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FINAL DEGREE PROJECT

IMMUNOGENIC PROPERTIES OF HEPATITIS A VIRUS IN TWO MOUSE STRAINS. PRELIMINARY RESULTS.

Main area	Physiology and Pathophysiology
Secondary areas	Immunology
	Microbiology

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ABBREVIATIONS

AEC: 3-amino-9-ethylcarbazole

ALT: alanine aminotransferase

Alum: aluminium hydroxide

APC: antigen-presenting cell

AST: aspartate aminotransferase

Az: azide

BrdU: 5-bromo-2'-deoxyuridine

CCiT-UB: Flow Cytometry Unit of the Scientific and Technological Centres of the University of Barcelona

CDC: Centers for Disease Control and Prevention

CK: cytokine

DC: dendritic cell

DMSO: dimethyl sulfoxide

eHAV: encapsulated hepatitis A virus

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunosorbent spot

FBS: foetal bovine serum

FSC: forward-scatter characteristics

HA: hepatitis A

HAV: hepatitis A virus

HAVCR: hepatitis A virus cellular receptor

HRP: horseradish peroxidase

IFN: interferon

Ig: immunoglobulin

IL: interleukin

Mab: monoclonal antibody

MSM: men having sex with men

NK: natural killer

NS: non-stimulated

OPD: *O*-phenylenediamine dihydrochloride

OSSMA-UB: Office of Safety, Health and the Environment of the University of Barcelona

OVA: ovalbumin

PAMPs: pathogen-associated molecular patterns

PBS: phosphate-buffered saline

RNA: ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

RT: room temperature

s.c.: subcutaneous

S.D.: standard deviation

SSC: side-scatter characteristics

Tc: T cytotoxic cells

TcCM: T cytotoxic central memory cells

TCID50: tissue culture infectious dose 50

TcEM: T cytotoxic effector memory cells

TCM: central memory T cells

TEM: effector memory T cells

Th: T helper cells

ThCM: T helper central memory cells

ThEM: T helper effector memory cells

TMB: 3,3',5,5'-tetramethylbenzidine

TNF: tumour necrosis factor

TRM: resident memory T cells

TSCM: stem-cell memory T cells

VLP: virus-like particles

A. ABSTRACT I RESUM

ABSTRACT

Hepatitis A (HA) is a viral disease caused by the hepatitis A virus (HAV) that occurs all over the world. It is transmitted by the faecal–oral route, and it is known that poor sanitary conditions increase the risk of HAV transmission.

As no specific treatment for HA is available, prevention with vaccination strategies is the key to controlling the infection. Most of the available vaccines are inactivated vaccines adjuvanted with aluminium hydroxide. The slow growth of HAV and the need for an adjuvant make the vaccines' economic cost relatively high and, what is more, there have been periods of vaccine shortages as demand has exceeded the production capacity.

Recently, it has been demonstrated that HAV can be found encapsulated in a lipid membrane that resembles exosomes. Exosomes have been suggested to take part in T-cell stimulation and can induce an immune response without the need of an adjuvant.

This study includes the preliminary experiments that will conclude with immunization with encapsulated HAV (eHAV) without adjuvant. In particular, herein we set up techniques to check an immunization protocol with HAV particles (with adjuvant) in two mouse strains (BALB/c and C75BL/6). These techniques include the proliferative capacity of spleen lymphocytes (ELISA), the antibody-secreting cells (ELISPOT) and the changes in the cell phenotype (flow cytometry). Although further studies remain to be done, the results obtained in the present study allow the defining of the mouse strain and obtention techniques to establish the immunization process.

RESUM

L'hepatitis A (HA) és una malaltia vírica, prevalent a tot el món, causada pel virus de l'hepatitis A (HAV). Es transmet per via fecal-oral, de manera que la falta d'higiene i condicions sanitàries incrementen el risc de transmissió d'aquesta malaltia.

Com que no es disposa d'un tractament específic, la prevenció a través de campanyes de vacunació és clau per controlar la infecció. La majoria de vacunes disponibles són vacunes de virus inactivat que utilitzen adjuvant. El creixement lent del HAV i la necessitat d'adjuvant fan que les vacunes tinguin un cost elevat i, a més, que de vegades es produeixi desproveïment degut a que la demanda excedeix a la capacitat de producció.

Recentment s'ha descobert que el VHA es pot trobar encapsulat en una membrana lipídica semblant als exosomes. S'ha suggerit que els exosomes poden estimular les cèl·lules T i induir una resposta immunitària sense necessitat d'adjuvant.

Aquest estudi inclou experiments preliminars que acabaran amb la immunització amb HAV encapsulat (eHAV) sense adjuvant. Concretament, hem posat a punt tècniques per determinar el protocol d'immunització amb partícules de HAV (amb adjuvant) en dues soques de ratolí (BALB/c i C57BL/6). Les tècniques inclouen la determinació de la capacitat proliferativa dels limfòcits de melsa (ELISA), de les cèl·lules secretores d'anticossos (ELISPOT) i dels canvis en el fenotip (citometria de flux). Tot i que els experiments han de continuar, els resultats obtinguts permeten definir la soca de ratolí a utilitzar i disposar de tècniques per establir el procés d'immunització.

B. JUSTIFICATION OF THE AREAS INTEGRATED IN THE PROJECT

To understand the pathogenicity of hepatitis A, it is necessary to know the different systems of the human organism. Additionally, to immunize animal models, it is necessary to have basic knowledge in anatomy and physiology. Thus, this project's main subject area is **physiology and pathophysiology**.

Moreover, in order to evaluate the immune response and characterize the immunogenic properties of the hepatitis A virus, it is crucial to understand how the immune system acts when an infection occurs, how it is activated and how the activation can be measured. Therefore, **immunology** is another important area for this project.

Lastly, hepatitis A is caused by a virus, so **microbiology** is needed to study the virus characteristics and behaviour once it enters the organism. This allows a better understanding of the results obtained. Additionally, knowledge in microbiology is necessary in order to work in safe conditions when processing samples that include viruses.

1. INTRODUCTION

Hepatitis A (HA) is a viral disease caused by the hepatitis A virus (HAV), which occurs all over the world [1,2]. This virus is transmitted through the faecal–oral route and humans are thought to be its principal host [1,3]. HA has high morbidity rates and can lead to fatal consequences, especially in older individuals [4]. As there is no specific treatment for HA, the fundamental management of this disease consists of its prevention through vaccination programmes [5,6].

1.1. PROPERTIES OF HEPATITIS A VIRUS

HAV is the sole member of the genus *Hepatovirus*, which is included in the *Picornaviridae* family [7,8]. HAV has a 27 nm icosahedral capsid that envelops a positive single-stranded ribonucleic acid (RNA) genome that only has one open reading-frame encoding for a single polyprotein [7–9] (**FIGURE 1**). This polyprotein suffers co-translational and post-translational proteolysis that lead to the separation of its three major regions: P1, P2 and P3. The P1 region provides the structural proteins of the viral capsid, which are VP1, VP2, VP3 and VP4 (only present in the non-mature virions). From the P2 and P3 regions, non-structural proteins are processed [7]. Also, the P3 region codes for proteins that are necessary for RNA synthesis and virion formation [7,10].

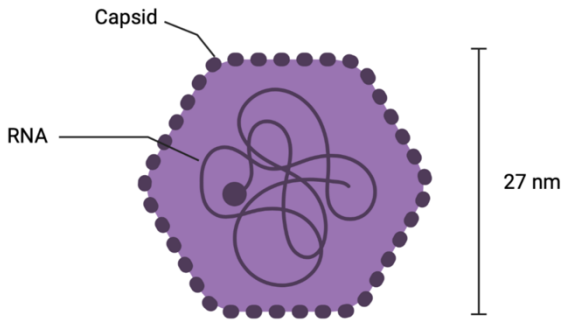

FIGURE 1: Hepatitis A virus (HAV) structure.
Figure made by the author based on reference [3].

HAV has some degree of genetic diversity at the capsid region, which can lead to antigenic variants that can be used to identify the origin of HA outbreaks [7,8,11]. Currently, six genotypes (I–VI) have been described based on the sequence of the VP1 region. Only the three first (I–III) genotypes affect humans, and the most prevalent ones worldwide are I and III. Conversely, only one serotype has been recognized, suggesting that there are severe structural constraints in the capsid that prevent more extensive substitutions necessary for the emergence of a new serotype [7,11)]

HAV is more stable in low pH, extreme temperatures (both heat and freezing) and other treatments than other picornaviruses [3,7,12]. This allows HAV to persist in faeces, food and other surfaces for several weeks, which facilitates its transmission [7,8].

Although virions were traditionally believed to be non-enveloped, recent data indicate the existence of both non-enveloped and lipid-enveloped forms, which are formed from host membranes and are similar to exosomes [8,13,14]. These lipid-enveloped forms are resistant to neutralizing antibodies and sensitive to chloroform [15]. This finding has implications in the human immune response, as will be later developed in this study [14].

1.2. TRANSMISSION OF HEPATITIS A VIRUS

HAV is transmitted through the faecal–oral route, which can happen through having close person-to-person contact with an infected person (such as caring for someone who is ill or using injection drugs). Moreover, it is also transmitted by having oral-anal sex with an infected person and through the ingestion by an uninfected person of water or food (especially seafood) that has been contaminated with the faeces of an infected person. Furthermore, the virus can not only survive in food, but also in inanimate objects contaminated by an infected person with dirty hands [2,3,6,16].

The most common infection source is through having close contact with an infected person. The virus is especially spread within families when an infected member with dirty hands prepares food for the other household members. It is also spread among school contacts. In these circles, HAV is rapidly spread because of its long incubation period: infected individuals are contagious for about one month before the onset of the symptoms and, once the symptoms appear, individuals are still contagious for one week. Furthermore, children younger than six years old tend to be asymptomatic, thus are considered to be the principal reservoir [1,2,6,8,17].

