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Javier Asín Ros

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Director/es

Pérez Rontome, Marta M.

Lujan Lerma, Lluis

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Lluís Luján Lerma
Marta María Pérez Rontomé

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PhD Thesis

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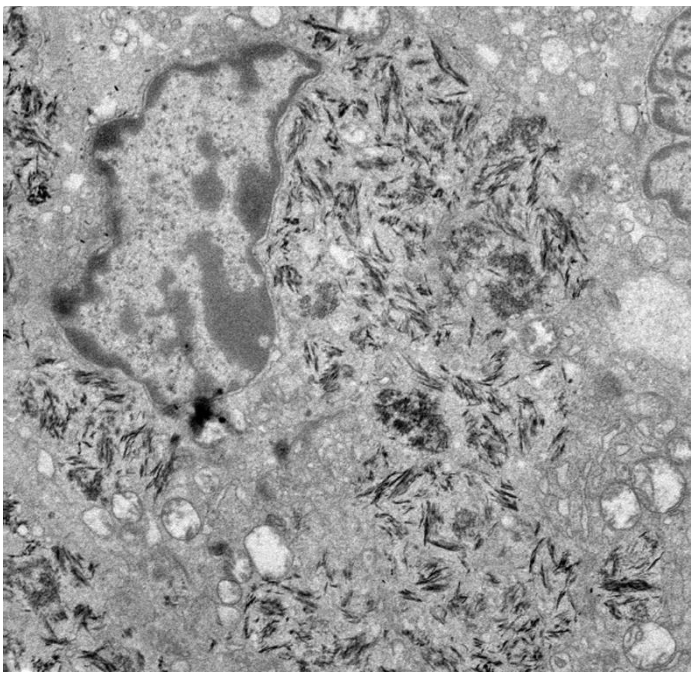

Javier Asín

Zaragoza, June 2019

Departamento de
Patología Animal
Universidad Zaragoza

Universidad de Zaragoza. Facultad de Veterinaria.
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CLINICO-PATHOLOGIC STUDIES IN SHEEP REPEATEDLY INOCULATED WITH ALUMINUM HYDROXIDE-CONTAINING VACCINES OR ALUMINUM HYDROXIDE ONLY

Memoria presentada por el Licenciado en Veterinaria Javier Asín Ros (Dipl. ECVP) para optar al grado de Doctor por la Universidad de Zaragoza

Dr. LLUÍS LUJÁN LERMA, Profesor Titular del Departamento de Patología Animal de la Universidad de Zaragoza y la Dra. MARTA Mª PÉREZ RONTOMÉ, Profesora Contratado Doctor del Departamento de Anatomía, Embriología y Genética Animal de la Universidad de Zaragoza,

Hacen constar:

Que la presente memoria de Tesis titulada "**Clinico-pathologic studies in sheep repeatedly inoculated with aluminum hydroxide-contaning vaccines or aluminum hydroxide only**" elaborada por JAVIER ASÍN ROS ha sido realizada bajo nuestra dirección y cumple con los requisitos exigidos para optar al título de Doctor con Mención Internacional en Veterinaria por la Universidad de Zaragoza.

Zaragoza, 18 de junio de 2019

Fdo.: Lluís Luján Lerma

Fdo.: Marta Mª Pérez Rontomé

"En cuestiones de cultura y de saber, sólo se pierde lo que se guarda; sólo se gana lo que se da"

Antonio Machado

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At the moment of its submission, this PhD thesis has produced two indexed publications:

- Asín J., Molín J., Pérez M., Pinczowski P., Gimeno M., Navascués N., Muniesa A., de Blas I., Lacasta D., Fernández A., de Pablo L., Mold M., Exley C., de Andrés D., Reina R. and Luján L. (2019). Granulomas Following Subcutaneous Injection With Aluminum Adjuvant-Containing Products in Sheep. *Vet Pathol*, **56**, 418-428.

- Asín J., Pérez M., Pinczowski P., Gimeno M., Luján L. (2018). From the bluetongue vaccination campaigns in sheep to overimmunization and ovine ASIA syndrome. *Immunol Res*, **66**, 777-782.

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SUMMARY

Over the years, vaccination strategies have contributed widely to global health by reducing the prevalence of many infectious diseases in animals and humans. Vaccines are used in most food-producing animal species as well as in companion animals. Specifically, in sheep, the use of vaccines against common diseases such as enterotoxaemia, abortions, respiratory processes or emerging diseases is widespread.

Most vaccines used in sheep and other species contain an adjuvant, which is a compound that enhances the immune response against the vaccine antigen, thus improving their effectivity. Indeed, most inactivated viral vaccines would not work properly without an adequate adjuvant. The list of available vaccine adjuvants for veterinary use includes mineral salts (i.e. calcium or aluminum [Al] salts), emulsions, toll like receptors agonists, cytokines, saponins, and a variety of polymers such as chitosan. However, Al salts, especially Al hydroxide, predominate over the others: Al hydroxide is actually the universal adjuvant used in sheep vaccines in the Spanish ovine industry.

Al has been used as a vaccine adjuvant for more than 50 years, but it is only recently when its mechanism of action has started being unraveled. Al leads to a Th2 immune response through the activation of the NLRP3 inflammasome system by the release of damage associated molecular patterns at the injection site. The unmatched effectiveness of this adjuvant has underestimated its risks for years. The neurotoxicity of Al is well-known and several studies have pointed to its detrimental effects on the central nervous system (CNS) when it is applied by the oral route. Studies on injected Al are less numerous, but rodent models have shown that this metal can be distributed throughout the body, accumulating in several tissues, including the CNS, leading to an array of symptoms and disease processes.

In sheep, one of the processes that has been related to the use of Al in vaccines is the so-called ovine autoimmune/inflammatory syndrome induced by adjuvants (ASIA syndrome). This syndrome was first described in human medicine and encompasses several entities which present a common triggering factor: the chronic stimulation of the immune system by an adjuvant. Ten years ago, a vaccination campaign against

bluetongue (BT) was implemented in sheep in Europe. This campaign included the application of more than 16 mg of Al per animal in a repeatedly vaccination schedule that was accomplished in less than a month. The BT outbreak was controlled but a broad spectrum of adverse effects was observed in parallel. Animals developed either a transient acute neurological reaction or a long-lasting chronic wasting disease. These two phases or clinical forms defined since then the ovine ASIA syndrome.

The present PhD thesis aims to study the effects of the repeated inoculation of Al hydroxide, either alone or combined into commercial vaccines, in a sheep model. Studies on the effects on animal ethology, morphological changes at the injection points and accumulation of Al in distant tissues are presented and pretend to establish the cornerstone for the study of the ovine ASIA syndrome.

The work is based on 3 groups of 26 lambs each at the end of the experiment, distributed in different conditions of housing, management and diet. Animals were subcutaneously inoculated with commercial vaccines containing Al hydroxide (Vaccine group), the same dose of Al in the form of Al hydroxide only (Adjuvant-only group) and phosphate buffered saline (PBS, Control group). Nineteen inoculations of the corresponding product were performed in 15 months. Clinical studies on animal cognition and behavior were performed when seven and sixteen doses had been applied, in summer and winter periods, respectively. These studies included: T-maze, open field and novel object recognition tests (cognition), home pen observations (individual and social behaviors) and a blood panel of stress biomarkers. *Post mortem* studies included gross, histo and ultrastructural pathology at the injection sites and regional lymph nodes, as well as analytical determinations of Al in regional lymph nodes and several areas of the CNS.

There were profound differences associated with the treatments in the home pen observations of social and individual behaviors. Vaccine and Adjuvant-only groups presented an increase in aggressive interactions and stereotypies, a decrease in affiliative interactions, and an increase in plasma cortisol levels. Furthermore, restlessness behavior and polyphagia were observed. Additionally, wool biting was patent in the Vaccine group, in which animals presented areas of wool loss and depilation associated with this aggressive interaction. These changes were more

marked with 16 than with 7 inoculations. On the other hand, the treatments had limited effects on animal cognition. This set of results suggests that the repeated inoculation of vaccines containing Al hydroxide or the equivalent amount of Al-hydroxide only induces changes in the inter-individual interaction patterns in sheep.

