously reported (Arifin et al., 2016; Duscher et al., 2011). Thus, our experimental study provides case-control groups and establishes a better association between liver pathological changes and serum biochemical alterations in *F. hepatica* experimental infection in cattle during a long period of time (30 wpi) (Zhang et al., 2005; Raadsma et al., 2008). However, immunological studies including humoral and cellular immune response elicited by *F. hepatica* should be further investigated to complement the circulating leukocyte population dynamics performed in this work.

It is worth noting that steers, regardless of *F. hepatica* infection, presented some variations in some of the red or white blood counts during the analysed period of time, although they remained in general between values of reference (Roland et al., 2014). This indicates that animals might be sensitive to other factors, independently of parasite infection. These may include seasonal or nutritional changes, variation in the distribution of eggs within a single faecal specimen, daily fluctuations of faecal production and consistency in the host (Valero et al., 2011; Mas-Coma et al., 2014; Roland et al., 2014), animal age or other factors influenced by the environment shared by the animals. Indeed, steers were free grass-fed up to day 110 after the infection and then transported to a feedlot facility where they were intensively feed with high energy diets. After transport, animals lost some weight (not shown). Therefore, both the transportation-induced stress as well as the nutritional changes during the experiment might have influenced the synthetic liver function and circulating blood and white cells, regardless of parasite infection.

TCZ is one of the antihelminthics for the treatment of fasciolosis, with a mechanism of action that involves disruption of the parasite tegument and causes severe damage to the reproductive system of *Fasciola* spp. (Fairweather et al., 2020; Coyne et al., 2020). Although effective, the increase of TCZ-resistant flukes in different parts of the world is an important drawback (Kelley et al., 2016; Fairweather et al., 2020; Kouadio et al., 2021). TCZ-treatment has shown to be effective, especially during the acute phase of fasciolosis, since it would prevent liver damage. Indeed, in recent studies the efficacy of TCZ was confirmed in cattle (Kouadio et al., 2021) but mainly in sheep and goats (Romero et al., 2019; Novobilsky et al., 2016; Shrimali et al., 2016), although the histopathological effects caused by the parasite in the liver were not studied in depth during the chronic stage of the infection. Indeed, one previous work analysed the serological and coproantigen ELISA and EPG in cattle experimentally infected with *F. hepatica* until 126 dpi with TCZ treatment, although no hepatic lesions were described (Brockwell et al., 2013). Interestingly, the authors found that steers infected with 500 metacercarie and treated at 84 dpi did not have detectable EPG or flukes in the liver (Brockwell et al., 2013). However, our results strikingly demonstrate that the administration of TCZ at 115 dpi in steers infected with 500 TCZ-sensitive metacercariae, although significantly reduced parasite burden in the liver, did not kill all the flukes in the liver, a fact that was accompanied by considerable hepatic damage. However, it remains to be determined whether TCZ treatment was associated with the presence of immature flukes in the livers of infected animals. Of note, animals treated with TCZ did not show any detectable faecal parasite eggs, likely due to the low sensitivity of the technique. An important issue is that in our study steers were maintained in snail-free establishments, preventing the parasite to continue its life cycle in the intermediate host. Thus, TCZ-treatment in the chronic phase of infection was associated with significant hepatic damage and would not resolve the economic losses due to confiscation of livers. In fact, condemnation of *Fasciola*-infected bovine livers at slaughter represents a significant loss of income for livestock in the world (Mas-Coma et al., 2019), including Uruguay (da Costa et al., 2019).

5. Conclusions

In conclusion, our work sheds light into the physiopathological parameters associated to both acute and chronic phases of fluke experimental infection in cattle, revealing the complexity of the host response to the infection, together with the effects of TCZ-treatments in chronically infected animals.

Declaration of interest

The authors declare no conflict of interests.

Animal welfare statement

Animal experimentation was carried out according to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences.

Author contributions

Monique Costa: Investigation, data curation and analyses, original draft of the manuscript; Anderson Saravia: Biological sample collection and parasitology and reviewing the manuscript; Diego Ubios: Animal care and biological sample collection; Pablo Lores, Valeria da Costa, María Florencia Festari, Mercedes Landeira and Santiago A. Rodríguez-Zraquia: data curation and analyses and reviewing the manuscript; Georgget Banchero: Conceptualization and reviewing the manuscript. Teresa Freire: Conceptualization, experiment design and supervision, analyses of data and writing, review and editing the manuscript.

Acknowledgements

We are highly grateful to Prof. Franklin Riet-Correa and Benjamin Doncel for his help and advice. We thank Marfrig Abattoir located in Río Negro and Tacuarembó, Uruguay. Financial supports were provided by Programa de Desarrollo de Ciencias Básicas (PEDECIBA), Agencia Nacional de Investigación e Innovación (SNI-ANII and FCE_1_2019_1_156295) to Teresa Freire.

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7 Characterization of the immune response in *F. hepatica*-infected cattle

This section details the findings related to objective 4.

The present chapter studies the cellular and humoral immune response of *F. hepatica* in experimentally infected cattle. We show unpublished results that complement the data discussed in the previous section.

Although the characterization of the immune modulation induced by the parasite in ruminants is scarcer than in rodents, the literature indicates an attenuation of the immune system with and an enhancement of the regulatory components (Byrne et al., 2019; Naranjo Lucena et al., 2017), generating a deficit in the immune response, likely increasing the risk of second infections. Most of the work has been carried out in sheep. It describes the immune response during the early stages of experimental fasciolosis or in naturally infected cattle in which the acute phase cannot be differentiated from the chronic infection. Results suggest that *F. hepatica* induces a mixed Th1/Th2 response during the acute phase, characterized by increased cytokines such as IFN-γ, IL-4, IL-10, and TGF-β (Ryan et al., 2020). In the chronic phase, there is an amplification of the Th2 response, which is associated with the suppression of Th1 (Flores-Velázquez et al., 2023).

However, there are currently no research reports on how the treatment of fasciolosis can affect the immunity of animals. Thus, the main aim of this part of this work was to investigate the humoral and cellular immune response generated in cattle by *F. hepatica* at different stages of the infection and to evaluate the effect on the immune response of the TCZ treatment.

7.1 Effects of *F. hepatica* on the cellular immune response in the acute phase of infection

To evaluate the systemic cellular immune response, we analyzed cell suspensions from PBMC by flow cytometry. Viable singlet cells previously incubated with different antibodies to identify the cell population were analyzed: CD4, a specific marker of helper T cells; CD21 to stain B cells; WC1 to identify γδ T cells; CD11c to identify DC. The gating strategy used to analyze each population is shown in the superior part of each Figure.

We conducted an analysis of two groups, the infected group and the control group, on three different occasions, days 0, 28, and 43 after infection. At 213 dpi, we analyzed three groups: infected, infected with TCZ treatment, and control. In this section, we will concentrate on day 43 post-infection, where we observed more significant differences.

Our results show an increase in the percentage of $CD4^+$ and $CD21^+$ in infected animals at 43 dpi (Figure 7-1A). The increase in $CD4^+$ cells can be attributed to their importance in activating various immune cells, including macrophages, eosinophils, and B cells, among others (Riollet et al., 2002.). On the other hand, the increase in $CD21^+$ cells coincide with the onset of specific antibody response to *F. hepatica* (results in the next section). In contrast, no changes in the percentage of $WC1^+$ cells were found at different stages of the infection among the groups. Last, an increase in the percentage of the $CD11c^+$ cell population was found between the control and infected groups, although this increase was not statistically significant (Figure 7-1D).