Apart from faeces, HAV is also found in the blood of infected individuals. Although possible, it is very rare to acquire HAV infection via blood transfusion or blood products originated from an infected donor [7,8,16].

For these reasons, risk factors for HA include poor sanitation, lack of safe water, living in a household with an infected person, being a sexual partner of someone with acute HA infection, being a man who has sex with other men (MSM), use of recreational drugs and travelling to areas with high endemicity without being immunized [2].

1.3. EPIDEMIOLOGY OF HEPATITIS A

HA is a public health problem all over the world, and HAV infection is the first cause of viral hepatitis worldwide [18,19]. Studies have shown that HA prevalence is linked to low income and difficult access to clean water and adequate sanitation [12,19,20]. Based on seroprevalence, geographic areas around the world can be classified into different patterns of high, intermediate, low or very low endemicity. The type of area will be determined by the socio-economic conditions, the probability of disease and mortality at older ages, and the probability of developing lifetime immunity [8,12,21].

In areas of high endemicity, 90% of the population is infected with HAV, and consequently acquires lifelong immunity by the age of 10 [21]. In these areas, there are practically no cases of HA among adults and, as a result, since children usually do not present symptoms, HA is not considered a major health problem [3,8,12,19,21].

In contrast, areas of low endemicity are high-income countries with good sanitary and hygienic conditions. As a result, the majority of the population does not get infected during childhood and therefore does not develop antibodies. Thus, many adults remain susceptible to HAV. The risk of severe symptoms and mortality increases in older ages, which is why HAV is considered a public health concern in areas of low endemicity if an outbreak occurs. Outbreaks in areas of low endemicity can be caused by imported food or travellers returning from areas of high endemicity [8,12,20,21].

The worst scenario is found in the areas with intermediate endemicity. In these areas, childhood transmission is less frequent, so there is an increased number of older children and young adults who are susceptible to becoming infected. These countries do not have the appropriate sanitary and socio-economic resources to prevent outbreaks and, as a consequence, both adolescents and adults get infected by HAV, which leads to a higher rate of severe clinical manifestations [8,12,20,21].

The epidemiology of HA has changed in recent years due to the socio-economic development and, consequently, the improved living conditions in many resource-poor countries and regions (**Figure 2**). This phenomenon, known as epidemiological transition, entails a decrease in the incidence of HA, resulting in a shift from high endemic areas to intermediate or low endemic areas [6,8,12,22]. Paradoxically, as seroprevalence decreases, the epidemiological transition results in an increase in the HA rates, fulminant liver failure and liver transplantation secondary to HAV infection [8,12].

1.4. Pathogenesis of hepatitis A

As previously stated, HAV is transmitted by the faecal–oral route. Several studies have demonstrated HAV antigen presence in the gastrointestinal tract, suggesting that after ingestion, HAV penetrates the gut mucosa and replicates in the epithelial cells of the gastrointestinal crypts [7,10,12]. Then, it reaches the liver, its target organ, via portal blood or after systemic circulation, and continues its replication in the hepatocytes and Kupffer cells [3,10,12].

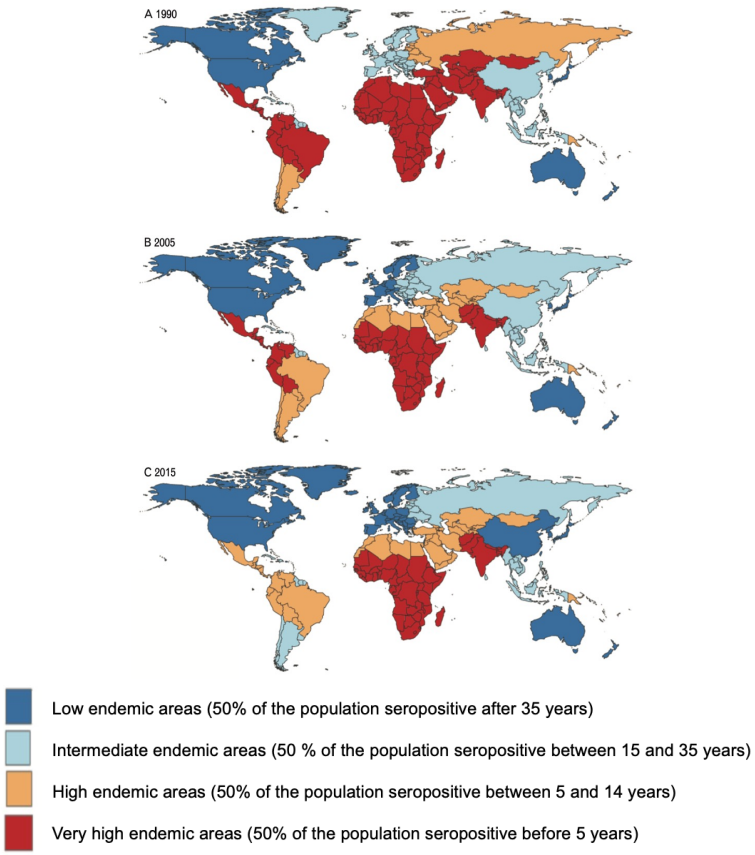

FIGURE 2: Changes in hepatitis A (HA) endemicity areas between 1990 and 2015. Each color represents an endemicity pattern based on the age in which 50% of the population has acquired seropositivity.

Adapted from reference [8].

Once in the liver, HAV attaches to a cell surface receptor called an HAV cellular receptor 1 (HAVCR-1). For this binding, calcium, proteins, sugars and lipids are required [7,23]. Additionally, other receptors, such as HAVCR-2 and the asialoglycoprotein receptor, are required for HAV to enter the cells [7]. However a recent study suggested that the HAVCR-1 receptor might not be necessary for HAV to enter to the liver [24]. As mentioned above, HAV particles can be found either free or coated. Free particles may use HAVCR-1 as described, while coated particles could use both HAVCR1 or the asialogycoprotein receptor to enter the cells [22].

HAV replicates slowly in the liver and does not cause cytopathic effects. However, it can cause liver cell damage due to both cellular and humoral immune responses [3,12]. For many years, the fact that HAV can be released to supernatant fluids without cell lysis remained unexplained. A recent study revealed that HAV hijacks cellular membranes to exit cells cloaked with a lipid membrane [15]. Therefore, HAV circulates in the blood as a quasi-enveloped virus but once it reaches the liver, its capsid is removed by the high concentration of bile acids [24]. After its replication, HAV is released through the bile to the stools as a non-enveloped virus. The faeces are considered to be an important transmission source, because they contain large quantities of viruses approximately 10 days before the onset of the symptoms and are still contagious for a week after the apparition of the symptoms [1,3,12,24]. This fact explains why the patient's infection source remains unknown in many cases [1].

1.5. CLINICAL MANIFESTATIONS OF HEPATITIS A

HA symptoms appear abruptly 2–6 weeks after exposure; on average they appear 30 days after the exposure and can last up to 2 months [3,5,8,16]. Symptoms can be attributed to the lesions produced by the immune response [3]. The severity of clinical manifestations is age-dependent: 70% of children under the age of 6 years are asymptomatic, while typical symptoms appear in 70% of infections during late childhood, adolescence or as adults [6,8,19]. The initial symptoms, indistinguishable from other viral hepatitis, are fever, fatigue, malaise, loss of appetite, nausea, vomiting, abdominal discomfort, dark urine, diarrhea, clay-colored stool, joint pain and jaundice [6,16,25]. Symptoms intensify 4–6 days before the icteric phase, which appears in 70–80% of adults but only in 10% of children. During the jaundice period, symptoms decrease and the disease is usually self-limiting [3,8].

Unlike other hepatitis viruses, such as hepatitis B virus or hepatitis C virus, there is no evidence of chronic liver disease and more than 99% of cases resolve completely. However, in 3–20% of clinical cases a relapse of symptoms, which are milder than the initial illness, has been reported [6,8,12]. Moreover, although rarely, other atypical clinical manifestations can appear, such as prolonged cholestasis, acute kidney injury and autoimmune hepatitis [6].

Lastly, it is important to consider that immunosuppressed patients and patients with chronic liver disease have an increased risk of developing fulminant HA, which is a rare complication that may require a liver transplant and has a mortality rate of 80% [3,6,12,25].

1.6. DIAGNOSE OF HEPATITIS A

HA is diagnosed by clinical symptoms and laboratory variables such as elevated levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and alkaline phosphatase [6,16] (**FIGURE 3**). The Centers for Disease Control and Prevention (CDC) defines HA as ‘an acute illness with a discrete onset of any sign or symptom consistent with acute viral hepatitis and jaundice or elevated total bilirubin levels or elevated serum ALT levels and the absence of a more likely diagnosis’ [16].

Serological testing must be done as a confirmatory laboratory evidence. These tests can include the detection of immunoglobulin (Ig) M and IgA anti-HAV antibodies, as they are developed just before or at the same time as the onset of the symptoms and can be detected in both asymptomatic and symptomatic patients. IgG anti-HAV antibodies increase later in the course of the infection and provide lifelong immunity (**FIGURE 3**). Additionally, nucleic acid amplification and sequencing techniques to detect HAV RNA can be performed in body fluids and faeces [12,16].