Vaccine and Adjuvant-only groups presented very persistent injection-site granulomas. More than seven granulomas per animal were recovered in more than 75 % of the lambs in the Vaccine group. Certain animals of this group presented granulomas corresponding to all the injections performed, pointing to a likely tendency in sheep to retain this metal in the body. Granulomas were composed of voluminous macrophages and multinucleated giant cells with granular to vacuolated cytoplasm. These cells presented an orangey, Al-selective positive fluorescence by lumogallion staining, which was confirmed by scanning transmission electron microscopy and energy dispersive X-ray spectroscopy. There were abundant intracytoplasmic aggregates of a spiculated electrondense material that was unequivocally identified as Al. Granulomas in Vaccine group presented higher lesion severity (i.e. higher frequency and severity of necrotic centers) and Al particle size than those in the Adjuvant-only animals, which may be associated with the longer persistency observed in the Vaccine group.

Regional lymph nodes in Vaccine and Adjuvant-only animals showed aggregates of Al-positive macrophages similar to those observed at the injection points. Indeed, Al levels in lymph nodes (established by transversely heated graphite furnace atomic absorption spectroscopy [TH GFAAS]) were much higher in these two groups than in Control group animals. This finding demonstrates that Al is actively translocated from the injection point to the regional lymph nodes, in which it accumulates, and suggests that most of the subcutaneously-injected Al is not eliminated from the body, at least in a short term. Furthermore, Al levels were even higher in the Vaccine group than in the Adjuvant-only animals, which suggests that sheep handles Al differently upon its presentation at the injection point.

Al levels established at the CNS (also by TH GFAAS) support the latter hypothesis. Vaccine and Adjuvant-only groups presented higher levels than the Control group in the lumbar spinal cord. The difference was more marked in the Adjuvant-only

group, which according to the lower levels observed in the lymph nodes and injection areas may suggest that injected Al distributes faster throughout the body when it is inoculated alone. Furthermore, the lumbar spinal cord is one of the points where lesions were observed in cases of ovine ASIA syndrome, therefore the higher Al levels found in this area are very suggestive of the likely role played by the metal in such lesions. On the other hand, few variations in the levels of Al at the parietal lobe were observed among the treatment groups, thus likely ruling out a direct neurotoxic effect of the inoculation schedule applied, at least at this location.

Some of the results obtained at the *post mortem* studies may explain part of the clinical changes observed in these animals. Behavioral changes in sheep are usually a result of an external stressor the animal cannot cope with. In this experiment, animals presented very persistent, Al-containing, subcutaneous, injection site granulomas, which may have led to the differences observed between the groups either by their simple presence (i.e. by causing a degree of physical discomfort), by increasing the Al-body burden or, most likely, by a combination of both mechanisms.

Altogether, the findings of the present PhD thesis point to the detrimental effects caused by the Al hydroxide used as a vaccine adjuvant in sheep. These effects are much more relevant than previously considered and suggest limiting (or even banning) the use of these salts as vaccine adjuvants. As it has been demonstrated in other species, avoiding Al in vaccines will most likely be of benefit also in sheep. This study justifies further research on alternative adjuvants, a field in need of extensive investigation, for providing better and safer vaccines for the future.

RESUMEN

A lo largo de los años, las estrategias de vacunación han contribuido ampliamente a la salud global mediante la reducción de la prevalencia de muchas enfermedades infecciosas en animales y humanos. Las vacunas se usan en la mayoría de animales de producción, así como en especies de compañía. Específicamente, en ovino, el uso de vacunas contra enfermedades frecuentes como enterotoxemia, abortos, procesos respiratorios o enfermedades emergentes es amplio.

La mayoría de vacunas usadas en ovino y otras especies contienen un adyuvante, que es una sustancia que aumenta la respuesta inmune hacia el antígeno vacunal, mejorando así su efectividad. Ciertamente, la mayoría de vacunas víricas inactivadas no funcionarían apropiadamente sin un adyuvante adecuado. La lista de adyuvantes vacunales disponibles para uso veterinario incluye sales minerales (p. ej. sales de calcio o aluminio [Al]), emulsiones, agonistas de receptores de tipo toll, citoquinas, saponinas y una variedad de polímeros, como el quitosano. Sin embargo, las sales de Al, especialmente el hidróxido de Al, predominan sobre otras: el hidróxido de Al es de hecho el adyuvante utilizado universalmente en vacunas para ovejas en la industria ovina española.

El Al ha sido utilizado como adyuvante vacunal durante más de 50 años, pero solo recientemente se ha comenzado a entender su mecanismo de acción. El Al desencadena una respuesta inmune de tipo Th2 mediante la activación del inflamasoma NLRP3 por medio de la liberación de patrones asociados a daño molecular (*damage associated molecular patterns*) en el punto de inyección. La incomparable efectividad de este adyuvante ha subestimado sus riesgos durante años. La neurotoxidad del Al es bien conocida y varios estudios han remarcado sus efectos negativos sobre el sistema nervioso central (SNC) cuando se aplica por la ruta oral. Los estudios sobre Al inyectado son menos numerosos, pero modelos murinos han mostrado que este metal puede distribuirse a lo largo del organismo, acumulándose en varios tejidos, incluyendo el SNC, y conducir a una serie de síntomas y enfermedades.

En ovino, uno de los procesos que ha sido relacionado con el uso de Al en vacunas es el síndrome autoinmune/inflamatorio inducido por adyuvantes (síndrome ASIA). Este síndrome fue inicialmente descrito en medicina humana e incluye varias entidades que presentan un factor desencadenante común: la estimulación crónica del sistema inmune por un adyuvante. Hace diez años, una campaña de vacunación frente a la lengua azul (LA) fue implementada en ovino a lo largo y ancho de Europa. Esta campaña incluyó la aplicación de más de 16 mg de Al por animal en un esquema de vacunación repetitivo que fue llevado a cabo en menos de un mes. El brote de LA fue controlado pero un amplio espectro de efectos adversos fue observado en paralelo. Los animales desarrollaron bien una reacción neurológica aguda o un proceso crónico debilitante. Estas dos fases o formas clínicas definen desde entonces al síndrome ASIA ovino.

Esta tesis doctoral pretende estudiar los efectos de la inoculación repetitiva de hidróxido de Al, solo o combinado en vacunas comerciales, en un modelo ovino. Se presentan estudios sobre los efectos en etología animal, cambios morfológicos en los puntos de inyección y acumulación de Al en tejidos distantes, y se pretenden establecer los pilares para el estudio del síndrome ASIA ovino.

Este trabajo está basado en 3 grupos de 26 corderos cada uno al final del experimento, distribuidos en diferentes condiciones de alojamiento, manejo y dieta. Los animales fueron inoculados por vía subcutánea con vacunas comerciales que contenían hidróxido de Al (grupo Vacuna), la misma dosis de Al en forma de hidróxido de Al solo (grupo Adyuvante-solo); y solución salina tamponada con fosfato (PBS, grupo Control). Se llevaron a cabo diecinueve inoculaciones del producto correspondiente en 15 meses. Se realizaron estudios clínicos sobre cognición y comportamiento animal cuando se habían aplicado 7 y 16 dosis, en verano e invierno, respetivamente. Estos estudios incluyeron: Tests del laberinto-T, campo abierto y reconocimiento de objeto novedoso (cognición), observaciones en corrales (comportamientos individual y social) y un panel sanguíneo con biomarcadores de estrés. Los estudios *post mortem* incluyeron patología macro, microscópica y ultraestructural en los puntos de inyección y linfonodos regionales, así como determinaciones analíticas de Al en linfonodos regionales y varias áreas del SNC.