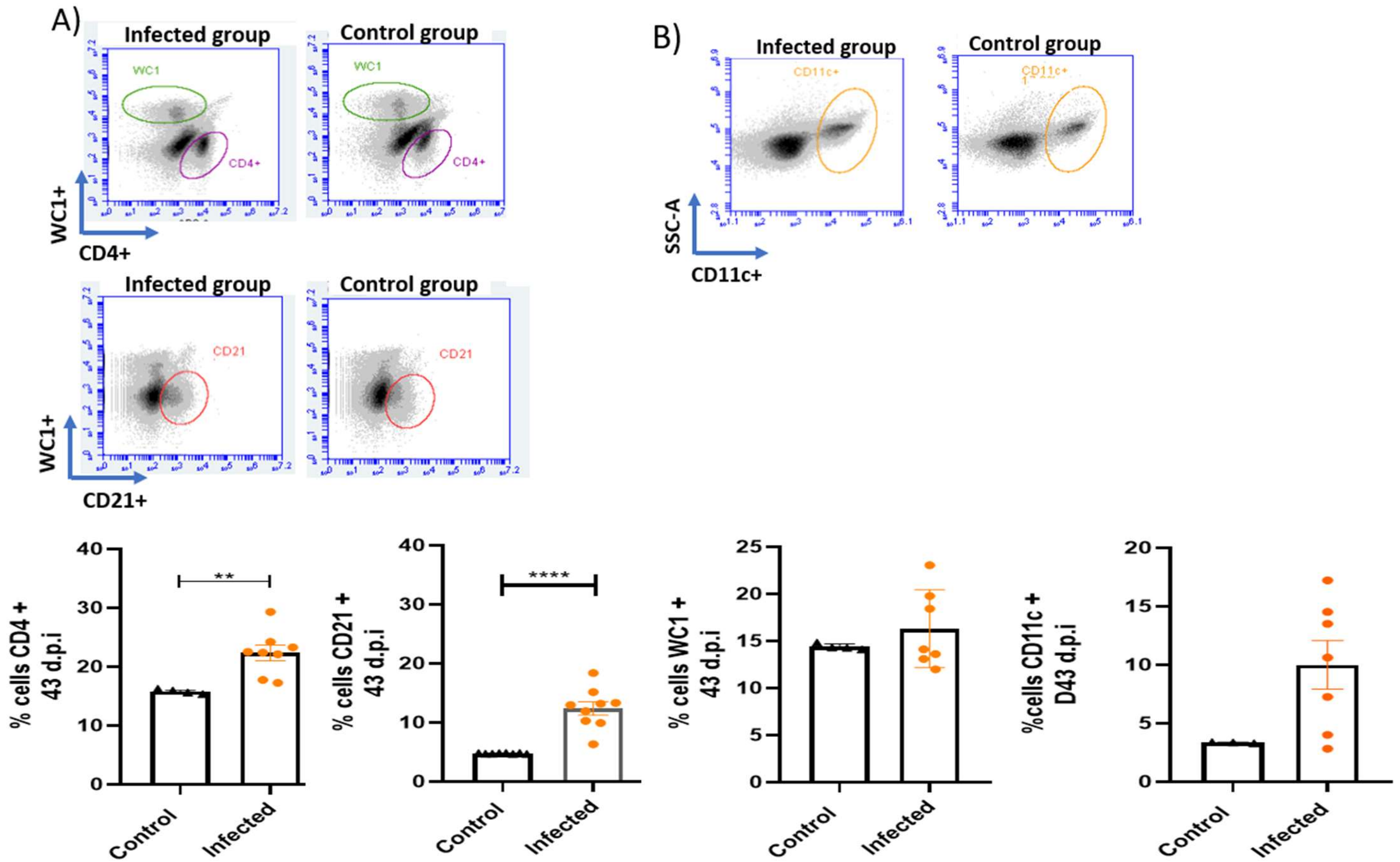


Figure 7–1. Identification of different cell populations in PBMCs in the acute phase of infection. Flow cytometry was performed in PBMC of infected and control animals at day 43 dpi. A) Percentage of $CD4^{+}$, $CD21^{+}$, $WC1^{+}$, and of $CD11c^{+}$ cells. Asterisks indicate significant differences (**** p<0.0001, *** p<0.0005, ** p< 0.005) performed by Unpaired T-test performed in GraphPad Prism version 6.04.

7.2 Specific humoral response against *F. hepatica*

To evaluate the immune response in cattle, we first analyzed the humoral response against *F. hepatica* during the experimental infection by measuring both total IgG and specific IgG against *F. hepatica.* During fasciolosis, the IgG antibodies can be found in sera and in the bile of infected animals, indicating their importance in developing tissue immunity against the parasite (Hughes et al., 1981).

We evaluated total IgG in sera using a sandwich ELISA. No significant differences were found between the groups (Figure 7-2A) at 0, 43, 87, and 213 dpi. On day 43, all animals presented a increase in the levels of total antibodies, independent of the infection. Environmental factors, such as nutritional conditions and stress, could explain this increase. Several studies indicate that other infections in cattle or pre- and post-partum changes affect the levels of this IgG in fluids (Harmon et al., 1976; Herr et al., 2011; Nielsen et al., 1978). However, no studies on total IgG in blood were previously reported for *F. hepatica* infection.

Parasite-specific IgG levels in sera were detected by an in-house ELISA using FhTE-coated plates. Figure 7-2B shows an increase of specific IgG from 28 and 43 dpi in both infected groups, coinciding with the increase of circulating B cells (Figure 7-1A). The antibody levels stayed elevated until 87 dpi. However, during the chronic phase of infection at 157 dpi, the specific IgG levels decreased in both infected groups, suggesting that once the flukes are inside the bile ducts, they escape the host immune system.

We also evaluated the avidity of specific IgG against *F. hepatica*. The term "avidity" describes the combined strength of multiple non-covalent binding interactions between antigenic epitopes and the antigen-binding sites of specific antibodies (Holec-Gąsior & Sołowińska, 2022). It is described that the affinity of antibody immune response typically increases progressively after immunization (Holec-Gąsior & Sołowińska, 2022).

Currently, many protocols are based mainly on a measurement of IgG avidity for the distinction between recent and past infection (Holec-Gąsior & Sołowińska, 2022). The avidity index can differentiate the acute or chronic stage in humans affected by fasciolosis. In our results (Figure 7-2C), throughout the infection, the avidity index remained elevated without differentiating the stage of the disease in the infected group. However, the avidity index substantially decreased in animals

treated with TCZ, suggesting that antibody avidity is reduced in cattle upon parasite elimination or reduction (Figure 7-2D).

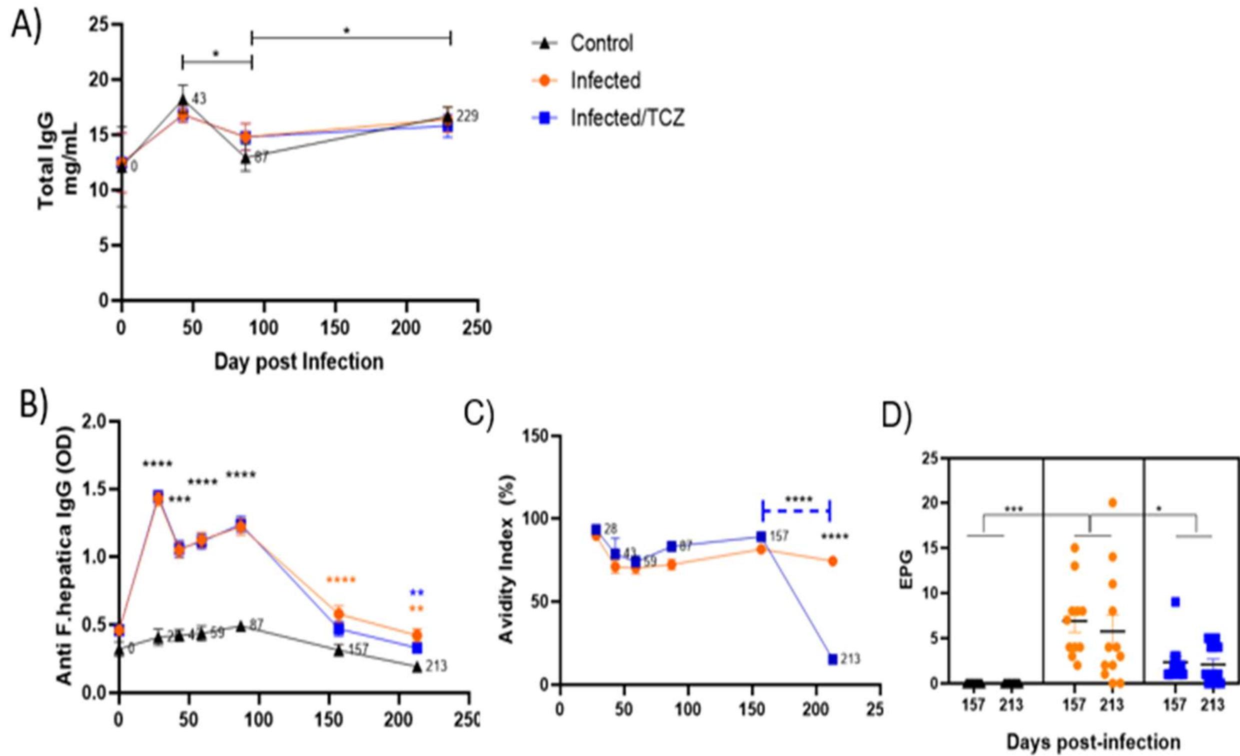


Figure 7–2. Humoral immune response during *F. hepatica* infection. A) total IgG in sera of infected (orange), Infected and TCZ-treated (blue), and control (black) animals measured by sandwich ELISA. B) Specific IgG anti-*F. hepatica* measured by indirect ELISA. C) Avidity index of specific IgG anti-*F. hepatica* determined by indirect ELISA in the presence of 7 M UREA. D) EPG in the chronic phase of infection. Asterisks indicate significant differences (**** $p<0.0001$, *** $p<0.0005$, ** $p< 0.005$, * $p<0,05$) performed by Mixed- effects analysis (A,and B), Two-way ANOVA (C), and Nested One-Way ANOVA (D) performed in