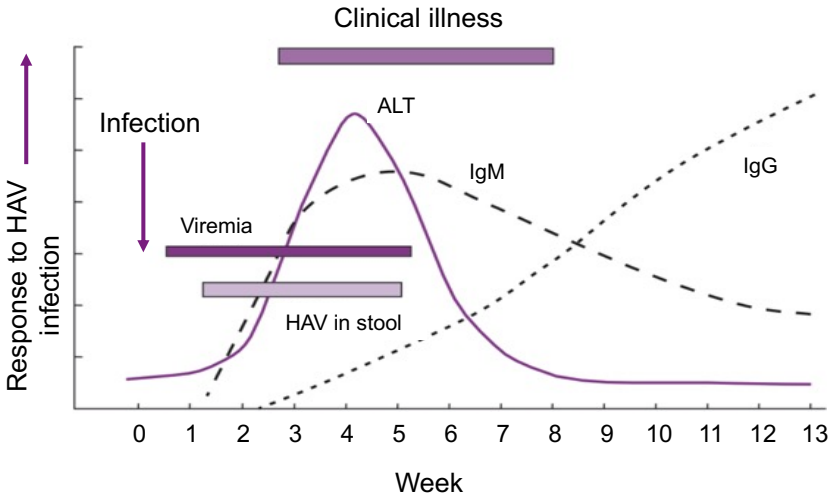

FIGURE 3: Immunological and clinical events associated with hepatitis A virus (HAV) infection and recovery.

Source: [26].

1.7. HEPATITIS A VACCINATION

There is no specific treatment for HA available, only symptomatic treatment. For this reason, prevention is key for the control of disease [5]. Certain drugs, such as paracetamol and medications against vomiting, should be avoided [2]. With regard to the prevention of HA, an effective measure is to improve sanitation and food and water supplies. However, the best way to prevent it is through vaccination [2,5,16].

Inactivated HAV vaccines have been available since the early 1990s. Currently, there are two types of HA vaccines available: inactivated and live-attenuated vaccines [12]. Inactivated vaccines are the most common worldwide and consist of viruses grown in cell culture, purified, inactivated with formalin and adsorbed to aluminium hydroxide (Alum) adjuvant. Nevertheless, these procedures are expensive and the HAV's poor growth in cell culture makes the vaccines' economic cost even higher. Also, in periods of high demand there have been vaccine shortages [25,27]. On the other hand, due to the successful use of the inactivated vaccines, the development of live-attenuated vaccines is hardly viable [25].

Both vaccines are safe and, thanks to the existence of a single serotype of HAV, are highly immunogenic [6,25,28]. In order to acquire long-term immunity, two doses are needed with an interval of 6–12 months, since protection with one dose only lasts for up to 2 years [6,8]. However, the period of time between the two doses can be extended to 18–36 months [12]. The first should be administered after 12 months of age, to avoid reactions if in the case of maternal antibodies having been passively acquired [8].

In highly endemic countries large-scale vaccination programmes are not recommended because most of the population have antibodies having acquired the HAV infection during childhood. Additionally, in low and very low endemic areas, vaccination programmes are only indicated for high-risk groups, such as travellers to areas of high endemicity, MSM, injection drug users and patients with chronic liver disease, among others [12]. In contrast, universal

vaccination programmes are indicated in those areas that are experiencing an epidemiological transition from high endemicity to intermediate endemicity. Therefore, recommendation for HA vaccinations depends on the epidemiological situation of each area [8,12].

1.7.1. IMMUNOGENIC PROPERTIES OF HEPATITIS A VACCINES

The defence against microorganisms is conducted through the effector mechanisms of the innate and adaptive immune systems. The innate immune system provides the initial defence, while the adaptive immune system provides a more potent and sustained response. In adaptive responses, a large number of effector cells and antibody molecules are generated. In the case of viruses, the innate response is characterized by type-I interferons (IFN) that inhibit viral replication of infected cells and by natural killer (NK) cells that kill infected cells. The adaptive immunity is characterized by antibodies (humoral response) and T lymphocytes (cellular response). Viral particles are phagocytosed by dendritic cells (DC), which act as antigen-presenting cell (APC), and present them to specific CD4+ T lymphocytes (Th cells) and $CD8^+$ T lymphocytes (Tc cells), which initiate the differentiation and activation process [29].

Most of the vaccines currently being used act by inducing the humoral immune response, as vaccines that stimulate the development of memory plasma cells and antibody production are considered to be the best [29]. However, it is becoming more apparent that T-cell-mediated immunity plays a key role in the control of viral infections [30].

The activation of Th cells and Tc cells by APC is a process that involves antigenic, costimulatory and cytokine (CK) signals. Once activated, Th and Tc cells modify intracellular signalling pathways that involve their activation, promoting proliferation, differentiation and migration of Th and Tc cells to inflamed tissues [30]. During the expansion phase, most activated Th and Tc cells acquire effector functions [31].

After the infection, most of the T cells are eliminated but a small fraction survives and persists as memory T cells even in the absence of antigen [30,31]. Memory T cells can be subdivided by distinct patterns of adhesion molecules and chemokine-receptors expressed on their cell surface. Traditionally, memory T cells are classified into two major categories based on their proliferation capacity, phenotypic features and migration potential: effector-memory T cells (TEM) and central memory T cells (TCM). Recently, another subtype of memory T cells has been described, called resident memory T cells (TRM), which might be further subdivided depending on the respective organ they reside in [31]. TEM have limited proliferation capacity but rapidly produce effector molecules and CK, such as IFN-γ and tumour necrosis factor (TNF). TCM exhibit a superior proliferation capacity and produce CK that are directly associated with better secondary expansion, such as interleukin (IL) 2 [30].

To classify the distinct T-cell subsets combinations of different markers are needed. Briefly, in humans, a typical marker combination for TEM is $CD45RO^+/CD62L^-/CD127^+/CD27^+/CCR7^-$,

for TCM the surface marker combination is $CD45RO^+/CD62L^+/CD127^+/CD27^+/CCR7^+$ and for TRM it is $CD45RO^+/CD62L^-/CD103^+/CD69^+$ [31]. Although humans and mice have analogous T-cell biology, T-cell subsets present some differences in the cell surface markers. In mice, the typical marker combination for TEM is $CD44^+/CD62L^-/CD127^+/CD27^+/KLRG1^+$, for TCM is $CD44^+/CD62L^+/$ $CD127^+/CD27^+/KLRG1^-$ and for TRM is $CD44^+/CD62L^-/CD103^+/CD69^+$. Recently, another subtype of memory T cells has been detected, which has a similar phenotype to I T lymphocytes: the stem memory T cells (TSCM). Further research is required to demonstrate whether vaccines can also stimulate TSCM production [30,31].

As stated before, HAV was considered a non-enveloped virus, but recently it has been discovered that HAV particles can hijack host cellular membranes to exit cells fully encapsulated in a lipid membrane that resemble exosomes [15,32]. Exosomes are small vesicles surrounded by a lipid bilayer formed through the fusion of multivesicular bodies with the plasma membrane that are released from several cell types like B cells and DC [33,34]. Although their physiological role is unknown, they have been suggested to take part in T-cell stimulation [33]. It has been shown that encapsulated HAV virions (eHAV) are responsible for DC activation and IFN production [22]. When DC are activated, they migrate to secondary lymphoid organs, where DC present pathogen-derived epitopes to Th and Tc lymphocytes, promoting their maturation [35]. Thus, exosomes play an important role in HAV infection and can be assessed to formulate a virus-like particle (VLP) vaccine. VLP vaccines are based on virus-derived proteins, which are assembled to form a particle [22,36]. These vaccines induce a particular immune response that involves both specific and non-specific pathways and leads to an activation of Th and Tc cells. Furthermore, VLP vaccines deliver pathogen-associated molecular patterns (PAMPs) that induce B-cell stimulation, something that adjuvants normally do. Thus, VLP vaccines could be formulated without adjuvants, although these may enhance the specific immune response [36].

Aluminium adjuvants, such as Alum, are the most used in HAV vaccines because they induce antibody production, although they have not been shown to induce cell-mediated immunity [12,37]. These adjuvants are known to induce side effects, such as erythema, granulomas and contact hypersensitivity, thus there is a desire to reduce their use in modern vaccines [37]. One of the reasons why eHAV virions could represent an alternative to the classic adjuvants is that it has been demonstrated that they enhance the immune response [36,38]. Additionally, the fact of not needing adjuvants would reduce the cost of the vaccines, and the use of a fast-growth eHAV strain described in the previous bibliography should avoid vaccine shortages [25,27]. Moreover, it has been reported that a single inactivated HAV vaccine dose can induce memory T cell production [39].

2. OBJECTIVES

This study includes preliminary experiments carried out as the first part of a project funded by La Marató-TV3 (ref. 201828-31) aimed at characterizing the potential of eHAV particles as possible vaccines for this disease.

The particular objectives of the current study were:

- To set up the immunization protocol to achieve an immune response to inactivated HAV together with adjuvant, in two strains of mouse.
- To set up techniques for determining the immune response, in particular the proliferative capacity, the number of antibody-secreting cells and changes in cell phenotype after activation.

To achieve these objectives, a reduced number of animals were used (belonging to mouse strains BALB/c and C57BL/6) and the immunization protocol followed in HAV was compared to that carried out in BALB/c mice that received ovalbumin in the same conditions.

3. MATERIALS AND METHODS

3.1. ETHICS, ANIMALS AND SECURITY MEASURES

This study was approved and conducted in strict accordance with the guidelines for the use of experimental animals of the Ethical Committee for Animal Experimentation at the University of Barcelona (ref. number 149/19) and the Generalitat de Catalunya (Project number 10761).

Six-week-old female BALB/c (BALB/cOlaHsd, n=12) and C57BL/6 (C57BL/6JolaHsd, n=4) mice (Envigo RMS, Spain) were used. The animals were housed in the Animal Experimentation Unit of the Faculty of Pharmacy and Food Science at the University of Barcelona, under controlled conditions of temperature (21 ± 2 ºC) and humidity (50%) and under a light/dark cycle of 12 h. After HAV inoculation, mice were housed in Type II biosecurity conditions (biosecurity room in the same Animal Experimentation Unit under conditions of negative pressure) and HAV was manipulated in a Class II microbiological safety cabinet located in the same room. The animals were provided with food and water *ad libitum*. Animals were handled by accredited researchers who followed the instructions of the Office of Safety, Health and the Environment of the University of Barcelona (OSSMA-UB), such as wearing two single-use coats over the lab coat, two vinyl gloves of AQL 1.5 quality (following regulations EN 455, EN 374 and EN 374-5 for bacteria, viruses and fungi handling), security glasses and FFP3 mask.