Se observaron cambios marcados asociados a los tratamientos en las observaciones de comportamientos sociales e individuales en corrales. Los grupos Vacuna y Adyuvante-solo presentaron un aumento en las interacciones agresivas y las estereotipias, una disminución en las interacciones afiliativas y un aumento en los niveles de cortisol en plasma. Además, se observó comportamiento agitado y polifagia. Adicionalmente, fue patente el mordisqueo de lana en el grupo Vacuna, donde los animales presentaron áreas de pérdida de lana y depilación asociadas a esta interacción agresiva. Estos cambios fueron más marcados con 16 que con 7 inoculaciones. Por otro lado, los tratamientos tuvieron efectos limitados en la cognición de los animales. Este conjunto de resultados sugiere que la inoculación repetitiva de vacunas con hidróxido de Al o la dosis equivalente de hidróxido de Al inducen cambios en los patrones de interacción inter-individual en ovino.

Los grupos Vacuna y Adyuvante-solo presentaron granulomas muy persistentes en el punto de inyección. Se recuperaron más de 7 granulomas por animal en más del 75 % de los animales del grupo Vacuna. Ciertos animales de este grupo presentaron granulomas que correspondían a todas las inyecciones llevadas a cabo, apuntando a una probable tendencia del ovino a retener este metal en el organismo. Los granulomas estaban compuestos de macrófagos voluminosos y células gigantes multinucleadas con citoplasmas granulares a vacuolizados. Estas células presentaban una fluorescencia positiva anaranjada, selectiva del Al, mediante la tinción de lumogallion, que fue confirmada mediante microscopía electrónica de transmisión y barrido y espectroscopía de rayos-X. Se observaron abundantes agregados intracitoplasmáticos de un material electrodenso espiculado que fue inequívocamente identificado como Al. Los granulomas en el grupo Vacuna presentaron mayor severidad lesional (p.ej. mayor frecuencia y severidad de centros necróticos) y mayor tamaño de partícula de Al que los animales del grupo Adyuvante-solo, lo cual puede estar asociado con la mayor persistencia observada en el grupo Vacuna.

Los linfonodos regionales en los grupos Vacuna y Adyuvante-solo mostraron agregados de macrófagos positivos a Al similares a los observados en los puntos de inyección. Ciertamente, los niveles de Al en los linfonodos (establecidos mediante espectroscopía de absorción atómica en horno de grafito de calentamiento transversal

[TH GFAAS]) fueron mucho mayores en estos dos grupos que en los animales del grupo Control. Este hallazgo demuestra que el Al es transportado de forma activa desde el punto de inyección al linfonodo regional, donde se acumula, y sugiere que la mayor parte del Al inyectado por vía subcutánea no es eliminado del organismo, al menos a corto plazo. Además, los niveles de Al fueron incluso más altos en el grupo Vacuna que en los animales del grupo Adyuvante-solo, lo que sugiere que las ovejas tratan el Al de forma distinta en función de su presentación en el punto de inyección.

Los niveles de Al establecidos en el SNC (también mediante TH GFAAS) apoyan la última hipótesis. Los grupos Vacuna y Adyuvante-solo presentaron mayores niveles que el Control en la médula lumbar. La diferencia fue más marcada en el grupo Adyuvante-solo, lo que de acuerdo con los niveles más bajos observados en linfonodos y áreas de inyección podría sugerir que el Al se distribuye más rápido a lo largo del cuerpo cuando se aplica solo. Además, la médula lumbar es uno de los puntos donde las lesiones fueron observadas en casos crónicos del síndrome ASIA ovino espontáneo, por lo que los niveles más altos de Al hallados en esta área son muy sugerentes del probable papel que juega el metal en el desarrollo de estas lesiones. Por otro lado, se observaron pocas variaciones en los niveles de Al en el lóbulo parietal entre los distintos grupos de tratamiento, por lo tanto, descartando probablemente un efecto neurotóxico directo del programa de inoculación repetitiva aplicado, al menos en esta localización.

Algunos de los resultados obtenidos en los estudios *post mortem* podrían explicar parte de los cambios clínicos observados en estos animales. Los cambios de comportamiento en ovino son habitualmente el resultado de un estresor externo al que el animal no se puede adaptar. En este experimento, los animales presentaron granulomas con Al muy persistentes en el punto de inyección, que podrían haber conducido a las diferencias observadas entre los grupos, bien por su simple presencia (p.ej. causando un grado de incomodidad física), por un aumento en la carga corporal de Al o, más probablemente, por una combinación de ambos mecanismos.

En conjunto, los hallazgos de esta tesis doctoral apuntan a los efectos perjudiciales del hidróxido de Al usado como adyuvante vacunal en ovino. Estos efectos son mucho más relevantes que lo que se consideraba y sugieren limitar (o

incluso eliminar) el uso de estas sales como adyuvantes vacunales. Como ya ha sido demostrado en otras especies, la eliminación del Al en las vacunas será muy probablemente también beneficioso en ovino. Este estudio justifica más investigación para proveer vacunas mejores y más seguras para el futuro.

1. INTRODUCTION

1.1 The use of vaccines in veterinary medicine

1.1.1 Vaccines and their contribution to global health

Vaccines have proven their efficacy in helping to reduce the incidence and prevalence of many infectious diseases in both human and animals; the most well-known examples of this is the eradication of smallpox and the cattle plague (Rinderpest) in humans and bovines, respectively (Strassburg, 1982; Roeder, 2011; Greenwood, 2014).

Edward Jenner developed a smallpox vaccine by substituting a sample of smallpox for fluid from a cowpox lesion. This material did not cause a severe infection in humans, but rather induced an immune response that gave cross-protection against the smallpox virus. Jenner´s findings laid the groundwork for the development of attenuated vaccines based on live microorganisms that are still used in the present day (Minor, 2015; Rusnock, 2016).

Rinderpest (cattle plague) was a morbilliviral disease in cattle that caused significant pandemics and economic losses both in Europe and Africa (Barrett and Rossiter, 1999; OIE, 2013a). Despite not being a zoonotic disease, its eradication in 2011 contributed tremendously to global health by providing healthy cattle to the families and communities who make a living based on this kind of animal husbandry, especially in developing countries. Vaccination was the cornerstone of the eradication programs (Njeumi *et al.*, 2012; Roeder *et al.*, 2013).

Other human and animal diseases that have a deleterious impact on global health are currently being controlled by vaccination, including measles, polio, and rotavirus in human medicine, and foot-and-mouth disease (FMD), *peste des petits ruminants* (PPR), rabies, and tuberculosis in domestic and wild animals (Cao *et al.*, 2016; Chambers *et al.*, 2017; Fooks *et al.*, 2017; Jorge and Dellagostin, 2017; Kumar *et al.*, 2017). It is reasonable to think that some of these diseases might be eradicated in the future, thanks to the contribution made by vaccines.

This unmatched effectiveness has led to the general perception that all infectious diseases can be defeated by vaccination, and that most – if not all – infectious diseases can be eradicated. Some authors in the early sixties even predicted a future without infectious diseases: "(…) it seems reasonable to anticipate that within some measurable time (…) all the major infections will have disappeared" (Cockburn, 1963). Time has already proven that this idea was not correct, given that over the years, not only have new infectious diseases come into being, but old ones have re-emerged (Morens *et al.*, 2008). One of the first developments to face these new and old infectious diseases is the generation of new vaccines, and their incorporation into the recommended vaccination protocols.