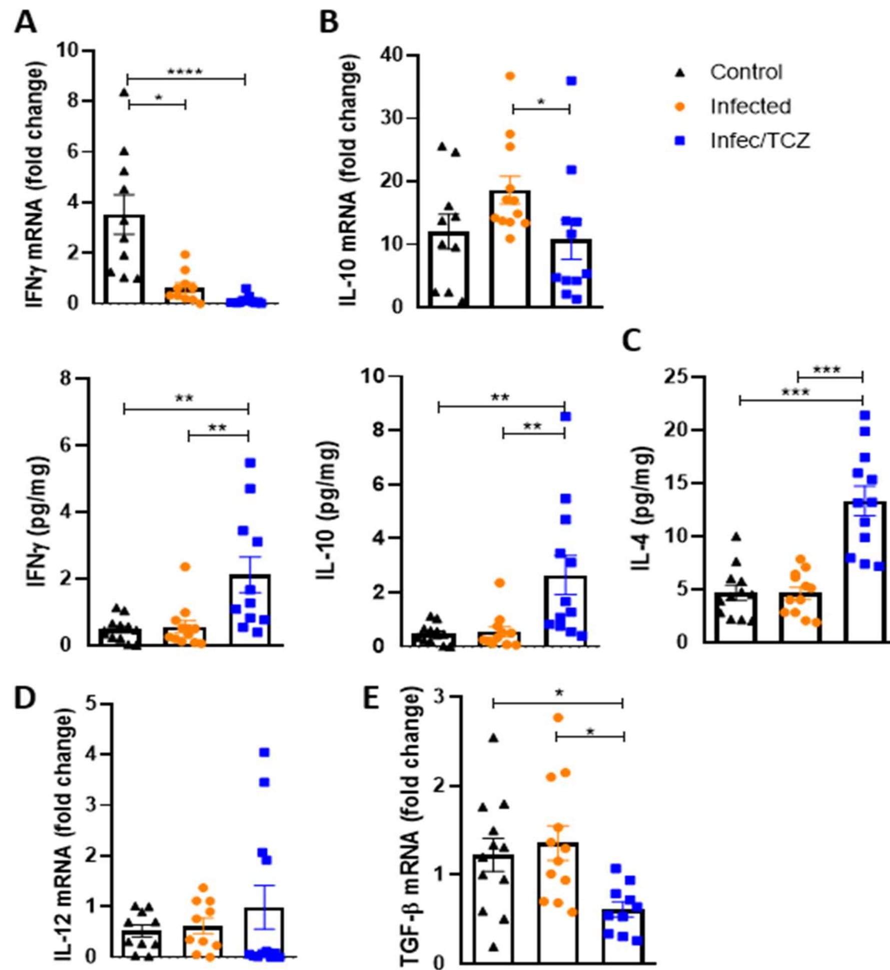
7.3 Cytokine production during *F. hepatica* infection

7.3.1 TCZ treatment in the chronic phase alters the cytokine profile in the liver and spleen of infected animals

To evaluate the cytokine production in organs, we analyzed the gene expression of IFN-γ, IL-10, IL-12, and TGF-β by qRT-PCR at the endpoint of the experiment. In addition, we performed a sandwich ELISA to quantify the IFN-γ, IL-10, and IL-4 in the liver and spleen.

q-RTPCR indicated a remarkable decrease in IFN-γ in the liver of both infected groups compared to the control (Figure 7-3A). However, a different result was observed by ELISA, where the TCZ/infected group showed significantly higher IFN-γ levels than the control and infected animals. In addition, significantly lower levels of IL-10 were found in the TCZ-treated group by qPCR. Nevertheless, once again, the livers from this group of animals presented higher IL-10 levels evaluated by ELISA (Figure 7-3B).

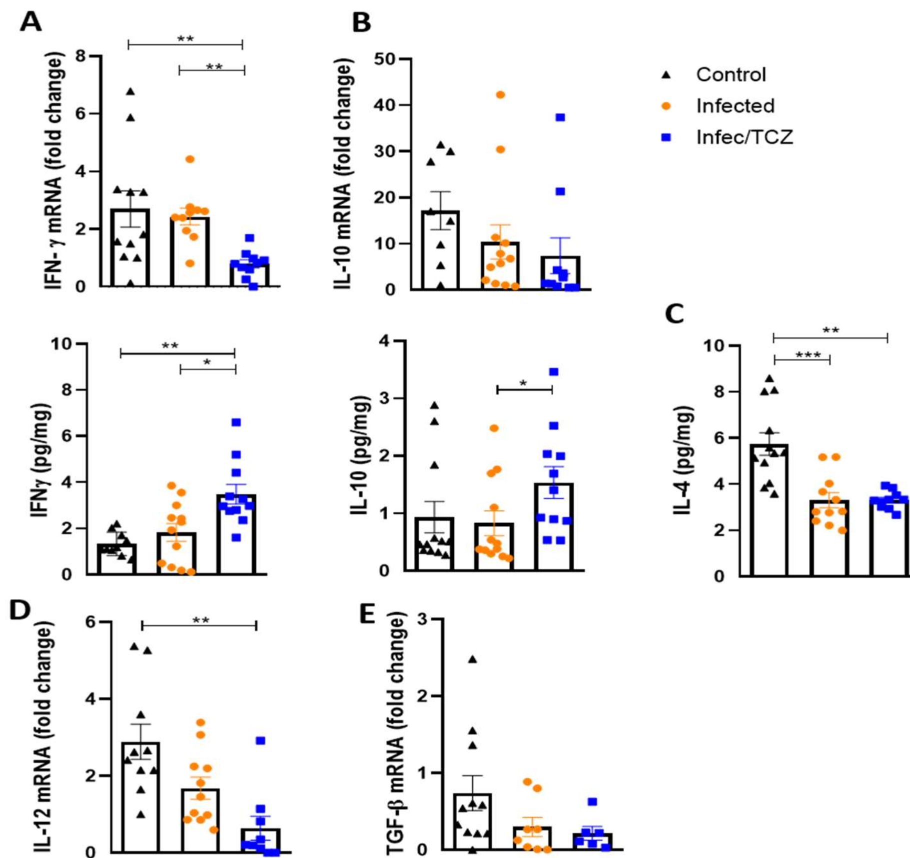
Figure 7-3C shows an increase in IL-4 production, detected by ELISA, in the group infected and treated with TCZ. No significant changes in IL-12 gene expression were observed (Figure 7-3D), whereas TGF-β expression was reduced in the TCZ-treated group (Figure 7-3E).



Figure–7–3. Cytokine production in liver. A) IFN- γ levels measured by qRT-PCR (upper plot) and ELISA (lower plot) in infected (orange), infected and TCZ-treated (blue), and control (black) animals. B) IL-10 levels measured by qRT-PCR (upper plot) and ELISA (lower plot). C) IL-4 levels measured by ELISA. D) IL-12 levels measured by qRT-PCR. E) TGF-β levels measured by qRT-PCR. Asterisks indicate significant differences (**** p<0.0001, *** p<0.0005, ** p< 0.005, * p<0,05) performed by One-Way ANOVA (D) in GraphPad Prism version 6.04.

Next, we analyzed cytokine levels in spleens The gene expression of IFN-γ was significantly reduced in the spleen of the TCZ-treated group compared to the liver (Figure 7-4A). However, the ELISA results indicated significantly higher levels in the spleens of the TCZ/infected group than in the control and infected animals. A similar result was obtained for IL-10 (Figure 7-4B).

In Figure 7-4B, the ELISA test results show significantly higher levels of IL-10 in the spleen of the infected/TCZ group compared to the infected group. Meanwhile, a decrease in IL-4 production was observed in the spleen of the infected groups (Figure 7-4C). A decrease in the gene expression of IL-12 was detected (Figure 7-4D) in the TCZ group, while no significant changes in TGF- β were detected (Figure 7-4E).



Figure–7–4 Cytokine production in spleen. A) IFN- γ levels measured by qRT-PCR (upper plot) and ELISA (lower plot) in infected (orange), infected and TCZ-treated (blue), and control (black) animals. B) IL-10 levels measured by qRT-PCR (upper plot) and ELISA (lower plot). C) IL-4 levels measured by ELISA. D) IL-12 levels measured by qRT-PCR. E) TGF-β levels measured by qRT-PCR. Asterisks indicate significant differences (**** p<0.0001, *** p<0.0005, ** p< 0.005, * p<0,05) performed by One-Way ANOVA (D) in GraphPad Prism version 6.04.

7.4 General conclusions

To summarize this chapter, it is worth noting that days 28 and 43 are important in *F. hepatica* infection. These days coincide with the parasite's migration through the liver parenchyma, which causes most of the pathology related to fasciolosis (Hoyle & Taylor, 2003).

Specifically, in our experiment, on day 43, there was an increase in $CD4^+$ and $CD21^+$ cell populations (as shown in Figure 7-1) that could be associated with increased parasite-specific antibodies (as shown in Figure 7-2B). During the chronic phase of *F. hepatica* infection in cattle, the TCZ treatment significantly impacts various aspects of the immune response. The treatment reduces the quantity and quality of specific antibodies against the parasite. However, it increases the levels of IFN-γ, IL-10 and IL-4 in the liver, which may be due to the promotion of liver tissue recovery.

8 Impact of *F. hepatica* infection on the immunity induced by vaccines

This section details the findings related to objectives 5.

Animal production relies on key pillars, including genetics, nutrition, management, and animal health. Animal health can be addressed through curative or preventive measures. The curative approach usually produces losses, while the prophylactic approach prevents them.

One commonly used measure is vaccination to prevent illness in a herd. In Uruguay, vaccines are normally used in cattle's first stage of life, mainly against *Clostridium* spp., bacterial anthrax, and respiratory diseases. Our country's only mandatory vaccination is against FMDV. The cattle population is systematically vaccinated twice a year and given to animals younger than two years (M. Costa et al., 2024).