3.2. PROCEDURE

To establish the immunization protocol for HAV and monitoring the mice immune response, a long-time protocol was followed in which samples were collected at different time points (**FIGURE 4**).

Mice were randomly distributed, according to weight, into four groups (n=4 per group):

- OVA-1: BALB/c mice
- OVA-2: BALB/c mice
- HAV-1: BALB/c mice
- HAV-2: C57BL/6 mice

On days 0, 14, 28 and 42, animals were immunized with ovalbumin (OVA, Merck Life Sciences, Darmstadt, Germany) or inactivated eHAV. Mice in groups OVA-1 and OVA-2 received 100 µL of a suspension containing 20 µg of OVA and 4 mg of Alum (Imject® Pierce, Rockford, IL, USA) by subcutaneous (s.c.) route in the intrascapular area. Mice in groups HAV-1 and HAV-2 received 100 µL of a suspension containing 100 µL of Alum and inactivated eHAV at a dose of 10^7 TCID50 (tissue culture infectious dose 50%) produced and titred by the Enteric Virus Group (Dr R.M. Pintó) in the Department of Genetics, Microbiology and Statistics of the Faculty of Biology (University of Barcelona).

Prior to immunization and on days 28, 42, 48 and 55, blood samples were obtained from the mandibular plexus (**FIGURE 4**). After plasma separation, it was preserved at -20 °C until it was used for the determination of specific antibodies. Throughout the study, the behaviour and weight of the animals were monitored.

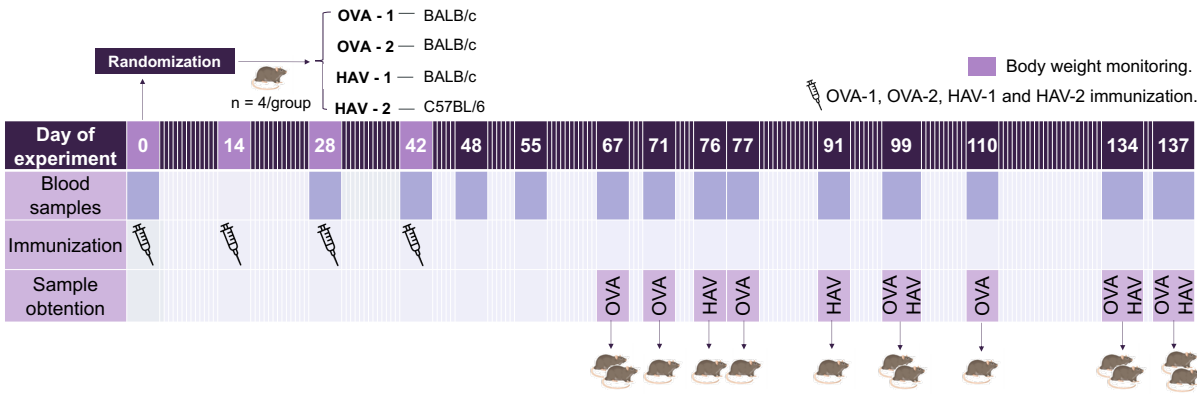

FIGURE 4. Experimental design. Animals were immunized on days 0, 14, 28 and 42. Blood samples were obtained, and body weight was monitored throughout the study.

At different time points (days 67, 71, 76, 77, 91, 99, 110, 134 and 137) a particular animal was randomly selected and blood (from heart) and spleen samples were collected under anaesthesia with isoflurane (Ecuphar, Barcelona, Spain) (**FIGURE 4**). Spleen was removed aseptically and placed in 3 mL of complete culture medium (Roswell Park Memorial Institute medium or RPMI, containing 10% foetal bovine serum (FBS), 1% glutamine, 1% penicillin and streptomycin solution, and 0.5% 50 µM 2ß-mercaptoethanol).

3.3. SPLEEN LYMPHOCYTE ISOLATION

After collecting the spleen, lymphocyte isolation was immediately performed. Spleen samples were treated in a biosafety cabinet that keeps the samples in sterile conditions. When working with HAV-immunized animals' samples, a cabinet that protects both the sample and the operator was used (Class II microbiological safety cabinet).

Lymphocytes were obtained after placing the spleen into a 40 µm cell strainer (Thermo Fisher Scientific, Barcelona, Spain) and smashing it using the plunger of a 2.5 mL syringe on a Petri dish. This process was carried out in cold conditions (on the top of ice) to maintain the viability of the cells. Cell suspension was collected with a sterile Pasteur pipette and deposited into a sterile 15 mL tube. The suspension was then centrifuged at 538 *g* for 10 min at 4 °C (Megafuge 2.0R centrifuge, Heraus). Thereafter, spleen erythrocytes were removed by osmotic lysis. After discarding the supernatant, the pellet was resuspended in 0.5 mL of sterile phosphate-buffered saline (PBS, pH 7.2) and then 8.5 mL of distilled water were added to make a hypotonic medium. The tube was immediately covered and inverted three times. Immediately after, 1 mL of sterile 10X PBS was added, the tube was capped, inverted five times and centrifuged again under the same conditions as before. The pellet was resuspended in 10 mL of complete medium.

Cell viability and concentration were assessed by a Countess™ Automated Cell Counter (Invitrogen, Thermo Fisher Scientific, Barcelona, Spain). For this, 10 µL of a 1/10 cell dilution were mixed with 10 µL of Trypan Blue (Invitrogen), placed in a Countess™ counting chamber slide and inserted into the machine.

3.4. CRYOPRESERVATION

In some cases, cells from spleen were frozen to be studied later. Cryopreservation is a process aimed at preserving cells without performing a permanent culture. This process was conducted according to bibliography [40,41].

Spleen lymphocytes were frozen after centrifuging the cells and resuspending the pellet in 1 mL of FBS (about 10^6 cells/mL) containing 10% dimethyl sulfoxide (DMSO, Merck Life Sciences, Darmstadt, Germany) that acts as a cryoprotectant. Cryotube vials were stored at –80 °C for several days or few weeks.

To thaw the cells, the cryotube content was diluted in warm complete medium (50 mL). Cell suspension was immediately centrifuged and cell viability and concentration were assessed following the process described in the previous section (3.3 Spleen lymphocyte isolation).

3.5. ANTI-OVA ANTIBODY DETERMINATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For the determination of specific anti-OVA antibodies, an indirect ELISA assay was carried out. For this purpose, 96-well polystyrene plates (Nunc MaxiSorp, Wiesbaden, Germany) were sensitized with 100 µL/well of an OVA solution (Merck Life Sciences) and incubated overnight at 4 °C in a humid chamber. After sensitization, remaining active sites in the plate were blocked by adding 200 µL/well of a 0.5% solution of gelatin (Merck Life Sciences) in PBS for 1 h. The plate was then washed three times with 200 µL/well of a washing solution (PBS with 0.05% Tween 20) and with a final wash with just PBS.

After blocking, diluted samples in PBS with 0.5% gelatin and 0.05% Tween 20 were added (100 µL/well) and incubated overnight at 4 °C in a humid chamber. After washing again, 100 µL/well of a detection antibody (biotinylated anti-mouse IgG, Merck Life Sciences) solution were added for 2 h (room temperature [RT] in a humid chamber). After washing, 100 µL/well of a 1/2000 dilution of ExtrAvidin®-peroxidase (Merck Life Sciences) were incubated for 30 min (RT in a humid chamber). Next, the plate was washed and 200 µL/well of an *O*-phenylenediamine dihydrochloride (OPD, Merck Life Sciences) solution in phosphate-citrate buffer (pH 5) with 0.04% of hydrogen peroxide at 30% (Merck Life Sciences) were added. The plate was left in agitation for 10–30 min. During this interval, pre-readings were taken at 450 nm. The reaction was stopped with 50 µL/well of 3 M sulphuric acid and the absorbance was read at 492 nm with a microplate photometer (Labsystems Multiskan^{MS}, Helsinki, Finland).

As stated in the Results section 4.2, ELISA for anti-OVA antibody quantification was set up by testing two dilutions of the detection antibody and two concentrations of OVA to sensitize the solid phase.

3.6. SPECIFIC SPLEEN LYMPHOCYTE PROLIFERATION ASSAY

The culture of spleen lymphocytes with specific stimulation to carry out a proliferation assay was conducted in 96-well U-bottom culture plates (TPP, Tissue Culture Plates, Switzerland). As habitually performed in these cases, we worked in quadruplicate, using stimulated and non-stimulated (NS) cells. Lymphocytes obtained according to the procedure described in Section 3.3, were tested at different concentrations. A cell suspension was added to each well and stimulated with either OVA or HAV at different conditions (with or without exosome, i.e. eHVA and HAV) and concentrations. Exosome was removed by treating HAV antigens with 1% nonidet-P40 or NP40 for 30 min at 37 ºC and three 30 s sonication cycles at 60 W. In wells containing NS cells, medium was added instead of the stimuli. The plates were incubated for 5 days at 37 ºC with a 5% of CO_2. As several conditions were tested, cell and stimuli concentrations used in each assay are described in the Results section.

To quantify the proliferative activity, a cell proliferation colorimetric kit (Merck Life Sciences) based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis by ELISA was used. For this, 20 µL/well of BrdU (1/100 dilution in labelling reagent) were added to the proliferative cells. This step was performed in the appropriate biosafety cabinet. After 2 h, the plate was centrifuged (10 min, 300 *g*), the supernatants were discarded (by aspiration in the biosafety cabinet) and the plate was dried with a hairdryer.