1.1.2. Vaccines used in animal species

Vaccination is a key procedure in controlling animal diseases with important zoonotic implications such as rabies and tuberculosis (Pastoret and Jones, 2004; Lutticken *et al.*, 2007; Meeusen *et al.*, 2007; Garrido *et al.*, 2011), and on top of this, intensive animal production and the availability of high-quality animal-based nutritional products for human consumption have also been fueled by the use of vaccines. Indeed, industrial animal production would not be possible without vaccines (van Oirschot, 1994), while vaccines also contribute significantly to the maintenance of a healthy status in companion animals (Horzinek, 2010; Day *et al.*, 2015) and the control of epizootic infectious diseases in wildlife species that can spread infections to other domestic animals and humans (Garrido *et al.*, 2011; Freuling *et al.*, 2013; Chambers *et al.*, 2017). This being so, vaccines are a key element in the maintenance of the so-called "One Health" concept.

Vaccination programs in livestock animals raised for industrial food production differ, depending on the geographical area, production systems, specific disease risks, and age and purpose of the animal population (i.e. adult animals raised to produce viable offspring versus feeder young animals bred for rapid slaughtering and meat production; Lacasta *et al.*, 2015).

In Spain, some of the diseases against which production animals are usually vaccinated include porcine circovirus, porcine respiratory and reproductive syndrome virus, porcine enzootic pneumonia, and/or porcine polyserositis in pigs; bovine viral diarrhea (BVD) and/or infectious rhinotracheitis in cattle; enterotoxaemia and/or pasteurellosis in sheep; myxomatosis and/or rabbit hemorrhagic disease in rabbits; and Marek's disease and/or gumboro in poultry (Maes *et al.*, 2008; Muller *et al.*, 2012; Spibey *et al.*, 2012; Segalés, 2015; Lacasta *et al.*, 2015; Boodhoo *et al.*, 2016).

Companion and sport animals also receive an important benefit from vaccination against canine distemper, canine parvovirus, and/or rabies in the case of dogs; feline panleukopenia and/or feline leukemia in cats; and equine influenza, tetanus and/or equine rhinopneumonitis in horses (Holmes *et al.*, 2006; Patel and Heldens, 2009; Stuetzer and Hartmann, 2014; Fooks *et al.*, 2017).

As well as these commonly used vaccines, new ones are implemented and added to the vaccination programs of each species when a newly emerging or re-emerging infectious disease appears in a specific area. Over the years, some of these diseases are bluetongue (BT), PPR, Rift Valley fever (RVF), sheep pox, West Nile, porcine and avian influenza, FMD, and Schmallenberg (Bhanuprakash *et al.*, 2006, Bhanuprakash *et al.*, 2009; Savini *et al.*, 2008; Kapczynski and Swayne, 2009; Amanna and Slifka, 2014; Díaz-San Segundo *et al.*, 2017; Faburay *et al.*, 2017; Kumar *et al.*, 2017; Stavrou *et al.*, 2017; Vincent *et al.*, 2017), among others. These necessary and newly implemented vaccination programs contribute to the global vaccine burden of the animals in the specific geographical areas and periods in which they are applied, thus possibly leading to an over-immunization effect (Trinca, 1976) that occurs in parallel to the desirable effects of protection.

1.1.3. Vaccination strategies in small ruminant medicine

Vaccination programs are one of the main practices in the modern sheep and goat industries. Vaccines against abortive agents (i.e. *Chlamydia abortus*, *Salmonella spp,* and *Brucella ovis*), clotridial diseases (mainly enterotoxaemia), and respiratory diseases (caused by various members of the *Pasteurellaceae* family) are by far the

most commonly used immunization products in disease management in Spain (Lacasta *et al.*, 2015).

As stated, the specific vaccines included in each program depend on the actual sanitary necessities of the area and flock of interest, and the time of the management cycle. In general, antiabortion vaccines are applied in the period before the mating season, whereas vaccines aiming to protect offspring by the passive transmission of antibodies through the colostrum (i.e. clostridial or respiratory diseases vaccines) are administered during the final third of pregnancy. Furthermore, newborn lambs and kids are vaccinated against the same diseases during the fourth-eighth week of life to guarantee their viability during the fattening process. Other diseases that can be controlled through vaccination in certain circumstances include mammary gland diseases (i.e. contagious agalactia), colibacilosis, paratuberculosis (Johne's disease), contagious ecthyma, caseous lymphadenitis, and/or foot-rot.

Vaccines against emerging and re-emerging diseases that cause outbreaks are implemented and added to these programs in specific periods of time. Due to the local field conditions in Spain, several compulsory vaccination campaigns against BT have been implemented several times over the last 20 years (de Diego *et al.*, 2014). Other diseases affecting small ruminants such as PPR and RVF are likely to appear in Spain in the following years (Sánchez-Vizcaíno *et al.*, 2013; Baazizi *et al.*, 2017), ensuring that new vaccination campaigns and strategies will be needed in order to control them.

The above detailed sanitary management practices mean that a single sheep can receive between two and four vaccines per year, and perhaps even more, depending on the specific health problems of a certain flock, or the implementation of compulsory vaccination campaigns against emerging infections in specific moments. In a seven-year lifespan, a single sheep can receive between 14-28 vaccine shots, while in specific periods of time, the application of more vaccines and/or other sanitary products (i.e. anthelmintic treatments) can be necessary due to management requirements, thereby further increasing the total load at a given point in time (Lacasta et al., 2015).

1.2. Vaccine adjuvants

1.2.1. The use and development of vaccine adjuvants

The word “adjuvant” is derived from the Latin verb “adjuvare,” which means “to aid.” This term is currently used in medical contexts to refer to a drug or method that enhances the effectiveness of another treatment by facilitating or helping in its action. Indeed, one of the most important constituent parts of a vaccine is the adjuvant, which in vaccinology, refers to any substance that is added to the vaccine preparation, and enhances its immunogenicity against the antigen (Coffman *et al.*, 2010; Leroux-Roels, 2010). Indeed, adjuvants are key constituents of vaccines, and certain products (i.e. inactivated viral vaccines) would not work properly without an adequate adjuvant.

The first steps in the development of vaccine adjuvants were taken by the French veterinarian Gaston Ramon, who added various substances such as calcium chloride and bacterial components to the diphtheria anatoxin vaccine for horses. Ramon realized that the local inflammatory reaction induced by these substances was related to an enhanced production of antibodies against the anatoxin (Ramon, 1924).

Since this discovery, research in vaccine adjuvants has become increasingly sophisticated and in recent years, the study and development of effective and safe adjuvants has come to be considered one of the main pillars of human and veterinary vaccine development (Coffman *et al*., 2010; Burakova *et al*., 2018).

1.2.2. Mechanisms of adjuvant action

Different mechanisms of action can be outlined, depending on the type of adjuvant. In most cases, adjuvants cause some degree of cellular damage that leads to the recruitment of inflammatory cells to the injection point, with a subsequent activation of innate and adaptive immune responses (Calabro *et al.*, 2011). Three fundamental mechanisms of action are described, as follows:

1.2.2a. Danger signals: The innate immune response is based on the recognition of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), which generate a non-specific immune response (Medzhitov and Janeway, 1997) by activating pattern recognition receptors (PRRs). As a result, the innate immune response is activated, which initially controls the pathogen replication, while the adaptive immune response is mounted, based on specific antibodies and memory T cells. Vaccine adjuvants that act by this mechanism mimic the effect of PAMPs or DAMPs by including components that activate PRRs or cause a certain degree of cellular damage, respectively.

1.2.2b. Administration and delivery vehicles: The deposition of antigen in the injection point is known as the "depot effect." This promotes a long-lasting and slow delivery of the antigen, which ensures antigen uptake by antigen-presenting cells (APCs) and, consequently, a continuous immune stimulation (Awate *et al.*, 2013).