F. hepatica infection increases the susceptibility to secondary infections, such as *Escherichia coli*, *Mycobacterium bovis*, *Salmonella dublin*, *Clostridium haemolyticum* and *Clostridium novy* (Byrne et al., 2019; Garza-Cuartero et al., 2014; Howell et al., 2018; Naranjo Lucena et al., 2017). Furthermore, *F. hepatica* infection in mice can reduce the effectiveness of a bacterial pathogen vaccine (Brady, O'Neill, et al., 1999). However, in cattle, there is only one published report on this topic, which the immunity induced by a respiratory vaccine in calves infected with *F. hepatica* (Krump et al., 2014). The authors did not find significant differences, but in this work, they worked with a younger population and used a lower dosage of *metacercariae* in the experimental infection than our conditions.

In this part of the thesis, we examined the impact of experimental fasciolosis on adult animals and the adaptive immune response generated through vaccination. During the experiment, we assessed the effectiveness of three different vaccines. The first two vaccines, FMDV and *Clostridium* spp, were administered in the first year of life and evaluated during the acute phase of infection. The third vaccine was a respiratory vaccine, against *P. multocida* and *M. haemolytica*, applied during the chronic phase of infection, as shown in Figure 8-1.

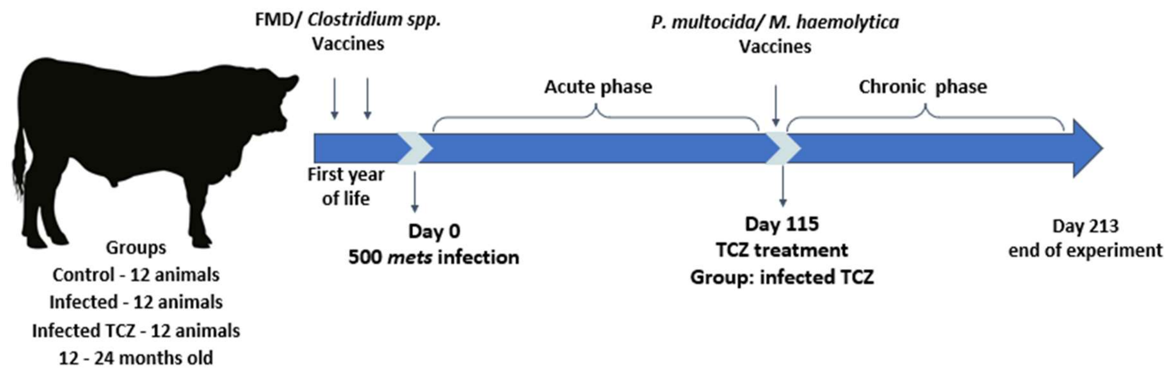


Figure 8–1. Experimental design to evaluated the impact of *F. hepatica* infection in different vaccines administered in cattle.

8.1 Acute fasciolosis impacts on the antibody immune response induced by the *Clostridium* spp vaccine

Clostridium spp is a very important Gram-positive anaerobic bacteria that causes severe economic losses in livestock. It is a causative agent of gas gangrene, necrotic enteritis, enterotoxaemia, black disease, and blackleg (Khiav & Zahmatkesh, 2021). The vaccination is one of the most effective methods to prevent this disease.

We evaluated the humoral immunity induced by the vaccine against *Clostridium* spp during the acute phase of *F. hepatica* infection. All animals were vaccinated against *Clostridium* spp during their first year of life, between 6 and 12 months before the experimental infection with *F. hepatica*.

To evaluate the humoral immune response generated by the vaccination, we developed an in-house ELISA to detect specific IgG against *Clostridium* spp in sera during the acute phase of infection, from 0 to 87 dpi. We found that infected animals presented a significant decrease in IgG levels against *Clostridium* spp. from 43 dpi. However, no significant differences were found at 87 dpi with respect to day 0 (Figure 8-2).

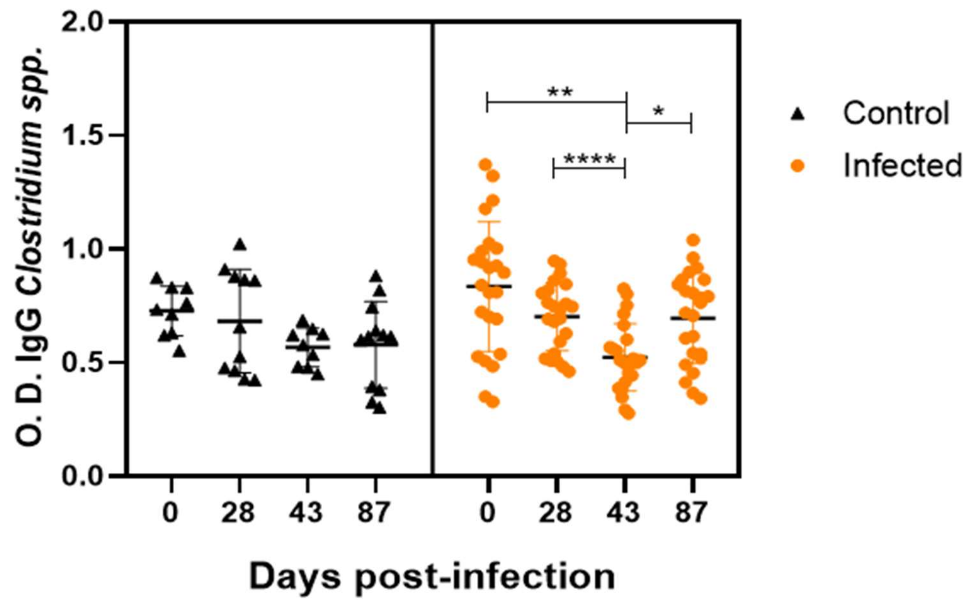


Figure 8–2. IgG levels against *Clostridium* spp. IgGs were detected by an indirect ELISA at 43 dpi in the control (black) and infected groups (orange). Asterisks indicate significant differences (**** p<0.0001, ** p< 0.005, * p<0,05) performed by Two-Way ANOVA in GraphPad Prism version 6.04.

8.2 Chronic fasciolosis impacts on the antibody immune response induced by bacterial respiratory vaccines

Bovine respiratory disease can be attributed to many pathogens and represents an important concern for all livestock categories (Callan & Garry, 2002). All animals used in our study were vaccinated against *P. multocida* and *M. haemolytica* during the chronic phase of the infection at 115 dpi. This date was chosen because animals had to be compulsory vaccinated at the entrance to the feedlot.

To evaluate the humoral immune response generated by the respiratory vaccines, we developed an in-house ELISA using plates coated either with *P. multocida* or *M. haemolytica* and detected specific IgG against the bacteria in sera at 157 and 213 dpi. Antibody levels against *M. haemolytica* were lower in the infected group, while the control and TCZ groups remained similar to those of the control group (Figure 8-3A). However, *P. multocida*-specific IgG decreased both in the infected group of animals at 213 dpi. (Figure 8-3B). This suggests that even with treatment and a reduction in parasites in the host, the antibody production is affected. Further studies are necessary in order to confirm these results.

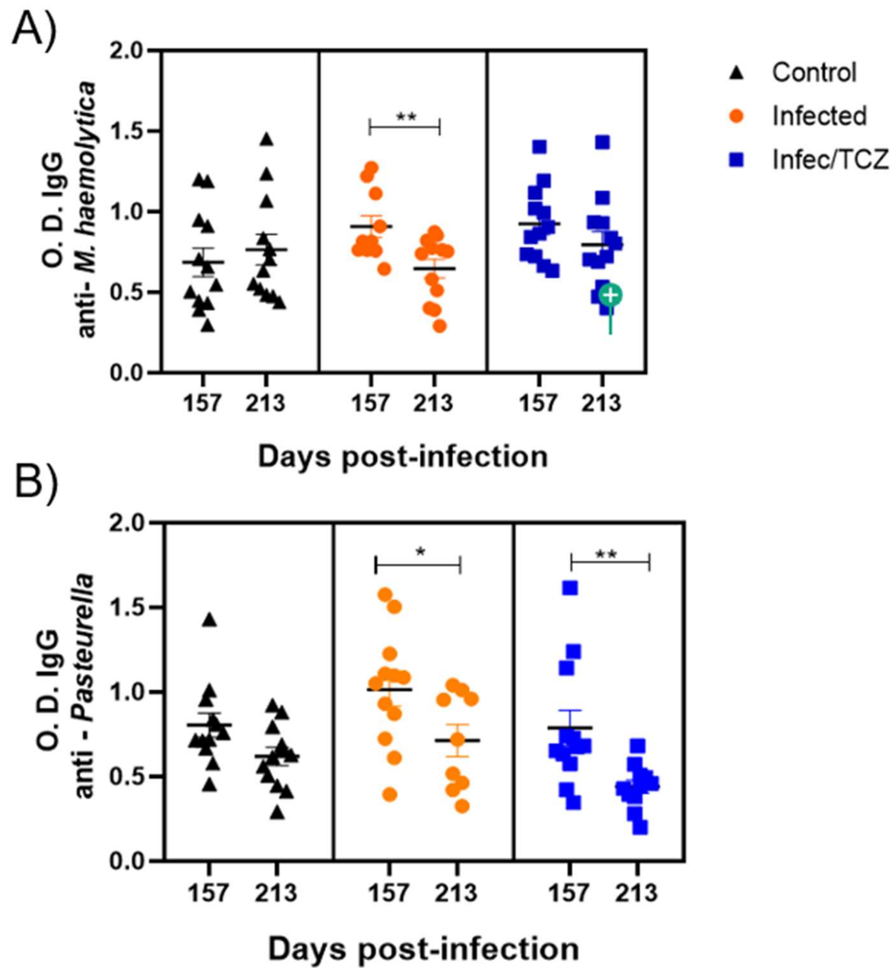


Figure 8–3. IgG levels induced by respiratory vaccine. A) IgG against *M. haemolytica*, detected by indirect ELISA at 157 and 213 dpi. B) Specific IgG anti- *P. multocida* detected by indirect ELISA at 157 and 213 dpi. Control (Black), Infected (Orange), Infected and TCZ-treated (Blue) groups. Asterisks indicate significant differences (** p< 0.005, * p<0,05) performed by Two-Way ANOVA (D) in GraphPad Prism version 6.04.