The next step entailed adding the fixation solution (FixDenat, 200 µL/well, 30 min, RT, in a humid chamber). Subsequently, the plate was emptied (not washed) and 100 µL/well of horseradish peroxidase-conjugated anti-BrdU antibody (Anti-BrdU-HRP, 1/100) were added. After 90 min of incubation (RT in a humid chamber), the plate was washed with washing buffer (3 times, 200 µL/well). Then, 100 µL/well of substrate solution (3,3',5,5'-tetramethylbenzidine [TMB]) were added, and the plate was incubated for 5 to 30 min (darkness, RT, under agitation). Pre-readings were taken at 595 nm. The reaction was stopped with 25 µL/well of 1M sulphuric acid and absorbance reading was done at 450 nm with a microplate photometer (Labsystems Multiskan^{MS}).

3.7. ENZYME-LINKED IMMUNOSORBENT SPOT (ELISPOT)

The ELISPOT assay was used to quantify the number of cells producing antibodies in the spleen of immunized animals.

The first phase entailed the sensitization of multiscreen-HA 96-well plates (Millipore, MAHAS4510) in a sterile environment. In this step, the plate was coated either with OVA (Merck Life Sciences, 100 µL/well, 50 or 100 µg/mL in coating buffer pH 9.6) or an anti-HAV antibody plasma (convalescent plasma, dilution 1/1000, 100 µL/well in coating buffer pH9.6). After incubating the plate overnight at 4 °C in a humid chamber, the plate was washed with sterile PBS (200 µL/well, 5 times for 2 min each one). The plates coated with anti-HAV antibody were then incubated with eHAV (100 µL/well, 10^5 TCID50 in PBS) for 2 h (RT in a humid chamber) and washed with sterile PBS (200 µL/well, 5 times for 2 min each one).

Plates coated with OVA and HAV were then blocked with complete medium (200 µL/well) for at least 1 h at 37 ºC. After discarding the medium, serial dilutions of spleen lymphocytes were added (100 µL/well) and incubated at 37 °C and 5% CO_2 for 18, 24, 48 or 72 h. In this period, lymphocytes from the immunized animals produced anti-OVA or anti-HAV antibodies that were caught around the cell. After incubation, cells were discarded and plates were washed (9 x 2-min washes in PBS with 0.25% Tween, plus 1 x 2-min wash with sterile distilled water). From this point on, no more sterile work was necessary.

To evidence antibodies secreted by stimulated lymphocytes, biotinylated anti-mouse IgG antibody (Merck Life Sciences, 100 µL/well) was added for 2 h (RT in a humid chamber) or overnight (4 °C in humid chamber). Then washes were done with PBS-Tw (5 x 2-min, 200 µL/well). Finally, ExtrAvidin®-peroxidase (Merck Life Sciences, 100 µL/well, 1/5000 in

PBS) was added for 1 h (RT in a humid chamber). After 5 x 2-min washes, the plates were dried and 3-amino-9-ethylcarbazole (AEC, Merck Life Sciences) substrate (100 µL/well) plus hydrogen peroxide were incubated for 15–30 min at RT. The plate was then washed with tap water (5 min) and left to dry in a dark and dry place.

The spots corresponding to each one-to-one antibody-secreting cell were coloured with a red-brown colour and were preserved for a long time. Spots were considered after being counted by an automatic counter (EliSpot reader AID, Autoimmun Diagnostika GMBH); the counter provided photos from each well and an automatic counting of spots. Antibody-secreting cell counts were confirmed by visual estimation.

3.8. MEMORY CELL DETECTION BY FLOW CYTOMETRY ANALYSIS

To determine the memory cells in OVA-immunized animals, spleen lymphocytes were OVA-stimulated and cell phenotype was established by immunofluorescence staining followed by flow cytometry analysis. Staining of extracellular (antibodies anti-CD3, anti-CD4, anti-CD44, anti-CD62L and anti-CD8 were used) and intracellular (anti-IFN-γ and anti-IL-2) molecules was carried out. All antibodies were conjugated to different fluorochromes and provided by Palex Medical SA, Barcelona, Spain).

Spleen lymphocytes from OVA-immunized mice (Section 3.3) were prepared at $6x10^6$ cells/mL and 500 µL/well were distributed into 12-well culture plates (TPP). We worked with stimulated and unstimulated cells according to the procedure described in previous literature [42]. For this, an OVA solution (25 or 50 µg/mL) or medium was added. The cells were incubated overnight at 37 °C with 5% CO_2. Prior to cell staining, 10 µL/well of brefeldin (Merck Life Sciences, 5 mg/mL) were added for 2 h. Subsequently, cells were collected and counted by a Countess™ automated cell counter (Invitrogen).

About 500,000 cells were deposited in a 5 mL tube and washed with 1 mL of complete medium. After that, cells were treated with 1 mL of PBS containing 2% FBS and 0.1% azide (PBS-FBS-Az, Merck Life Sciences) and centrifuged (535 *g*, 5 min, 4 °C) before their Fc receptors were blocked by adding 100 µL/tube of a 1/10 dilution of rat serum (10 min, 4 °C). After washing again, cells were labelled following an incubation (for at least 20 min, 4 °C, protected from light) with saturating concentration of an appropriate dilution of a fluorochrome-conjugated antibody against an extracellular molecule (**TABLE 1**). Different control tubes were also included. Afterwards, cells were washed with 1 mL of PBS.

TABLE 1: Rat anti-mouse monoclonal antibodies (Mab) used in this study: target molecules (extracellular and intracellular), fluorochrome and working dilution

	Extracellular staining					Intracellular staining	
Anti-mouse Mab	CD3	CD4	CD8	CD44	CD62L	IL-2	IFN
Fluorochrome	APC-Cy7	PerCP-Cy5	FITC	PE	APC	BV711	BV421
Dilution	1/5	1/20	1/20	1/5	1/20	1/5	1/5

For intracellular labelling, the cells must be fixed and permeabilized by means of a fixation/permeabilization solution (Life Technologies, SA, Madrid, Spain, 600 µL/tube, 30 min, 4 °C in the dark). Then, cells were washed with 1 mL of the permeabilization buffer (Life Technologies) and centrifuged (535 *g*, 5 min, 4 °C). After discarding the supernatant, 10 µL of the intracellular Mab appropriate dilutions (**TABLE 1**) were added for 30 min (4 °C, protected from light). Finally, a double washing was performed with PBS. Samples were kept in 300 µL of PBS, at 4 ºC in the dark until processing. Analyses were performed using a Gallios Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB) and data were assessed by the FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA). Results are expressed as percentage of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC).

3.9. DATA ANALYSIS

The results obtained are expressed as the mean ± standard deviation (S.D.) and were plotted with the SigmaPlot 12.1 program. Statistical analysis was performed with Microsoft Excel. To calculate the statistically significant differences between the different study groups, the Student's t-test was used. In all cases, differences with $p < 0.05$ were accepted as significant.

4. RESULTS

4.1. ANIMAL SUPERVISION

During the study, animals' welfare was checked according to the approved procedure. It was established by body weight, animal behaviour and appearance. No change was observed that involved discarding any animal. As is well known by animal researchers, C57BL/6 mice are more aggressive and fidgety than BALB/c mice, which led to more difficult handing.

4.2. ANTI-OVA ANTIBODY DETERMINATION

An ELISA technique was set up to quantify anti-OVA antibodies. To do so, three ELISA assays were performed. The objective of the first assay was to establish the optimal dilution of the samples and the detection antibody. In the second assay, the main purpose was to determine the most suitable concentration of the capture antigen. The third assay was done to assess the evolution of the anti-OVA antibody titre over the study period.

4.2.1. ESTABLISHMENT OF WORK DILUTIONS OF SAMPLES AND DETECTION ANTIBODY

First, samples from the OVA-1 and OVA-2 groups at different time points were analysed at two dilutions (1/50 and 1/300) applying two conditions of detection antibody (anti-mouse IgG-Biotin, 1/2000 and 1/8000). Results are summarized in **TABLE 2.**

TABLE 2: Results obtained from plasma pools corresponding to OVA-1 and OVA-2 groups (diluted 1/50 and 1/300) at different time points from immunization (days 0, 28, 42 and 48 of the study) using two different dilutions of the detection antibody. Results are expressed in absorbance values ± S.D. (n=2). * $p < 0.05$ vs day 0

Day	Anti-mouse IgG-biotin (1/2000)		Anti-mouse IgG-biotin (1/8000)	
	Sample dilution 1/50	Sample dilution 1/300	Sample dilution 1/50	Sample dilution 1/300
0	0.070 ± 0.004	-	0.051 ± 0.001	-
28	1.276 ± 0.027*	1.251 ± 0.032	0.546 ± 0.032*	0.577 ± 0.019
42	1.265 ± 0.016*	1.228 ± 0.004	0.638 ± 0.005*	0.600 ± 0.012
48	1.298 ± 0.134*	1.417 ± 0.044	0.652 ± 0.019*	0.681 ± 0.004

As can be seen, anti-OVA antibodies were already detected by day 28, since the absorbance values were significantly higher than the values on day 0 ($p < 0.05$ vs day 0). The absorbance results did not show significant differences between samples diluted 1/50 and 1/300 but were significantly higher when working with the more concentrated detection antibody ($p < 0.05$ 1/2000 vs 1/8000). These results prompted us to establish an intermediate working dilution of the detection antibody at 1/5000.

4.2.2. ESTABLISHMENT OF CAPTURE ANTIGEN CONCENTRATION

After the first assay, two OVA conditions were tested to coat the plate (10 and 50 µg/mL). In this assay, anti-OVA antibodies from plasma samples were also titred. The results can be seen in **TABLE 3.**

These results allowed us to establish that a solution with 10 µg/mL of OVA was enough to coat the plate for anti-OVA antibodies detection as absorbance values were non-statistically significant or even higher than those obtained with 50 µg/mL of OVA.