1.2.2c. Activation/maturation of APCs: Of the cells recruited to the inoculation point, APCs are especially relevant because they participate in the three signals necessary for a fully developed immune response: they present the antigen to the T lymphocytes (signal 1); they modify and determine the kind of final response by co-stimulation (signal 2); and they produce cytokines (signal 3) necessary for the recruitment of other immune cells. Signals 2 and 3 mostly depend on the actual danger signal induced by the pathogen or by the adjuvant, and in this way, adjuvants are able to determine the type of induced immune response (Th1, Th2, or Th17). Via these signals, T cells are activated, undergo clonal expansion, and become antigen-specific effector T cells that either eliminate infected cells (cytotoxic T lymphocytes) or produce more cytokines that induce the maturation of other immune cells (T helper lymphocytes). Adjuvants specifically increase the antibody response or the cell-mediated immune response by this mechanism, thereby noticeably reducing the time necessary for a full-mounted response, the number of immunizations needed, and the amount of antigen that needs to be included in the vaccine preparation (Gerdts, 2015).

A single adjuvant can act concurrently by a different combination of these mechanisms, depending on its physicochemical and immunogenic properties (i.e. some

mineral salt-based adjuvants can induce both a transient depot effect and a danger signal; Gupta *et al.*, 1995; Toussi and Massari, 2014). Further research and knowledge on both the innate and adaptive immune responses is promoting the development of new adjuvants that are exploring new mechanisms of action, and are likely to have broad relevance in the future (Yu et al., 2017; Soares et al., 2018).

1.2.3. Types of vaccine adjuvants in veterinary medicine

Most of the vaccine adjuvants used in veterinary medicine are applied in immunization strategies based on inactivated or subunit vaccines that are poorly immunogenic but safer (Foged, 2015). Normally, live and attenuated vaccines do not require an adjuvant, but can cause safety problems as a result of the reactivation of the infectious agent (Warren-Gash *et al.*, 2017). Reactivation of the agent in live or attenuated vaccines has produced outbreaks of animal diseases such as BT or viral laryngotracheitis in ruminants and poultry, respectively (Hughes *et al.*, 1991; Savini *et al.*, 2014).

The list of approved and used adjuvants for veterinary medicine is much broader than for human medicine (Macy, 1997; Spickler and Roth, 2003). Due to safety concerns, some compounds are prohibited for use in human vaccines (i.e. emulsions or saponin-based adjuvants such as Quil A). Combinations of adjuvants are created in order to derive benefit from the properties of a number of adjuvants at the same time, leading to genuine compounds with unique properties of immunopotentiation, as well as antigen-delivery properties (Heegaard *et al.*, 2016; Burakova *et al.*, 2018).

Two major groups of adjuvants can be outlined, based on their predominant mechanism of action: immunostimulants (i.e. saponins, Toll-like receptors [TLRs] agonists or cytokines), and delivery and deposit agents (i.e. emulsions, microparticles, and mineral salts). The main adjuvants available for use in veterinary vaccines include:

1.2.3a. **Mineral salts**: This group includes aluminum (Al) salts and calcium phosphate. These compounds induce high titers of IgG and IgE, as well as eosinophil activation, through a type Th2 immune response. This makes them good candidates for

antibacterial and parasitic vaccines, but poorly immunogenic for intracellular organisms (i.e. viruses; Gupta *et al.*, 1995).

1.2.3b. Emulsions: Formed by the mixture of two immiscible liquids (oil and water), in which one of the compounds of the emulsion forms small droplets that are dispersed into the other. The antigen is entrapped in one of the phases, which guarantees a degree of slow release after the injection. There are various presentations in this regard, depending on the proportion of the two phases of the emulsions and the specific location of the antigen (Burakova *et al.*, 2018):

-Water-in-oil (W/O), formed by the dispersion of small water droplets within an oil phase. Antigen is included in the oil phase, and they have a strong depot effect. Freund's adjuvants are the most well-known examples of these adjuvants, while others such as Montanide™ incomplete (SEPPIC, France) are extensively used in commercial veterinary vaccines (Ibrahim Eel-S *et al.*, 2015).

-Oil-in-water (O/W), formed by the dispersion of oil droplets in an aqueous phase. Antigen is included in the water phase. O/W adjuvants do not induce as strong a depot effect as W/O adjuvants, but in this case, the oil droplets also promote chemokine-based immune cell recruitment and differentiation of other immune cells. MF59 and Montanide ISA, Emulsigen® are available examples of this group (Barnett *et al.*, 1996).

-Water-in-oil-in-water (W/O/W), formed by a continuous aqueous media that contain dispersed oil droplets, which at the same time, contain smaller water droplets. Antigens are included in both phases, thus promoting both a rapid and prolonged release. Few W/O/W adjuvants are currently available in the market. Montanide ISA 201 and 206 (SEPPIC, France) belong to this group, and have been used in veterinary vaccines (Bouguyon *et al.*, 2015).

1.2.3c. TLRs agonists: TLRs are membrane receptors of macrophages and APCs. They recognize specific components of microbes and danger signals (i.e. PAMPs and DAMPs), and activate the innate immune response, inducing the expression of certain cytokines involved in T cell differentiation. This being so, TLRs are key players in the

generation of antigen-specific immunity (Vidya *et al.*, 2018). Many components with TLR activity are licensed for use as vaccine adjuvants, i.e. components of bacterial LPS such as Monophosphoryl lipid A (Steinhagen *et al.*, 2011), bacterial lipoprotein, and flagellin (Hajam *et al.*, 2013), or *Pseudomonas aeruginosa* membrane lipoprotein OprI (Rau *et al.*, 2006); however, the use of these compounds in veterinary vaccines is still limited.

1.2.3d. Cytokines: Cytokines are immunoactive compounds that have an important role in cell signaling and T cell activation (Dinarello, 2007). The incorporation of cytokines such as IFN-ɣ, IL-18, and IL-7 has been successfully tested in various veterinary vaccines (Hung *et al.*, 2010; Fan *et al.*, 2016; Huo *et al.*, 2016).

1.2.3e. Saponins: Saponins also have immunoactive activity. They are composed of several hydrophilic glycoside moieties attached to a lipophilic triterpene derivative. Quil-A, alone or combined with cholesterol and phospholipids in immune-stimulating complexes (i.e. ISCOMS), is the most well-known saponin-based vaccine adjuvant (Kensil, 1996). They are capable of stimulating CD4+ and CD8+ T cells, and aid in antigen phagocytosis by APCs. Veterinary vaccines based on this kind of adjuvants are continuously being tested, with promising results (Orbegozo-Medina *et al.*, 2018), and even employed routinely in licensed vaccines (i.e. equine influenza vaccines; Paillot, 2014).

1.2.3f. Polymers: These are compounds used as a delivery vehicle for an antigen-controlled release (Ferreira *et al.*, 2013). Chitosan is a well-known example of this type of adjuvant, which works as an antigen carrier and an immunostimulant, mainly through macrophage mannose receptor activation (Shibata *et al.*, 1997; Kumar *et al.*, 2004). Chitosan-based vaccine adjuvants have demonstrated their effectiveness in FMD experimental vaccines (Çokçalişkan *et al.*, 2014). Other synthetic polymers tested and/or used for veterinary vaccines include polyacridic acid polymers (carbomers, i.e. Carbopol®) and polyphosphazene polymers (Fachinger *et al.*, 2008; Dar *et al.*, 2012). These compounds remain very promising vaccine adjuvants that are able to induce a cell-mediated type of immune response, and are very interesting for viral pathogens (Burakova *et al.*, 2018).

1.2.4. Al adjuvants

In 1926, Alexandre T. Glenny and colleagues discovered that injecting diphtheria toxoid precipitated with an Al salt (potassium alum) brought about a significant increase in the immune response against the toxoid in laboratory rabbits and guinea pigs (Glenny *et al.*, 1926). Since then, Al salts have been widely used both in human and veterinary immunization products, becoming possibly the most commonly used vaccine adjuvant due to its unmatched effectiveness and inexpensive price (Lindblad, 2004; Gołos and Lutyńska, 2015).