8.3 *F. hepatica* infection modifies IgG1 specific immune response to foot-and-mouth disease virus induced by vaccination

The main results of this chapter are contained in the attached publication (Art 4).

Costa, M., Mansilla, F., Sala, J. M., Saravia, A., Ubios, D., Lores, P., Capozzo, A. & Freire, T. (2024). Fasciola hepatica infection modifies IgG1 specific immune response to foot-and-mouth disease virus induced by vaccination. *Vaccine*, *42*(3), 541-547.

In this section we investigated whether *F. hepatica* infection in cattle affects the long-term immunity provided by the commercial FMD-inactivated vaccine currently in use in our country. This work was published in Vaccine in January 2024.

FMD is a highly contagious vesicular viral disease that affects cloven-hoofed animals. It causes death only in young animals and high morbidity in adults. The circulation of the virus has led to numerous restrictions on the movement and commercialization of derived products, resulting in significant economic losses (Tewari et al., 2020).

Vaccinating susceptible populations is a commonly used strategy to control the spread of FMDV in endemic regions, such as Africa, Asia, and South America. In Uruguay, the vaccination of animals younger than 2 years old is mandatory, using a commercial vaccine composed of inactivated viral particles of A24/Cruzeiro and O1/Campos strains in an oil-adjuvanted suspension.

To prevent future outbreaks, maintaining high levels of neutralizing IgG antibodies against FMDV is essential in livestock. In fact, the protection against the virus is mediated mainly by antibodies, which correlate with high levels of protection (Balamurugan et al., 2005; McCullough et al., 1992). Our objective was to analyze the antibody immune response triggered by FMDV vaccination during *F. hepatica* infection.

8.3.1 Results

8.3.1.1 *F. hepatica infection alters IgG1 levels against FMDV*

First, we analyzed the immune response elicited by the vaccination against FMDV in the control and infected groups (Figure 1B). We used an indirect ELISA assay using purified A24/Cruzeiro strain 146S particles as capture antigens to evaluate the titers of IgG, IgG1, and IgG2. This work was performed under the supervision of Dr. Alejandra Capozzo. Also, we performed a commercial ELISA to detect non-structural proteins of the virus to rule out the possibility of a current FMDV infection. No animals were detected as positive (data not shown).

Figure 3A shows that both groups had high levels of total IgG against FMDV. Figure 3 shows that specific IgG1 levels decreased in the infected group 28 dpi compared to the control group. However, the infection did not alter the total IgG2 titers (Figure 3C). It is important to highlight that the commercial vaccine induced higher levels of specific IgG2 than IgG1 in all animals evaluated. However, the reported literature indicates that the isotype IgG1 is the one associated with protection against the disease, while IgG2 are predominantly associated with recovery from the illness (Brito et al., 2014; Capozzo et al., 1997). Thus, the decrease of IgG1 titers at 28 dpi could indicate lower levels of protection induced by the vaccination.

8.3.1.2 *Acute fasciolosis alters FMDV-specific IgG1 avidity*

We performed the antibody avidity to correlate with the protection generated by FMDV vaccination. Figure 4A shows no differences detected in the avidity index of specific total IgG ab from both groups, which is in agreement with the previous results (Figure 3A). However, a small decrease in the avidity of specific IgG1 at 28 dpi was detected in the infected group (Figure 4B).

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Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Fasciola hepatica infection modifies IgG1 specific immune response to foot-and-mouth disease virus induced by vaccination

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A R T I C L E I N F O

Keywords:
Fasciolosis, immune modulation
FMD vaccine
Vaccine efficacy
IgG avidity

A B S T R A C T

Fasciola hepatica, a worldwide distributed helminth, has a robust immunoregulatory effect in the host, increasing the susceptibility to secondary infections. Foot and mouth disease (FMD) is a highly contagious acute vesicular viral disease effectively controlled by vaccination in endemic regions. Despite the evidence of immunoregulatory effects, the impact of fasciolosis on the immune response induced by FMD vaccination in cattle has never been assessed. Our objective was to evaluate whether the infection by *F. hepatica* in cattle influences the long-term immunity elicited by the currently used commercial FMD-inactivated vaccines. Aberdeen Angus steers negative for *F. hepatica* were vaccinated twice against FMD virus (FMDV) during the first 6 months of age using a commercial oil vaccine formulated with A24/Cruzeiro and O1/Campos strains. When maternal antibodies against *F. hepatica* were weaned (18—20 months of age) animals were divided into groups of 12 and infected or mock-infected with 500 metacercariae/animal. Individual serum samples were collected at 0-, 28-, 59-, 87- and 157-days post-infection (dpi). Indirect ELISAs were used to detect A24/Cruzeiro specific bovine IgG and IgG subtypes. The total IgG antibody levels and avidity against FMDV did not show significant differences between all the groups. The commercial vaccine induced higher IgG2 than IgG1 titers in vaccinated animals. Anti-FMDV IgG1 levels significantly decreased in the infected group at 28 dpi. In addition, the avidity of IgG1 FMDV-specific antibodies at day 28 in the infected group was reduced compared to the control. These results show that *F. hepatica* infection modified anamnestic responses against FMDV, reducing serum IgG1 titers and avidity. To our knowledge, this is the first report of immune-regulation of *F. hepatica* altering the immune response of FMD vaccines, one of the most globally used animal vaccines.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious acute vesicular viral disease that affects clovenhoofed animals, causing death only in young animals and high morbidity in adults. The circulation of FMD virus (FMDV) in susceptible livestock imposes severe restrictions on the movement and trade of animals and derived products, causing severe economic losses to the affected countries [1]. FMDV is endemic in many regions of Asia, Africa, and South America, where vaccination of susceptible populations is widely used as a major control strategy. Uruguay is free from the disease with the cattle population vaccinated systematically twice a year in animals younger than 2 years old. The commercial vaccine is composed of oil-adjuvanted suspensions of inactivated viral particles of A24/ Cruzeiro and O1/Campos strains.

Maintaining high levels of total antibodies against FMDV is paramount to prevent future outbreaks. Indeed, protection against FMDV is mediated mainly by antibodies [2,3] and the antibody levels induced after vaccination have been correlated to the protective levels achieved after challenge. These correlations performed along the years allow us to predict protection elicited by vaccination without the need of infecting

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https://doi.org/10.1016/j.vaccine.2023.12.067
Received 19 August 2023; Received in revised form 15 December 2023; Accepted 20 December 2023
Available online 6 January 2024
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Please cite this article as: Monique Costa et al., *Vaccine*, https://doi.org/10.1016/j.vaccine.2023.12.067

animals, complying with animal welfare requirements and the 3R concept [4]. Other parameters of the antibody response have also been related to protection, such as the antibody avidity and IgG 1 and 2 isotype ratio [5]. Thus, it is possible to predict the protective status of vaccinated animals by characterizing the profile of the antibody response.

Fasciolosis, a highly prevalent neglected disease [6], causes substantial economic losses worldwide of approximately 3 billion dollars per year [7–9] by reducing fertility, milk production and weight gain as well as leading to secondary infections [10–12]. In cattle, *Fasciola hepatica* causes liver damage due to the migration of immature flukes and obstruction of bile ducts by adult parasites [13]. This leads to the condemnation of livers affected by chronic infections.

During infection, *F. hepatica* modulates the host immune response characterized by the presence of regulatory dendritic cells [14–17], alternative activation of macrophages [18,19], and an adaptive immune response characterized by T-helper type 2 (Th2) and regulatory T cell (Treg)-associated cytokines [17,20,21]. Th2 cells induce cell cytotoxicity by eosinophils, basophils and mast cells while Tregs attenuate pro-inflammatory immune responses. Previous studies have also demonstrated that the expression of immunoregulatory molecules during the infection, such as heme oxygenase-1, renders dendritic cells with an immunoregulatory function [21,22].

Since *F. hepatica* is an immunoregulatory pathogen it could also affect the immunity induced by vaccines. A previous work on the evaluation of the effect of *F. hepatica* infection on respiratory vaccine responsiveness in calves did not reveal any differences in the antibody responses to the vaccine antigens in the presence or absence of *F. hepatica* infection [23]. However, no studies have been carried out to analyze the antibody immune response triggered by FMDV vaccination during *F. hepatica* infection. Thus, the main goal of this work was to evaluate whether and how vaccine-induced immunity is affected by *F. hepatica* infection in cattle.

Our results indicate that parasite infection reduces IgG1 FMDV-specific titers and avidity of systemic antibodies at 28 days after infection (dpi), indicating that parasite infection affects the immunity induced by FMDV vaccination.