TABLE 3: Results obtained from pooled samples from groups OVA-1 and OVA-2 (serially diluted between 1/300 and 1/19200) at different time points from immunization using two capture antigen conditions (10 and 50 µg/mL). Results are expressed in absorbance values ± S.D. (n=2). * $p < 0.05$ vs 10 µ/mL OVA

Day	[OVA] (µg/mL)	Sample dilution						
		1/300	1/600	1/1200	1/2400	1/4800	1/9600	1/19200
28	**10**	2.65 ± 0.03	2.64 ± 0.01	2.60 ± 0.04	2.67 ± 0.01	2.44 ± 0.11	2.22 ± 0.17	1.66 ± 0.24
	50	2.55 ± 0.01*	2.56 ± 0.03	2.58 ± 0.04	2.58 ± 0.05	2.38 ± 0.06	2.09 ± 0.08	1.59 ± 0.27
42	**10**	2.57 ± 0.05	2.58 ± 0.06	2.62 ± 0.05	2.76 ± 0.01	2.59 ± 0.17	2.45 ± 0.10	2.26 ± 0.10
	50	2.57 ± 0.05	2.61 ± 0.02	2.63 ± 0.12	2.70 ± 0.01*	2.63 ± 0.02	2.53 ± 0.06	2.26 ± 0.10
48	**10**	2.62 ± 0.05	2.66 ± 0.03	2.60 ± 0.02	2.72 ± 0.08	2.47 ± 0.02	2.49 ± 0.11	2.24 ± 0.21
	50	2.45 ± 0.23	2.50 ± 0.10	2.50 ± 0.25	2.52 ± 0.19	2.42 ± 0.25	2.32 ± 0.30	2.64 ± 0.39

4.2.3. ASSESSMENT OF THE EVOLUTION OF ANTI-OVA ANTIBODY LEVELS

Once the ELISA technique was set up, the assay was carried out to establish the time course of the anti-OVA antibodies throughout the study. This assay was conducted with plasma samples diluted at 1/240,000. Samples were obtained on days 0 (before immunization), 28 (after 2 immunizations), 42 (after 3 immunizations), 48, 55, 67 (after 4 immunizations) and the day of the animals' euthanasia (days 71, 77, 99 and 110). Results are shown in **FIGURE 5**.

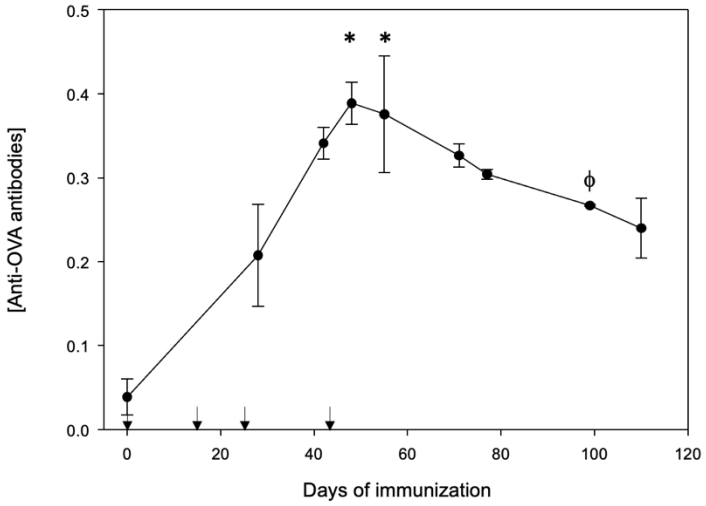

FIGURE 5: Time-course of plasma anti-OVA antibodies throughout the study. Results are expressed in absorbance values ± S.D. (n=4-8). Arrows indicate the immunization days (0, 14, 28 and 42). Differences $p < 0.05$: * days 48 and 55 vs days 0, 28 and 42, ϕ day 99 vs days 48 and 55.

As shown in **FIGURE 5**, the anti-OVA antibodies titre gradually increased during the first seven weeks, in which four immunizations were injected (each one two weeks away) and achieved maximum values on days 48–55 ($p < 0.05$ vs previous days). After day 55, anti-OVA antibody titres tended to decrease until the last day of the study ($p < 0.05$ day 99 vs days 48–55).

4.3. VIABILITY OF LYMPHOCYTES FROM SPLEEN

Cell viability was assessed in each experiment in which we obtained spleen lymphocytes from any animal both from OVA and HAV groups. Cell viability ranged from 65–88%. Likewise, cell viability was assessed after thawing cells. In this case, cell viability was about 85%. In all cases, to carry out the assays with cells, we only considered live cells.

4.4. LYMPHOCYTE PROLIFERATION ASSAY FOR OVA-SENSITIZED GROUPS

To establish the optimal procedure for measuring the lymphocytes' proliferative ability, several proliferation assay conditions were tested.

4.4.1. ESTABLISHMENT OF THE CELL CONCENTRATION FOR CELL CULTURE

In the first assay, three different cell concentrations were stimulated under two OVA conditions. One hundred µL/well of a cell suspension (3×10^6 cells/mL, 5×10^6 cells/mL or 6×10^6 cells/mL) were stimulated with 100 µL/well of an OVA solution (25 or 50 µg/mL) for 96 h. Cells tested were spleen lymphocytes obtained aseptically from a mouse of the OVA-1 group at day 67 of the study. Results can be seen in **TABLE 4.**

TABLE 4: Absorbance values expressed as stimulated/NS ratio. Cells were stimulated for 96 h under two OVA conditions. Results are expressed as mean ± S.D. (n=4)

	OVA condition	
Cell concentration	**25 µg/mL**	**50 µg/mL**
3 x 10^6 cells/mL	2.606 ± 0.512	3.096 ± 0.556
5 x 10^6 cells/mL	5.027 ± 0.459	4.757 ± 0.246
6 x 10^6 cells/mL	3.251 ± 0.261	3.197 ± 0.143

No significant differences were observed in any of the conditions tested ($p > 0.05$). From these results we established that a concentration of 3 x 10^6 cells/mL and stimulation with 25 µg/mL of OVA for 96 h was enough to quantify the proliferative activity of spleen lymphocytes in immunized animals.

4.4.2. ASSESSMENT OF FROZEN CELLS

Once the stimulation conditions were established, an assay was performed to test the proliferative activity in frozen cells and compare it with that of fresh cells. In this assay, spleen lymphocytes of mice from the OVA-1 group were used. Frozen cells (obtained at day 110 of the study) and fresh cells (obtained at day 134 of the study) were used. In both cases, 75 µL/well of a cell suspension (3x10^6 cells/mL) were incubated for 96 h with 25 µL/well of 25 µg/mL OVA solution. Results are summarized in **FIGURE 6.**

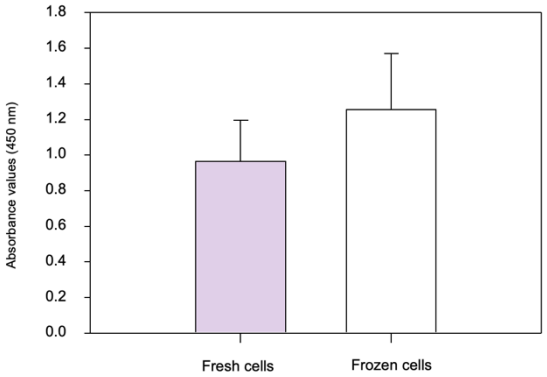

FIGURE 6: Absorbance values representing the proliferative activity of fresh and frozen cells. Results are expressed as mean ± S.D. (n=4).

After performing this assay, we concluded that the proliferation ability of spleen lymphocytes in OVA-immunized mice can be assessed in either fresh or frozen cells since no statistically significant differences were observed ($p > 0.05$).

4.5. LYMPHOCYTE PROLIFERATION ASSAY FOR HAV-SENSITIZED GROUPS

As for the OVA-immunized groups, several assays were performed with the HAV-immunized animals' cells to determine the optimal proliferation assay conditions. The first assay served to establish the virus conditions. The second assay was applied to compare the proliferative ability of spleen lymphocytes between the two mice strains used in the study (BALB/c and C57BL/6). The third assay was performed to test the proliferative ability of BALB/c mice's spleen lymphocytes.

4.5.1. ESTABLISHMENT OF VIRUS CONDITIONS

In the first assay, 100 µL/well of a cell suspension ($3x10^6$ cells/mL) were stimulated under two HAV conditions (with or without exosome, i.e. eHVA and HAV) and at three different HAV concentrations (10^5, 10^6 and 10^7 TCID50, 25 µL/well). Cells tested were spleen lymphocytes obtained aseptically from a HAV-1 mouse at day 76 of the study. Results can be seen in **FIGURE 7**.

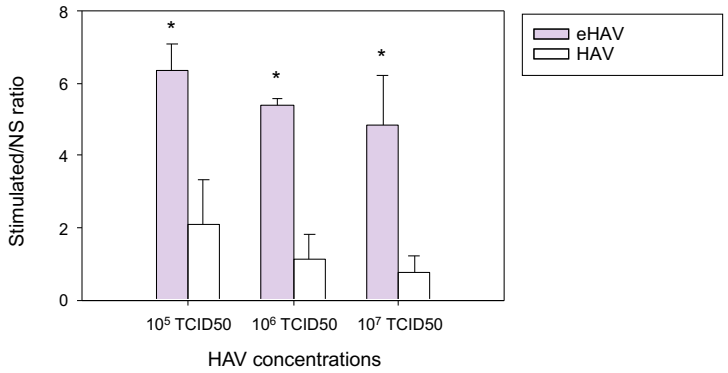

FIGURE 7: Absorbance values expressed as stimulated/NS ratio. Cells were stimulated for 96 h under two HAV conditions and at three different HAV concentrations. Results are expressed as mean ± S.D. (n=3). * $p < 0.05$ vs HAV

In all cases, cells stimulated with eHAV showed a significantly higher proliferation ability ($p < 0.05$). These results prompted us to establish eHAV at 10^5 TCID50 as the optimal condition for the proliferation assay in HAV-immunized animals.