A remarkable fact concerning the use of Al adjuvants is that they have been used for several decades, without their exact mechanism of functioning being known. Initially, Glenny postulated that Al salts worked exclusively by means of a long-lasting depot effect that permitted continuous presentation of antigen and immunostimulation (Marrack *et al.*, 2009). This theory was supported by initial research that indicated that the precipitated toxoid persisted much longer than the soluble toxoid (Glenny *et al.*, 1931), and that ablated seven-week-old Al-induced injection site nodules were able to induce immunity in different guinea pigs (Harrison, 1935). Later, White *et al.* (1955) partially supported the depot theory by showing that there was significant production of antibody-containing plasma cells in the Al-induced granuloma and in the local lymph node. However, they also demonstrated that these cells were not present in the regional lymph nodes after three weeks, possibly indicating that no more antigen was being presented at that time.

More than 50 years after those first approaches to the mechanisms of action, several researchers have demonstrated that the depot effect theory was a simplistic explanation (Hutchison *et al.*, 2012) and, over the last 20 years especially, research into Al adjuvants has significantly increased, giving rise to striking and previously unknown discoveries about their mode of action (HogenEsch *et al.*, 2018).

Al salts activate the innate immune system, leading to a Th2 immune response (Kool *et al.*, 2008). The direct cytotoxicity of Al salts leads to the release of DAMPs as uric acid, which rapidly converts into monosodium urate (MSU) crystals that are phagocytosed by resident cells (Eisenbarth *et al.*, 2008). In addition, Al salts are

phagocytosed. Both MSU crystals and phagocytosed Al induce lysosomal destabilisation and cathepsin B release (Jacobson *et al.*, 2013), which co-activates the NLRP3 inflammasome, leading to caspase-1 activation and subsequent IL-1ß, IL-18, and IL-33 production (Li *et al.*, 2007, Li *et al*., 2008; Sokolovska *et al.*, 2007).

Multiple independent works have supported these mechanisms, since several proinflammatory cytokines (Il-1ß, CCL2, and CCL11, among others) might be detectable after the injection of Al adjuvants into experimental mice, along with the recruitment of various innate inflammatory leukocytes to the injection site (Kool *et al.*, 2008; Sharp *et al.*, 2009).

Various insoluble Al salts are currently available as adjuvants in human and veterinary medicine, including: i) Al hydroxide [$Al(OH)_3$] or Alhydrogel; ii) Al phosphate [$Al(PO4)_3$] or Adju-Phos; iii) Al potassium sulphate [AlK(SO4)2] or Alum; and iv) Al hydroxide and magnesium hydroxide [Al(OH)3 + Mg(OH)2] or Imject Alum (Marrack *et al.*, 2009).

Alum was the salt employed by Glenny and colleagues in their first studies on Al (Glenny *et al.*, 1926), and it did not establish a strong relationship with the antigen that was just precipitated. Conversely, Al hydroxide and Al phosphate capture the antigen by direct absorption, establishing several types of close chemical interactions between the antigen and the adjuvant (Marrack *et al.*, 2009), therefore these two salts have almost totally replaced Alum in human and veterinary vaccines. Imject Alum is an experimental salt that has so far not been applied to any commercial vaccine (Hem *et al.*, 2007). Each salt has different physicochemical properties, which give them specific characteristics. Remarkably, the different particle size of these salts has been demonstrated to influence its interaction with phagocytic macrophages and cytotoxicity (Morefield *et al.*, 2005; Harris *et al.*, 2012; Kolade *et al.*, 2015; Shardlow *et al.*, 2016; Art *et al.*, 2017).

Al hydroxide (Al oxyhydroxide or Alhydrogel®) is currently the most relevant adjuvant for clinical use. It is a crystalline compound with a significantly higher degree of protein absorption than Adju-Phos, and with fewer cytotoxic effects (Mold *et al.*, 2016). Rabies vaccines for dogs, cats, and livestock are good examples of veterinary

vaccines with Alhydrogel® as an adjuvant (Yang *et al.*, 2013). Other species such as porcine and poultry also have benefited from the immunopotentiating properties of these compounds in their species-specific vaccines (Pini *et al.*, 1965; Sellers and Herniman, 1974; Norimatsu *et al.*, 1995), although currently, in these species they are not used as extensively as other adjuvants. In the case of ruminants, Al hydroxide is by far the most common adjuvant in vaccines in the Spanish management conditions; in the Aragón Spanish region, virtually all vaccines used for sheep contain Al hydroxide as an adjuvant. These products include bacterial vaccines (i.e. anti-abortive vaccines such as those against *Chlamydia abortus* and *Salmonella spp.*), toxoid-based vaccines (i.e. clostridial vaccines against enterotoxaemia), and viral inactivated vaccines (i.e. BT vaccines). In general, these vaccines contain a dose of 2-4 mg of Al per ml. Considering the health management practices (two-four vaccinations per year), the estimated amount of Al a single sheep may receive over its entire productive life (a seven-year lifespan) is about 70 mg, an amount that can vary significantly, depending on the health management of specific flocks and animals, punctual sanitary risks, compulsory vaccination campaigns, and farmer demands (Lacasta et al., 2015).

1.3. Adverse reactions to veterinary vaccines and adjuvants

1.3.1. Types of vaccine-associated adverse events (VAEs)

Veterinary vaccines are highly regulated products, with very controlled testing requirements. The European Union requirements for veterinary vaccine testing are outlined in Annex I of the European Commission Directive 2009/9/EC (Official Journal of the European Communities, 2009) and the European Pharmacopoeia (Council of Europe, 2016). These requirements include the performance of overdosage studies in the most sensitive target species, and the route of administration that contains the highest risk of adverse effects. The administered dose should be twice the recommended for inactivated vaccines, and ten times for live vaccines. These studies should also be accompanied by titer determination to establish the vaccine safety and efficacy profiles. The accomplishment of these requirements implies that currently, veterinary vaccines offer great safety indexes. However, as for any medical compound

with a biological action, its use entails a percentage of risk of adverse reactions occurrence in a variety of animal species. In general, however, these reactions are considered mild and of low occurrence (Ohmori *et al.*, 2002; Moore *et al.*, 2005a, 2005b; Moore *et al.*, 2007; Moore and HogenEsch, 2010; Tung *et al.*, 2015; Valli, 2015).

It is important to note that in companion animals, VAEs are viewed in an individual-based manner, whereas in production animals, these reactions can be more easily detected because these animals are vaccinated based on groups; that is, the entire flock is vaccinated at the same time, irrespective of individuals and their specific conditions. For instance, in Atlantic-farmed salmon, systemic autoimmunity is detected after the use of oil-adjuvanted vaccines in thousands of animals (Koppang *et al.*, 2008; Haugarvoll *et al.*, 2010), while in broiler chickens, several farms have reported concurrent cases of bronchopneumonia in chicks associated with in an in-ovo fowlpox vaccination (Williams *et al.*, 2010).

Several classifications of VAEs in veterinary have been proposed. These include similar categories that can be summarized as follows (Martinod, 1995; Roth, 1999):

1.3.1a. Injection-site reactions: Injection-site reactions are possibly the most commonly reported VAEs, and are usually local allergic reactions that show areas of swelling, edema, sterile abscesses, or granulomas. They are most commonly related to the use of adjuvant-containing vaccines, and emulsion-based adjuvants and Al salts are frequently reported as the main cause implicated in these lesions (Straw *et al.*, 1990; O'Toole *et al.*, 1995; Macy, 1997; Cox and Coulter, 1997; Aucouturier *et al.*, 2001; Spickler and Roth, 2003; Mutoloki *et al.*, 2006; Sesardic, 2006; Day, 2007). Interestingly, injection site reactions have been implicated in the development of injection-site sarcomas in cats, and several studies have connected components such as the Al adjuvant or feline leukemia virus antigens to the development of sarcomas. Indeed, adjuvant-containing vaccines are considered one of the main risk factors in the development of these tumors in cats (Hartmann *et al.*, 2015; see 1.7).