2. Materials and methods

2.1. Ethics statement

Animal handling and experiments were carried out in accordance with strict guidelines and regulations from the National Committee on Animal Research, as described in [24] (protocol number 0009/11).

2.2. FMD vaccination

Cattle were vaccinated twice against FMDV with the full dose (5 ml) Oleolauda bivalent vaccine from Paraguay (series 5967700A) formulated with A24/Cruzeiro and O1/Campos strains administered intramuscularly in the first six months of age (Fig. 1A). The vaccination was performed in the context of the compulsory national program of vaccination against FMDV in Uruguay.

2.3. Parasite infection and collection of samples

Six- to eighteen-month-old male Aberdeen Angus steers (young, neutered ale cattle) were used in this experiment. They were kept outdoors and fed with high-quality pasture and water *ad libitum*. Experimental infections (n = 24) were carried out with 500 metacercariae (Ridgey Laboratories, England) per animal, spread in saline solution,

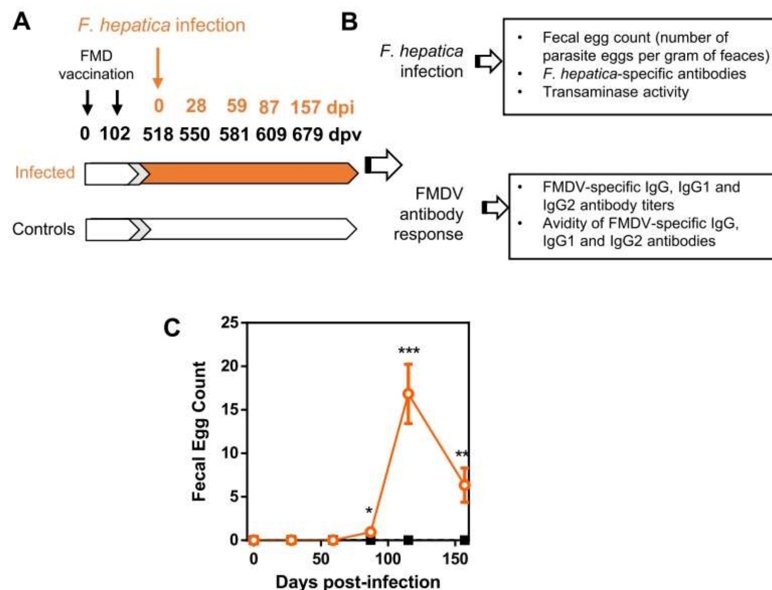

Fig. 1. Fecal egg count (FEC) in *F. hepatica* infected and control steers. A) Thirty-six animals were vaccinated twice with FMDV vaccine. After the first vaccination, animals were either infected with 500 *F. hepatica* metacercariae per animal (n = 24) or used as controls (n = 12). Fecal and blood samples were obtained before the infection (day 0) and at 28, 59, 87 and 157 days post-infection (dpi). These dates corresponded to 518, 550, 581, 609 and 779 days post-vaccination (dpv). B) Parasite egg counts in *F. hepatica* infected (orange) and control (black) animals. Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inserted into gelatine capsules (Torpac®) and delivered orally using a dosing gun, seventeen months after the first FMD vaccination (Fig. 1A). As control, non-infected steers (n = 12) were maintained under the same conditions as infected animals during the experiment in a separate space. Fecal and blood samples were collected before (day 0) and at 28, 59, 87, 115 and 157 after infection. *F. hepatica* egg counts per gram in feces were determined individually using the sedimentation technique [25].

2.4. Transaminase activity

Plasma samples from infected and control animals were used to determine aspartate aminotransferase (AST) and gamma glutamyl-transferase (GGT) activity levels using an automatized spectrophotometer (Dimension RxL Max integrated chemistry system; Siemens).

2.5. Evaluation of F. hepatica- specific antibodies

Parasite specific antibodies were detected by an in-house ELISA [26] using protein lysates from adult parasites. Briefly, adult worms obtained from the bile ducts of infected bovine livers, were mechanically disrupted and sonicated. After centrifugation at 40,000 × *g* for 60 min, supernatants were collected and dialyzed against PBS. The obtained lysate (FhTE) was resuspended on PBS containing a cocktail of protein inhibitors (Sigma-Aldrich, St. Louis, MO) and dialyzed against PBS for 24 h, before determining protein concentration by bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO).

FhTE (1 μg/well) in 50 mM carbonate buffer (pH 9.6) was coated overnight at 4 °C in ninety-six-well microtiter plates (Nunc, Roskilde, Denmark). After blocking with 1 % gelatin in PBS, three washes with PBS containing 0.1 % Tween-20 were performed. Serially diluted sera in buffer (PBS containing 0.1 % Tween-20 and 0.5 % gelatin) were added to the wells for 1 h at 37 °C. Following three washes, wells were treated for 1 h at 37 °C using sheep anti-bovine IgG peroxidase-conjugate (Biorad, CA) and o-phenylenediamine-H_2O_2 was added as substrate. Plates were read photometrically at 492 nm in an ELISA spectrophotometer (Labsystems Multiskan MS, Finland).

2.6. FMDV-specific IgG subtypes

A24/Cruzeiro-specific bovine IgG isotypes were detected by indirect ELISAs as reported by Lavoria et al. [4], using 146S purified viral particles as capture antigen. Plates were revealed using anti-IgG1 and -IgG2 peroxidase conjugate antibodies (Biorad, CA) and 2,2̂azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) as chromogen. Serum samples were run in two-fold serial dilutions starting at 1:50 and isotype antibody titers were expressed as the highest dilution of the serum reaching an optical density (OD) equal to the mean OD obtained from all pre-immune negative sera ± 2 standard deviations (SD).

2.7. Avidity of FMDV-specific antibodies

FMDV-specific total IgG and IgG avidity were determined using a single dilution indirect ELISA performed as described previously [4]. Briefly, A24/Cruzeiro 146S coated plates were blocked, washed and serum samples (diluted 1:50) were run in duplicates. One of the wells was washed with PBS and the other with PBS containing 6 M Urea to detach low-avidity binders. The presence of specific antibodies was revealed using peroxidase-conjugates against bovine total IgG and IgG1 (Biorad, CA). OD values for samples and controls were corrected by subtracting mean blank OD values (cOD). The avidity index was estimated by the ratio between Urea and PBS treated samples (cOD) multiplied by 100.

2.8. Antibodies against non-structural (NS) protein

Antibodies against the highly conserved 3B non-structural protein were determined using a commercial kit following the manufacturer's instructions (PrioCHECK™ FMDV NS Antibody ELISA Kit, ThermoFisher Scientific).

2.9. Liquid-phase blocking (LPB) ELISA

Total anti-FMDV A/24 Cruzeiro antibody responses were assessed by LPB-ELISA performed as stated by the WOAH Manual [27], using strain-specific rabbit antisera to capture inactivated virus and a monoclonal antibody pool as detection antibodies, followed by an anti-mouse peroxidase conjugate (Jackson, USA). Antibody titers were expressed as the reciprocal log10 of serum dilutions giving the 50 % of the absorbance recorded in the virus control wells without serum.

2.10. Data analysis

Antibody titers induced after vaccination and measured by LPBE were analyzed to fit the expected protection (EPP). The EPP estimates the likelihood that cattle would be protected after the homologous FMDV challenge based on LPBE titers measured before the challenge (at 60 dpv) and results from the homologous challenge performed using the PP method on 16 naive cattle [27, 53]. EPP values for the A24/Cruzeiro and other strains were calculated from correlations between LPB-ELISA [27, 53]. The EPP ≥ 75 % (EPP75) values serve as a reference of antibody titers associated with the protection at the population level against the homologous challenge with the vaccine strains. A24/Cruzeiro LPBE EPP75% titer is 1.90.

Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test or student *t* test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3. Results

3.1. F. hepatica experimental infection induces specific IgG antibodies

To study and characterize the specific memory immune response against FMDV during acute fasciolosis in bovines, we experimentally infected steers with 500 *F. hepatica* metacercariae per animal that previously received two vaccinations against FMDV (Fig. 1A). After experimental infection, animals showed a significant increase in fecal egg counts (FEC) from day 87 onwards (Fig. 1B) that eventually lead to liver damage [24]. As expected, non-infected animals did not display detectable FEC during the experiment (Fig. 1B). In addition, *F. hepatica* specific-antibodies were observed after 28 dpi in infected animals, while no specific antibodies were elicited in control animals (Fig. 2A).

Considering that the parasite infects the liver and that the transaminase activity levels in plasma can correlate with liver dysfunction and with FEC [24], we analyzed the AST and GGT enzyme activity in plasma (Fig. 1A). AST activity was increased from 28 dpi (Fig. 2B), while GGT increased after 59 dpi (Fig. 2C). The two different increased kinetic profiles were in accordance with previous reports and could account for their organ-specific expression [28] or function. Indeed, while AST, an intracellular enzyme, usually indicates hepatocellular injury, GGT suggests biliary tract obstruction (cholestasis) [28]. All infected animals showed significantly higher hepatic damage and fibrosis at sacrifice while control steers showed normal livers [28]. Altogether, these results demonstrate the induction of a humoral immune response specific to the parasite and hepatic dysfunction associated with parasite infection.