4.5.2. COMPARISON BETWEEN MICE STRAINS

A second proliferation assay was carried out to compare the cells' proliferation ability between the two mice strains used in this study. One hundred µL/well of a cell suspension ($3x10^6$ cells/mL) were stimulated under a eHAV solution (25 µL/well) with the HAV conditions established in the previous assay (10^5 TCID50). Cells tested were spleen lymphocytes obtained aseptically from HAV-1 and HAV-2 mice at day 91 of the study. Results are summarized in **FIGURE 8**.

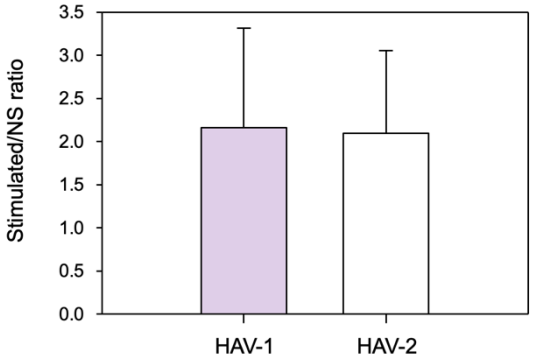

FIGURE 8: Absorbance values (Stimulated/NS ratio) of the cells stimulated for 96 h with HAV with exosome at 10^5 TCID50. Results are expressed as mean ± S.D. (n=4).

This assay showed no statistically significant differences between the two mice strains ($p > 0.05$).

4.6. ANTIBODY-SECRETING CELLS OF ANTI-OVA ANTIBODIES

For enumerating the antibody-secreting cells an ELISPOT assay was used. A first assay was performed to establish the optimal concentration of the capture OVA solution. Afterwards, a second assay was performed to establish the incubation period of cells and to compare the antibody production between fresh and frozen cells. Concurrently, this second assay was also performed with HAV. The spots representing the secreting cells were manually counted. The criterion that was established for counting the cells was the presence of a halo around the spot, as can be seen in **FIGURE 9** and **FIGURE 10**.

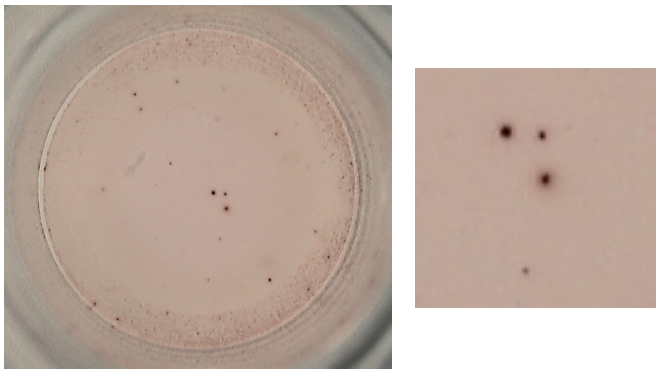

FIGURE 9: ELISPOT assay for anti-OVA antibody-secreting cells. Detail of positive cells

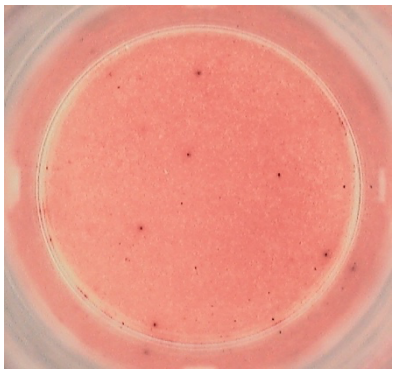

FIGURE 10: ELISPOT assay for anti-HAV antibody-secreting cells

4.6.1. ESTABLISHMENT OF OVA CONCENTRATION

The first ELISPOT assay was performed under two OVA conditions (50 and 100 µg/mL). Spleen lymphocytes (obtained from an OVA-2 mouse at day 110 of the study) were serially diluted (2, 1, 0.5 and 0.25 x 10^6 cells/mL) and incubated for 18 h. Results are summarized in **TABLE 5**. These results did not show any statistically significant differences between coating the plate with OVA at 50 or 100 µg/mL ($p > 0.05$). Nevertheless, since it seemed that the number of spots tended to be slightly higher using OVA at 100 µg/mL, we performed a second assay with this condition varying the incubation time of the cells.

TABLE 5: Counts of anti-OVA antibody-secreting cells. Results are expressed as the mean of spots per well or per 10^6 cells ± S.D. (n=6) considering all cells incubated under the same OVA condition

	Stimuli condition	
Cell amount	**OVA 50 µg/mL**	**OVA 100 µg/mL**
2 x 10^5 cells	3.50 ± 0.96	4.50 ± 2.81
1 x 10^5 cells	2.67 ± 1.70	2.50 ± 1.71
0.5 x 10^5 cells	1.00 ± 1.15	1.83 ± 1.34
0.25 x 10^5 cells	0.50 ± 0.76	0.83 ± 0.90
Total (spots/10^6 cells)	12.27	15.47

4.6.2. ESTABLISHMENT OF INCUBATION PERIOD AND ASSESSMENT OF FROZEN CELLS

The second ELISPOT assay was performed with spleen lymphocytes obtained from an OVA-1 mouse at day 137 of the study and with frozen cells obtained from an OVA-2 mouse at day 110 of the study. Cells were serially diluted (1, 0.5, 0.25 and 0.125 x 10^6 cells/mL) and incubated for 24, 48 or 72 h. Results are summarized in **TABLE 6**.

TABLE 6: Counts of anti-OVA antibody-secreting cells. Results are expressed as the mean of spots per well or per 10^6 cells ± S.D. (n=2) considering all cells incubated under the same condition

	Fresh cells			Frozen cells		
Cell amount	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
1 x 10^5 cells	11.00 ± 7.07	6.00 ± 2.83	8.50 ± 0.71	8.00 ± 2.83	8.00 ± 4.24	11.00 ± 1.41
0.5 x 10^5 cells	2.50 ± 2.12	4.50 ± 0.71	5.50 ± 0.71	3.50 ± 0.71	4.50 ± 0.71	4.50 ± 2.12
0.25 x 10^5 cells	2.0 ± 0.00	1.50 ± 0.71	2.00 ± 2.83	1.00 ± 1.71	2.00 ± 1.41	3.50 ± 0.71
0.125 x 10^5 cells	0.00 ± 0.00	3.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00
Total (spots/10^6 cells)	8.27	8.0	8.53	6.67	8.0	10.13

The results of the ELISPOT assay for enumerating anti-OVA secreting cells allow us to conclude that this technique could be applied either in fresh or frozen cells and incubating the cells for 24–72 h without statistically significant changes between these conditions ($p > 0.05$).

4.7. ANTIBODY-SECRETING CELLS OF ANTI-HAV ANTIBODIES

ELISPOT assay was applied to spleen lymphocytes from two HAV-1 animals obtained at day 137 of the study. Cells were serially diluted (1, 0.5, 0.25 and 0.125 x 10^6 cells/mL) and incubated for 24, 48 or 72 h. Results are summarized in **TABLE 7.**

TABLE 7: Counts of anti-HAV antibody-secreting cells. Results are expressed as the mean of spots per well or per 10^6 cells ± S.D. (n=2) * $p < 0.05$ vs mouse B

Cell amount	**24 h**		**48 h**		**72 h**	
	Mouse A	**Mouse B**	**Mouse A**	**Mouse B**	**Mouse A**	**Mouse B**
1 x 10^5 cells	12.00 ± 4.24	0.50 ± 0.71	7.00 ± 1.41	2.50 ± 2.12	12.50 ± 0.71*	3.00 ± 1.41
0.5 x 10^5 cells	6.00 ± 0.00*	1.00 ± 1.41	5.00 ± 0.00	1.50 ± 2.12	5.00 ± 2.83	1.50 ± 2.12
0.25 x 10^5 cells	1.50 ± 0.71	0.00 ± 0.00	2.50 ± 0.71	2.00 ± 1.41	4.50 ± 0.71	1.50 ± 1.12
0.125 x 10^5 cells	0.50 ± 0.71	0.00 ± 0.00	0.50 ± 0.71	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total (spots/10^6 cells)	10.67*	0.80	8.00	3.20	11.73*	3.20

As can be seen, the two analysed spleen samples showed a different behaviour in the ELISPOT assay. Whereas mouse A displayed a considerable number of antibody-secreting cells (about 8–11/10^6 spleen lymphocytes), mouse B showed hardly any. Considering the time of incubation, 24 h is enough to detect such cells and longer periods seem to decrease the sensitivity.

4.8. MEMORY CELLS

The established lymphocyte populations were defined as follows: T cells ($CD3^+$), Th cells ($CD4^+$ inside CD3+), and Tc cells ($CD8^+$ inside CD3+). In each subset, cells were classified as TEM ($CD44^+$ $CD62L^-$), TCM ($CD44^+$ $CD62L^+$) or naïve cells ($CD44^-$ $CD62L^+$). Additionally, cells with intracellular IFN-γ in TEM subsets and cells with intracellular IL-2 in TCM subsets were established (**FIGURE 11**). The percentages of both TEM and TCM in Th (ThEM and ThCM) and Tc (TcEM and TcCM) subsets after the stimulation with two different OVA conditions are summarized in **TABLE 8**.