1.3.1b Systemic reactions: Systemic, mild, and transient unspecific VAEs are also frequently reported after the application of a vaccine (Soos, 1987; George *et al.*, 1988; Smith *et al.*, 1990; Dalgleish and Love, 1993; Yeruham *et al.*, 1994; Dixon *et al.*,

1996; Twigg *et al.*, 1997; Ellis and Yong, 1997; Gaskell *et al.*, 2002; Newton *et al.*, 2005; Ramsay *et al.*, 2005). Transient increases in body temperature are common and stated on vaccine labels as expectable VAEs. The appearance of hyperthermia has significant effects, especially in production animals, with drops in the milk yields from dairy cows (Scott *et al.*, 2001; Bergeron and Elsener, 2008). Feedlot lambs and calves have demonstrated an increase in rectal temperature, especially after booster vaccinations (Troxel *et al.*, 2001; Cerviño *et al.*, 2011), which may have an effect on their final weight at the end of the feeding process.

Abortions and/or temporary fertility reduction due to an increase in body temperature have also been reported in cows (Nusinovici *et al.*, 2011), while other effects may include vomiting or unspecific neurological signs (Martinod, 1995). In any case, the mild and transient nature of these effects ensures that they are usually deemed acceptable consequences of vaccination, especially considering the effects of the diseases they are intended to counter (Nusinovici *et al.*, 2011). Nevertheless, these effects should be taken into account in the economic balances calculated by farms where vaccination strategies are applied, such as the risk/benefit balance of applying a vaccine against *Clostridium spp*. in lambs twice; they may gain less weight due to a higher increase in body temperature and subsequent anorexia, but they will be fully protected, and fewer animals in the batch will die of the disease, due to the higher antibody titers provided by the two shots (Cerviño *et al.*, 2011).

1.3.1c Allergic reactions: Hypersensitivity reactions against the antigen or other components of the vaccines can occur in animals.

-Type I hypersensitivity reactions due to the introduction of IgE can lead to anaphylaxis. Anaphylaxis is a relatively frequently reported event as an adverse reaction to canine vaccines. Animals may show suggestive signs such as a skin rash, hypotensive shock, dyspnea, or edemas (Greene and Levy, 2011).

-Type II hypersensitivity, or antibody-mediated reactions, can occur when vaccines contain antigens of normal cells and structures. In this case, the animal develops antibodies against these structures, and the immune system may react against them, when they are encountered throughout the body. This kind

of reaction has been proposed to play a role in feline chronic kidney disease (Brown *et al.*, 2016). Certain viral vaccines are produced using feline kidney cell lysates (Crandell-Rees feline kidney cell lysates), and the production of antibodies against such lysates has been demonstrated in experimental conditions (Lappin *et al.*, 2005, Lappin *et al.*, 2006; Whittemore *et al.*, 2010). Indeed, the role of these findings in the development of feline chronic interstitial nephritis deserves further investigation.

-Type III hypersensitivity, or immune complex type reactions, can occur either when circulating antibodies against the vaccine antigen are present at the moment of vaccination, or when a vaccinated animal becomes infected, and vaccine-induced antibodies encounter the replicating agent (antigen). A well-known example is the induction of anterior uveitis and corneal edema in dogs vaccinated against canine adenovirus (Curtis and Barnett, 1983). Immune-complex reactions have been proposed to play a role in the development of subcutaneous vasculitis and necrotizing panniculitis after rabies vaccination in dogs and cats (Mauldin and Peters-Kennedy, 2016).

-Type IV hypersensitivity or delayed-type reactions imply the stimulation of antigen-specific lymphocytes and their replication at the injection point, cytokine production, and migration of macrophages or other leukocytes to the injection site. There are few reports of veterinary vaccines-induced allergic reactions of this category, but this type of hypersensitivity may play a role in the granulomatous reactions observed at the injection point, after the injection of certain vaccines (Lauren *et al.*, 2016).

1.3.1d. Immune system alterations: Autoimmunity and immunosuppression are two alterations of the immune system that have resulted from the use of vaccines several times in the past.

The prevalence of autoimmune conditions in companion animals has increased in the last 30 years, and a causal relationship with vaccination procedures has been proposed (Dodds, 1999; Gershwin, 2018). Indeed, autoimmunity linked to the

overstimulation of the immune system by the use of veterinary vaccines has been recently reviewed in the context of genetic predisposition (Gershwin, 2018).

Duval and Ginger (1996) suggested a link between autoimmune hemolytic anemia (AMHA) and vaccination in dogs, while other authors have reported confirmed or suspected cases of immune-mediated polyarthritis in dogs following vaccination (Kohn *et al.*, 2003). Nevertheless, links between canine immune-mediated polyarthritis/AMHA and vaccination have recently been questioned by other groups (Idowu and Heading, 2018; Moon and Veir, 2019). A multivalent canine vaccine was shown to be able to induce autoantibodies in experimental beagles, although clinical signs were not observed after 20 weeks of study (HogenEsch *et al.*, 1999). In addition, an increase in anti-thyroglobulin antibodies in pet and research dogs after vaccination has been demonstrated (Scott-Moncrieff *et al.*, 2002), but the same group was not able to associate this change with autoimmune thyroiditis in a subsequent work (Scott-Moncrieff *et al.*, 2006). An increase in IgE antibodies to pollen in inbred atopic dogs has been induced by vaccination (Frick and Brooks, 1983), while the appearance of immune-mediated neuropathies has been associated with vaccination in dogs: a post-vaccinal polyradiculoneuritis has been reported in puppies (Schrauwen and Van Ham, 1995; Gehring and Eggars, 2001), and a Guillain-Barré-like syndrome with muscular atrophy due to denervation has been associated with the use of an inactivated rabies vaccine (Quiroz-Rothe *et al*., 2005).

In farm animals, the most recent and dramatic example of vaccine-induced autoimmunity was the case of bovine neonatal pancytopenia (BNP). A specific BVD vaccine (PregSure® BVD, Pfizer Animal Health) was associated with increased cases of neonatal calves that showed severe coagulation and bone marrow disorders, as well as generalized bleeding and death (Boes, 2011). Alloantibodies against bovine MHC I and immature platelets, granulocytes, and monocytes in the bone marrow were demonstrated (Deutskens *et al.*, 2011; Assad *et al.*, 2012). Remarkably, this disease was induced by alloantibodies generated by the vaccinated mothers, and transmitted to the calves by the colostrum (Bridger *et al.*, 2011; Bell *et al.*, 2013; Benedictus *et al.*, 2015). This occurrence highlighted the risks of using allogenic cell lines for vaccine

production, which may lead to the development of autoimmune reactions (Benedictus and Bell, 2017).

On the other hand, some modified live vaccines (MLV) have been reported to induce immunosuppression by suppressing the lymphocyte response to mitogens (Datz, 2010). In dogs, a combined multivalent MLV against canine adenovirus and canine distemper virus (CDV) has been shown to be able to induce blastogenesis suppression of peripheral lymphocytes (Phillips *et al.*, 1989), while a MLV against canine parvovirus induced a similar effect (Mastro *et al.*, 1986). In bovines, a BVD vaccine strain has been shown to suppress neutrophil function (Roth and Kaeberle, 1983). Although reported only occasionally, these immunosuppressive effects seem to be minimized when healthy dogs are vaccinated (Datz, 2010).

1.3.1e. Residual pathogenicity and inadequate inactivation: The residual pathogenicity of agents used in MLV is always a concern, but it is uncommon in manufactured approved vaccines. Bovine herpesvirus-1 (BHV-1) infection can occur after the vaccination of cattle with ineffectively attenuated vaccines against bovine rhinotracheitis and influenza (Bryan *et al.*, 1994), while respiratory symptoms have been observed in poultry after the administration of an infectious laryngotracheitis vaccine (Picault *et al.*, 1982). Reversion to virulence of CDV within MLV is a well-known effect of these products, which may produce the so-called “post vaccinal encephalitis,” especially if such vaccines contain the CDV Rockborn strain (Demeter *et al.*, 2010; Martella *et al.*, 2011).