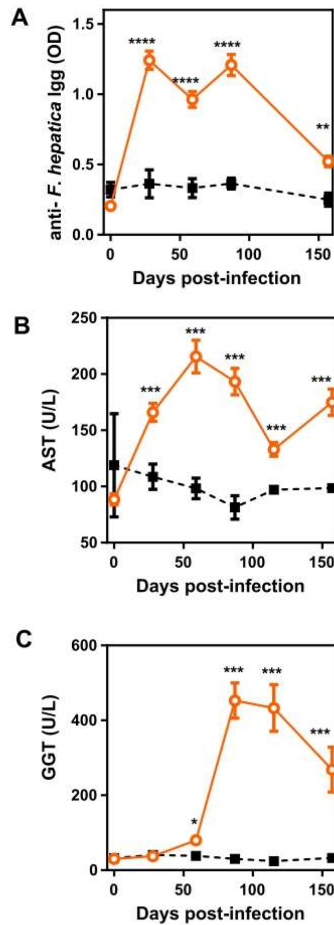

Fig. 2. *F. hepatica* specific antibodies and transaminase levels in plasma of *F. hepatica* infected (orange) and control (black) cattle. A) Parasite-specific IgG levels were detected by ELISA. B) Aspartate transaminase (AST) in plasma. C) Gammaglutamyl transferase (GGT) in plasma. Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

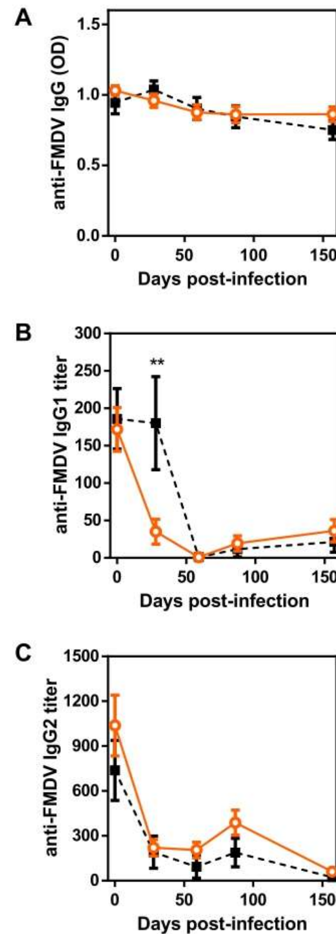

Fig. 3. FMDV-specific antibody immune response in *F. hepatica* infected (orange) and control (black) steers. IgG (A), IgG1 (B) and IgG2 (C) antibodies specific to FMDV A24/Cruzeiro strain were detected by indirect ELISAs using 146S purified viral particles as capture antigen. Asterisks indicate statistically significant differences between infected and control animals calculated with two-way Anova followed by a Tukey multiple comparison test: **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Differential FMDV-specific IgG1 immune response induced by vaccination in F. hepatica infected and control animals

Next, we analyzed the immune response elicited by FMDV vaccination in the two groups of animals (Fig. 1B). IgG titers against FMDV were evaluated with an indirect ELISA assay using purified A24/Cruzeiro vaccine strain 146S particles as capture antigen. High levels of total IgG specific to FMDV were found in infected and control steers, without significant differences between them (Fig. 3A). In addition, no differences in the total anti-FMDV antibody titers, assessed by LPB-ELISA, were observed (Supplementary File 1). In all cases, antibody levels decreased to titers below protective levels at nine months after completing the vaccination schedule (Supplementary File 1). Of note, no antibodies directed to non-structural proteins of the virus were detected, ruling out the possibility of a current FMDV infection (not shown).

Then, we analyzed FMDV-specific IgG1 and IgG2 isotype antibody titers. Interestingly, FMDV-specific IgG1 antibodies decreased in infected animals at 28 dpi in comparison to non-infected steers (Fig. 3B). However, no significant differences were detected in the IgG2 antibody titers (Fig. 3C) during *F. hepatica* infection. Of note, higher FMDV-specific IgG2 than IgG1 titers were observed in vaccinated animals.

3.3. F. hepatica infection impairs FMDV-specific IgG1 avidity

Apart from the IgG1 and IgG2 titers, other parameters such as antibody avidity can be correlated with protection induced by FMDV vaccination [4,29]. In agreement with the results shown above, no

differences were detected in the avidity of FMDV-specific IgG antibodies from infected and control groups (Fig. 4A). However, a small decrease in the avidity of FMDV-specific IgG1 antibodies (Fig. 4B) was detected at 28 dpi.

4. Discussion

F. hepatica has powerful immunoregulatory properties that can modify an immune response or exert a bystander effect on concurrent infections in the host [11,30–32]. For instance, cell mediated immune responses against *Mycobacterium bovis* are downregulated in animals infected with liver fluke [11]. *F. hepatica* co-infection with bacteria can suppress a protective T helper 1 (Th1) immune response [33,34] and increase the risk of zoonotic *Escherichia coli* infections [11,35]. In addition, the diagnosis and pathological progression of secondary infections can be modulated by co-infection with *F. hepatica* [36]. Indeed, cattle co-infected with *F. hepatica* and *M. bovis* display reduced responsiveness to skin and interferon gamma (IFN-γ) tests, affecting bovine tuberculosis diagnosis [33,37–39].

In this work we demonstrate that both FMDV-specific IgG1 titers and avidity are impaired in FMD-vaccinated animals with acute fasciolosis. These results suggest that the humoral immune response induced by the vaccine against FMD is modified during the acute phase of *F. hepatica* infection, likely due to its immunoregulatory properties. In fact, the vaccine usually triggers isotype switching towards a predominance in FMDV-specific levels, which in turn, is associated with protection against the disease [5,29]. Thus, the decrease of anti-FMDV IgG1 titers at day 28 after parasite infection could indicate lower levels of protection induced by the vaccine. Interestingly, although *F. hepatica* infection was associated with a decrease of anti-FMDV IgG1 titers at 28 dpi, no differences were observed in IgG2 levels during the evaluation period of time. This could be related to different T cell differentiation and the concomitant differential production of cytokines [40]. Furthermore, a reduction of specific IgG2 levels during *F. hepatica* infection has been previously reported in cattle [41].

To our knowledge, this is the first work in studying the impact of fasciolosis on the antibody immune response induced by FMD vaccination. A previous report, however, established no alteration in the specific total antibody response to respiratory bacterial and viral vaccination [23]. Both *F. hepatica* infected and control animal groups developed similar antibody profiles to bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI3) and *Mannheimia haemolytica* vaccination [23]. In agreement with these results there were no effects of *Ostertagia ostertagi* and *Cooperia* spp. infection on the respiratory vaccine response in calves [23] or to bovine viral diarrhea virus 1 (BVDV-1) vaccine in cattle [42]. Major differences between these works and ours include vaccination and infection timing and conditions of parasite infection. While in the present study steers were vaccinated twice prior to parasite challenge with 500 metacercariae, Krump and collaborators administered the respiratory vaccine two weeks after experimental parasite infection, performed with a lower parasite challenge dose (150 metacercariae) [23]. On the other hand, De Seram et al. [43] vaccinated cattle with the respiratory vaccine prior to feedlot entry and studied whether naturally-acquired helminth infection impacted in the antibody immune response against the BVDV-1 vaccine antigen. Last, Schutz et al. performed vaccination before experimental co-infection with *O. ostertagi* and *Cooperia* spp [42].

The results found in this work also suggest that the memory immune response triggered by FMD vaccination is hampered with *F. hepatica* infection. However, further studies are needed to relate the observed changes in the vaccine induced antibody response with protection against FMD. Affinity maturation and class switching of antibodies occur with repeated exposures to the same antigen during the immune response. In addition, immune-suppressive cytokines, such as those produced by helminths, may reduce vaccine-induced humoral and cell-mediated immunity responses [44–47]. Thus, the characterization of the cellular immune response, along with the antibody response, is needed to understand the molecular and cellular mechanisms that are altered by *F. hepatica* infection. Memory B cell activation or function [48–50] as well as antibody integrity [51,52] could be compromised upon parasite infection.

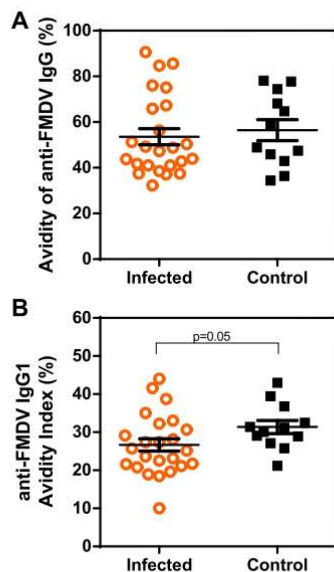

Fig. 4. Avidity of FMDV-specific antibodies in *F. hepatica* infected (orange) and control (black) steers. Anti FMDV- A24/Cruzeiro total IgG (A) and IgG1 (B) avidity (day 28 dpi), was determined using an indirect ELISA in the presence or absence of urea to detach low-avidity antibody binders. The statistically significant difference between infected and control animals was calculated with the student *t* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions

The results obtained in this work indicate that *F. hepatica* infection can affect the antibody response induced by FMD vaccination by reducing the quality and quantity of FMDV-specific IgG1. This effect coincides with the onset of the anti-parasite-specific antibodies, suggesting an association with the immunoregulation induced by the parasite. Thus, this study highlights the need to explore the role of the immune regulation exerted by *F. hepatica* infection in cattle to ensure the efficiency of livestock FMD vaccination programs in endemic settings.