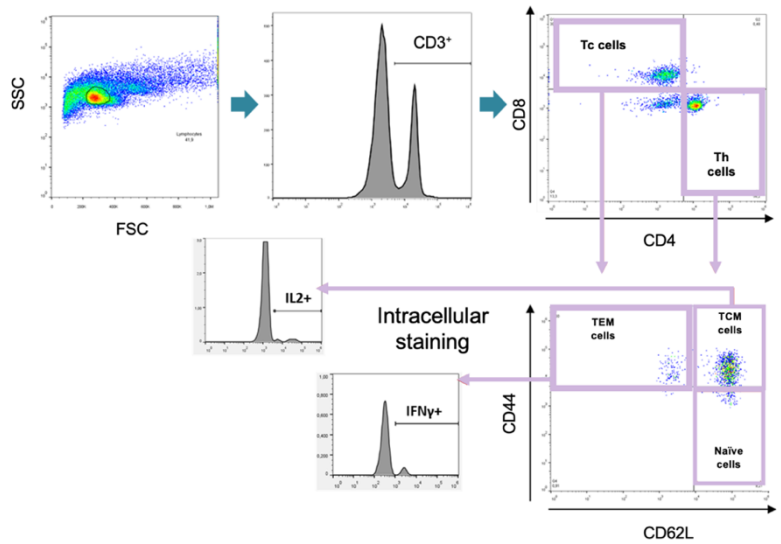

FIGURE 11: Selection method applied in flow cytometry analysis to establish the lymphocyte population of interest.

TABLE 8: Percentage of lymphocyte characterization. Cells were stimulated overnight under two OVA conditions (25 and 50 µg/mL). Results are expressed as the mean ± S.D. (n=2). * $p < 0.05$ vs NS cells for a particular subset

	Stimuli condition		
Cell phenotype	**NS cells**	**OVA (25 µg/mL)**	**OVA (50 µg/mL)**
Th cells ($CD3^+CD4^+$)	23.40 ± 11.20	25.40 ± 7.10	14.85 ± 3.15
Tc cells ($CD3^+CD8^+$)	8.94 ± 0.54	8.76 ± 2.34	3.86 ± 0.71*
Th/Tc ratio	2.70 ± 1.42	3.36 ± 1.71	3.83 ± 0.11
ThEM cells ($CD3^+CD4^+CD44^+CD62L^-$)	73.90 ± 11.00	70.95 ± 15.55	88.75 ± 0.65
ThEM IFN$^+$ cells	1.15 ± 0.11	1.54 ± 0.47	1.32 ± 0.23
ThCM cells ($CD3^+CD4^+CD44^+CD62L^+$)	21.87 ± 12.44	24.17 ± 15.43	7.94 ± 0.99
ThCM cells IL-2$^+$ cells	2.78 ± 1.37	6.40 ± 2.59	1.93 ± 0.39
Naïve Th cells ($CD3^+CD4^+CD44^-CD62L^+$)	1.41 ± 0.75	2.58 ± 2.00	0.70 ± 0.27
TcEM cells ($CD3^+CD8^+CD44^+CD62L^-$)	43.15 ± 2.25	39.90 ± 5.00	23.30 ± 1.60*
TcEM IFN$^+$ cells	7.11 ± 1.47	7.42 ± 1.76	6.28 ± 0.55
TcCM cells ($CD3^+CD8^+CD44^+CD62L^+$)	24.00 ± 4.50	29.95 ± 7.55	22.20 ± 0.50
TcCM cells IL-2$^+$ cells	3.58 ± 2.80	2.62 ± 2.01	0.00 ± 0.00
Naïve Tc cells ($CD3^+CD8^+CD44^-CD62L^+$)	23.10 ± 4.80	22.25 ± 1.65	30.55 ± 2.45

The flow cytometry analysis revealed that all the searched populations could be quantified. Although we only analysed cells from two animals under stimulation and non-stimulation conditions, some trends could be observed. The highest OVA stimulus (50 µg/mL) decreased the total Tc cell proportion and also the percentage of Tc effector memory cells.

5. DISCUSSION

The aim of this study was to establish an immunization protocol in two mouse strains and to set up the techniques needed for assess such immunization. As a control to determine whether the designed protocol succeeded in immunizing the animals, an OVA immunization was used. The easiest way to check immunization success is by quantifying specific antibodies. To quantify anti-OVA antibodies, an ELISA assay was set up based on previous studies with rats [43]. After optimizing the technique to our conditions, we determined the anti-OVA antibodies in the plasma samples obtained along the study. According to the bibliography [43], antibodies already appeared after two immunizations and increased gradually.

Clonal expansion or lymphocyte proliferation is essential for both cellular and humoral immune responses, as it is needed to produce sufficient B and T antigen-specific effector cells to fight the infection. Further, a few lymphocytes will remain after infection as memory cells [4,44]. Thus, the assessment of spleen lymphocytes' proliferation ability allows the immune response of mice to be quantified, which will be directly associated with the antibody titre that will be produced. We set up a proliferation assay for both OVA- and HAV-immunized animals. First assays, carried out in OVA-sensitized groups, showed that cells exhibited the same proliferative ability under all the conditions we tried, so we decided to perform this assay with the simplest conditions. Moreover, we assayed the proliferative ability in frozen cells and no significant differences were found with fresh cells. This finding allows the planning of *in vitro* experiments in a more flexible and relaxed way after sample collection. In the case of HAV-sensitized groups, the proliferation assay was set up according to reported studies [39,45,46]. We observed that the stimulation of lymphocytes with eHAV leads to increased proliferation rate compared to HAV. This could be explained by the fact that exosomes can activate DC and promote lymphocytes' maturation and further clonal expansion [22,33,35]. Nevertheless, more studies remain to be done to establish the optimal conditions for this assay as exosomes may bias the results.

We also established the optimal conditions for performing an ELISPOT assay to quantify the anti-OVA and anti-HAV antibody-secreting cells based on previous literature [47,48]. In previous studies, this technique has been used to evaluate the induction of HAV-specific immunity [4]. Firstly, from the ELISPOT assay of OVA-immunized mice, we observed that the technique can be performed using either fresh or frozen cells, which is important because, since the day of sample collection and cell isolation from a group of animals is often a long day, we may not be able to begin the ELISPOT assay. Moreover, as there are no significant

differences between the different cell incubation periods assessed (24, 48 or 72 h), in further studies we can adapt the cell incubation period to the needs and weekday. The ELISPOT assay for HAV-immunized mice showed a great variability between the two analysed animal samples. These results indicate that the assay must be performed in a large number of animals and it would be interesting to correlate its results with the anti-HAV antibody titres.

HAV infection causes a strong T-cellular immune response in humans, which is why T-cell-mediated immunity is key to controlling the viral infection [30,33,49]. Tc cell responses were the first cellular immune responses described for HAV. Surprisingly, when studies were conducted, it was established that the control of the infection was more related to Th cells than to Tc cells – in fact, Th response is activated prior to Tc response [50,51]. In this study, we assessed the phenotype of spleen lymphocytes of OVA-immunized mice. Although we could barely see differences because of the reduced sample size we used, we set up the optimal conditions to detect both extracellular and intracellular markers. According to the previous bibliography, one dose of the currently commercialized HAV vaccine can induce the production of TCM cells, which are related to a better clonal expansion [30,39,49]. Thus, we expect to detect a significant increase in both TEM and TCM cells in further studies.

Finally, the animal strain to be used in the next studies had to be decided. Previous bibliography suggested that BALB/c and C57BL/6 mice are the most suitable strains for developing immunization protocols (52–54). It is well known that BALB/c and C57BL/6 mice express different immune responses, C57BL/6 mice induce a Th cell type 1 (Th1) immune response, characterized by higher levels of IFN-γ and IL-2, while BALB/c mice induce a Th2 immune response. These differences lead to a higher replication of HAV in BALB/c hepatocytes [45,55]. Thus, in this study the immune response of BALB/c and C57BL/6 mice was evaluated to see which strain best fitted the designed protocol. No significant difference in the spleen lymphocytes' proliferation ability between the two mice strains was found. As lymphocytes' proliferation is the basis of a proper immune response [29], if there were no significant differences in lymphocyte proliferation, there would not be significant differences in the secretion and titre of antibodies generated by the two strains of mice. Thus, we decided to use BALB/c mice in future studies because, from our experience, the animal handling of BALB/c mice was better than for C57BL/6 strain. Moreover, many other authors also work with BALB/c mice for studies related to HA immunization protocols [4,44,46,56,57].

Overall, we set up the conditions to check both humoral and cellular immune responses to HAV in mice. We worked with inactivated HAV together with Alum. As recent studies have shown that exosomes can replace classical adjuvants [14,36,38], further studies remain to be undertaken to assess the immune response using inactivated HAV with or without Alum.

6. CONCLUSIONS

This study allowed the setting up of experimental assay conditions to assess:

- Plasma anti-OVA antibodies by ELISA.
- Specific proliferative response from both fresh and frozen spleen lymphocytes.
- Antibody-secreting cell counts by ELISPOT, from both fresh and frozen spleen lymphocytes.
- Effector and central memory T cells by immunofluorescence and flow cytometry from spleen lymphocytes.

In addition, from the results obtained we can conclude that:

- Specific anti-OVA antibodies already appeared two weeks after a second immunization and gradually increased with the third and the fourth immunizations. Thereafter, the antibody titre remained although a tendency to decrease 8 weeks after the last immunization was observed.
- Specific proliferative response to OVA and HAV can be quantified after 4 weeks of the last immunization.
- Specific proliferative response to eHAV was higher when the spleen lymphocytes were stimulated with eHAV rather than naked HAV.
- Antibody-secreting spleen lymphocytes were detected after 10 weeks of finishing the immunization process and even later.
- The performed extracellular and intracellular stainings allowed us to quantify all the searched populations for the detection of ThEM, ThCM, TcEM and TcCM and IL-2 and IFN-γ production.
- The immune response to HAV in BALB/c and C57BL/6 mice strains did not differ according to their specific proliferative response.

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