6. Animal welfare statement

Animal experimentation was carried out according to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences.

CRediT authorship contribution statement

Monique Costa: Data curation, Investigation, Methodology, Writing – review & editing. **Florencia Mansilla:** Data curation, Investigation, Methodology. **Juan Manuel Sala:** Investigation, Methodology. **Anderson Saravia:** Investigation, Methodology. **Diego Ubios:** Investigation, Methodology. **Pablo Lores:** Investigation, Methodology. **Alejandra**

Victoria Capozzo: Data curation, Formal analysis, Funding acquisition, Supervision, Writing – review & editing. **Teresa Freire:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank INIA La Estanzuela for animal handling and Marfrig Abattoir located in Río Negro and Tacuarembó, Uruguay.

Financial supports were provided by Programa de Desarrollo de Ciencias Básicas (PEDECIBA), Comisión Sectorial de Investigación Científica and Agencia Nacional de Investigación e Innovación (SNI-ANII and FCE_1_2019_1_156295) to Teresa Freire. Funding by GFRA Research to Florencia Mansilla was also provided. M. Costa was funded by ANII and CAP. A. V. Capozzo is a researcher of CONICET, Argentina.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2023.12.067.

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8.4 Conclusions

This section demonstrates that *F. hepatica* can alter the immunity induced by vaccination of different pathogens in the acute and chronic phases of infection. The IgG anti-*Clostridium* ssp. and FMDV-specific IgG1 titers were impaired in animals with acute fasciolosis. Our results suggest that the humoral immune response induced by both vaccinations is modified during acute F. *hepatica* infection, probably due to immunoregulatory mechanisms and liver damage generated by the parasite at this stage.

In the chronic phase of infection, the *P. multocida* and *M. haemolytica*-specific titers are impaired in infected animals. Also, the TCZ treatment was ineffective in recovering antibody levels against *P. multocida*, demonstrating that even lower parasite loads or partial recovery of liver tissue can still reduce the immune response induced by vaccination.

Additional research is needed to ascertain the extent of the influence of fasciolosis on the immunity provided by the vaccination for FMDV. It is crucial to analyze the consequences of an active infection on the vaccination program in calves and its potential impact on shielding them against the virus.

General Discussion

9 General discussion and perspectives

In this thesis we focused on studying the immune-regulatory strategies that *F. hepatica* induces in peritoneal APCs during infection in mice using an experimental infection model. Additionally, we examined the biochemical parameters, cellular and humoral immune responses, and the impact of fasciolosis on vaccination in cattle infected with *F. hepatica*.

Our primary objective was to investigate the relationship between the presence of HO-1$^+$ cells in fasciolosis and the differentiation or expansion of Tregs through cellular and molecular mechanisms in a mouse model. Our findings indicate that F4/80$^+$HO-1$^+$ cells play a role in reducing the production of ROS/RNS in the inflammatory environment, which suggests that HO-1 has a protective effect on the oxidative function of APCs. This particular cell population is responsible for producing IL-10, a cytokine necessary for parasite establishing in the host.

We found that the cells recruited to the peritoneal cavity of *F. hepatica*-infected mice are CD11c$^+$ F4/80$^+$ HO-1$^+$ CCR2$^+$ MGL2$^+$ and could consist of a population of monocyte-derived DCs or SPM with regulatory properties. Moreover, the MGL2$^+$ cell depletion showed partial resistance to the infection and did not experience an increase in CD4$^+$/CD25$^+$FoxP3$^+$ Tregs induced by the infection. However, the parasite molecules that trigger this immunoregulatory pathway on MGL2$^+$ cells during *F. hepatica* infection have not been identified in this study. The identification of the proteins carrying carbohydrates present in a parasite lysate that are recognized by MGL2 could be carried out by affinity chromatography (pull down) using a recombinant MGL2 protein. This is strategy has previously been used to identify MGL ligands from tumor cells (Pirro et al., 2018).

The role of HO-1 in *F. hepatica* infection is not entirely understood in cattle and humans, the natural hosts. In this thesis, one of the primary initial objectives was to investigate further the role of HO-1 in the immune response in cattle, mainly using PBMCs stimulated with HO-1 inhibitors and stimulants. However, the use of frozen PBMCs was limited in cell culture due to significant decrease in cell viability.

During the acute phase of infection, primarily during the 4 – 6 wpi, is when the most important events for the host occur, including the liver damage generated by the parasite migration through the parenchyma and followed by an early increase of AST in sera. Furthermore, on day 43, an increase in $CD21^{+}$ cells in blood associated with high parasite-specific antibodies in the infected animals was found. Additionally, the percentage of eosinophils increases on this same day, a parameter that was correlated with the number of flukes recovered in our animals.

During this same period (acute phase), the immunoregulatory effects generated by the parasite affect the immune response induced by vaccines. The animals infected with *F. hepatica* have a diminished antibody immune response against *Clostridium* spp (total IgG at 43 dpi) and Foot and Mouth Disease virus (IgG1 and avidity at 28 dpi). Both vaccines were administered to the animals during their first year of life, 6 and 12 months, respectively, before the experimental infection with *F. hepatica*, and not during the infection.

The results of our study have provided insights into the long-term immunity generated by the current vaccines and shed light on how the immune modulation caused by a helminth parasite would affect the humoral memory response triggered by vaccines. However, further research is needed to understand the impact of *F. hepatica* infection on the immunity induced by previous or subsequent vaccination. This information could be essential to determine vaccination timing or fascioloside treatment in our country.

Another limitation of our work was the inability to investigate innate cell receptors involved in recognizing *F. hepatica* glycans in bovine hosts during the acute phase of the infection, such as MGL2, which was studied in mice. The implication of CLRs can be further explored by culturing cells with FhTE in the presence of Ethylenediaminetetraacetic acid (EDTA), a calcium-chelating agent. By chelating calcium with EDTA, we could eliminate partial carbohydrate binding and determine the importance of C-type lectin receptors in parasite recognition (Aspberg et al., 1995; Rodríguez et al., 2015). However, it would be advisable to use MGL-specific blocking antibodies in order to block this (and not other) receptor, which are not available for bovine MGL.

The chronic phase of *F. hepatica* infection also significantly impacts on various aspects of the host. in this phase flukes reach sexual maturity inside the bile ducts. Unfortunately, in consequence of the low viability of PBMC, it was not possible to accurately characterize the type of cell population involved in this phase of the infection.

In the beginning of the chronic phase, infected animals showed an increase in fecal EPG, with a peak at 115 dpi, associated with an increase in GGT activity. The livers from the infected group were characterized by a high degree of fibrosis, hyperplasia of bile ducts, and a pale color. Also, the levels of specific antibodies to *F. hepatica* decreased at 157 dpi in the infected group, suggesting an escape of the host immune system.

Our study found that treating TCZ during the chronic phase effectively reduced parasite burden, fecal EPG, and the levels of GGT and AST in sera. This is important, as the effects of TCZ treatment on the liver have yet to be studied in depth during the chronic phase of the infection (Romero et al., 2019). However, while the treatment clearly reduced parasite burden, it did not avoid hepatic damage or eliminated all flukes.

The TCZ treatment also impacted in the quantity and quality of specific antibodies produced against the parasite, and modified the cytokine profile in the liver. The group of treated animals showed an increase of the levels of IFN-γ, IL-10, and IL-4, and may occur due to the stimulation of the Th2 response, which, in turn, promotes fibrosis and the recovery of liver tissue damage and liver regeneration.

During our study, we also conducted RT-qPCR to measure HO-1 levels in liver at the endpoint of the experiment. One of the few studies on hepatica HO-1 in bovines naturally infected with *F. hepatica* the livers showed increased levels of HO-1 mRNA expression with infection (Carasi et al., 2017). However, we observed no significant variations between the groups (Data not shown). A possible explanation might be a different time of sampling in relation with parasite infection. Also, in the study carried out by Carasi et al., samples of injured liver tissue were used. In contrast, we collected random liver tissue samples for our study in experimentally infected animals (chronic phase, 213 dpi).

To improve the accuracy of our results, performing a spatial gene expression analysis at different points in time of infection would be a better option to elucidate and understand the gene expression profile in the structure of liver tissue during the infection. This technique would generate a complex

map where different gene expressions, including the HO-1 expression, could be visualized in areas more or less damaged by the parasite.

Chronic infection caused by *F. hepatica* also appears to impact the immune response induced by vaccines administered simultaneously. Antibodies against respiratory bacterial agents decreased as the infection progressed, while control animals had constant levels. Even with treatment and a reduction in parasites in the host, antibody production is still affected. However, further studies are necessary to confirm these results. One of the tests necessary to confirm our results would be to culture PBMC in the presence of the vaccine antigen, IL-21 and CpG (Wood, et al., 2004) to induce specific antibody secretion, which can be later be evaluated by ELISA.

In summary, this doctoral thesis provides valuable insights of the immunoregulatory mechanisms induced by *F. hepatica* in mice and cattle, a topic on which little information is available. The study delves into the molecular mechanisms that facilitate the parasite's survival in the hosts. The results obtained from this research contribute to the knowledge of how the parasite can impact the response induced by vaccines, which can be useful in future research projects. Ultimately, this can lead to a reduction in economic losses caused by the decrease in weight gain of livestock and the confiscation of livers, while also improving animal health and well-being.

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