Similarly, inadequate inactivation of the virus in inactivated vaccines may also cause VAEs. FMD and equine encephalitis have been associated with inadequately inactivated vaccines (Fedida, 1986; Brown, 1993).

1.3.1f. Contamination of vaccines: The contamination of components intended for the production of veterinary vaccines with biologically active agents has been occasionally reported (Bolin *et al.*, 1994; Makoschey *et al.*, 2003). Indeed, this mechanism has been implicated in fatal disease a number of times; i.e., clostridial disease in ruminants accidentally infected by *Clostridium sordelli* (Téllez *et al.*, 2006); BVD in cattle induced by a contaminated-BHV-1 vaccine (Barkema *et al.*, 2001; Falcone

et al., 2003); and even BT disease in dogs arising from a contaminated MLV canine vaccine has been described (Akita *et al.*, 1994).

1.3.1g. Genetic recombination: Organisms submitted to genetic modification are starting to be applied in veterinary and human vaccines, and constitute a very active field of research (Nascimento and Leite, 2012; Naim, 2013). Some of the biological systems used in the production of these vaccines may reverse to virulence, acquiring deleted genes via recombination (Henderson et al., 1990; Martinod, 1995).

1.3.2. VAEs related to over-immunization.

In addition to the above-mentioned general mechanisms of VAEs, there are other possible effects that may be induced by the way vaccines are used. Among these, the repetitive and intensive use of vaccines has been highlighted as the most common practice leading to over-immunization in animals (Dodds, 1999). Over-immunization or hyper-immunization can be defined as the hyperstimulation of the immune system by an excessive vaccine load that may cause a wide array of side-effects, most of them still not well understood (Tsumiyama *et al*., 2009).

Vaccines might cause over-immunization through repeated inoculations over a defined period of time, or by the use of several vaccine doses at a single point in time. There have been several recent reports of VAEs linked to over-immunization in human medicine (Cadusseau *et al.*, 2014; Nellore and Randall, 2016), although the risks of hyper-immunization were already being underlined as early as 1976 (Trinca, 1976).

In veterinary medicine, concurrent vaccinations and repetitive inoculations have been related to an increase in the proportion of VAEs in dogs and cats. In a retrospective cohort study including 1,226,159 vaccinated dogs, a significantly increased risk of VAEs within three days of the vaccination was observed, as the number of vaccine doses administered in a single visit increased. Furthermore, each additional vaccine in following visits to the veterinarian significantly increased the risk of VAEs (Moore *et al.*, 2005b, 2005c). In another parallel study encompassing 496,189 cats, a similar conclusion was reached, since the risk of VAEs increased significantly, as the number of vaccines administered per single office visit increased (Moore *et al.*,

2007). A similar relationship was found in another retrospective study including 3,587 ferrets: the incidence rate of VAEs increased in parallel to the cumulative number of CDV and/or rabies vaccinations performed (Moore *et al.*, 2005a).

Furthermore, it is also known that booster vaccination in the neonatal period in puppies and kittens may increase the risk of VAEs (Day, 2007). Interestingly, there has been a report of immune-mediated membranous glomerulonephritis in a dog vaccinated seven times by the owner without veterinary supervision (Ortloff *et al.*, 2010).

1.3.3. VAEs related to Al adjuvants

As explained above, vaccine adjuvants are occasionally involved in the development of VAEs, especially injection site reactions caused by oil- and/or Al-based adjuvants

Al is a well-known neurotoxic metal (Li *et al.*, 2009; Caito and Aschner, 2015) that has been implicated in the generation of reactive oxygen species in the central nervous system (CNS), among other effects (Halliwell, 1992). There are several examples of Al neurotoxicity that have occurred in the past:

-Al dementia or Al encephalopathy in hemodialysis patients is a process that used to occur in patients under hemodialysis treatment, related to the use of dialysis baths that contained Al. Affected patients also showed osteomalacia with fractures and anemia. The use of Al-free dialysis fluid has almost eliminated the occurrence of dialysis dementia, but this disease still remains the most widely known example of the neurotoxic effects of Al (Alfrey *et al.*, 1976; Wills and Savory, 1985).

-Al intoxication may also be observed in patients with chronic kidney disease and glomerular filtration rates <30ml/min/1.73 m^3 who are administered Al-containing phosphor binders (Rob *et al.*, 2001). There have been several reports of Al toxicity in people exposed to other Al solutions (i.e. Al hydroxide) with concurrent renal disease (Malaki, 2013). Individuals develop neurological signs such as tremors, myoclonic

spasms, and cognitive impairment due to accumulation of Al in the brain (McDermott *et al.*, 1978).

-In July 1988, an incident that took place in Camelford (UK) produced the worst episode of massive population poisoning in the history of the United Kingdom. Twenty tonnes of Al sulphate were discharged into the drinking water supply, and 20,000 people exposed to concentrations of Al that were up to 3,000 times over the acceptable limit, while other toxics such as copper and sulphuric acid were also filtered into the drinking water. Short- to long-terms signs of intoxication were observed among people in the area, and many inhabitants demanded an investigation into the increase in neurodegenerative diseases, anxiety, and other neurological disruptions that were supposedly observed (McMillan *et al.*, 1993; Owen and Miles, 1995). Interestingly, the effects of this event in the animal population were also studied; much of the Al sulphate was spilled into near rivers, killing thousands of fish, but on the other hand, the contamination of the water supply had limited detrimental effects on livestock in the area (Allen and Sansom, 1989). In any case, this issue generated broad controversy and vigorous scientific debate and even today, studies of tissues from Camelford patients yield clues about the effects of massive human exposure to Al (Exley and Esiri, 2006; King *et al.*, 2017; Mold *et al.*, 2019a, 2019b).

-Injected Al as a part of immunization products has also been implicated in VAEs. Macrophagic myofasciitis (MMF), a neuromuscular condition (see 1.5.1b), is one of the most well-known examples of this (Gherardi *et al*., 1998; Gherardi *et al*., 2001; Gherardi and Authier, 2003), while other VAEs, mainly restricted to the injection point, include granulomas and persistent subcutaneous nodules, urticaria, subcutaneous sarcoidosis, progressive circumscribed sclerosis, and cutaneous pseudolymphoma (Chong *et al.*, 2006; Hansen *et al.*, 2008; Marcoval *et al.*, 2008; Ozden *et al.*, 2009; El Shabrawi-Caelen *et al.*, 2009; Kreft *et al.*, 2011). In murine models, Al adjuvants have been implicated in the development of motor neuron degeneration (Petrik *et al.*, 2007), its translocation to the CNS has been demonstrated (Khan *et al.*, 2013; Crépeaux *et al.*, 2015; Eidi *et al.*, 2015; Crépeaux *et al.*, 2017), and neurobehavioral changes have been observed in rodents (Crépeaux et al., 2017).

1.4. The BT vaccination campaigns

One of the best examples of over-immunization-related VAEs in the context of a veterinary vaccination is the secondary reaction observed in sheep and cattle following the massive compulsory vaccination campaigns against BT that occurred in Europe at the end of the first decade of the 21st century.

BT is a viral, non-zoonotic, insect-borne, systemic disease of ruminants that causes serious sanitary and economic problems, and is especially pernicious in sheep (Maclachlan, 2011). It is a systemic process characterized by fever, mucosal hemorrhages, depression, edemas, and generalized cyanosis, which this last sign best observed in the tongue. Morbidity can reach almost 100 %, and mortality oscillates between 2 % and even 70 % in certain cases (OIE, 2013b).

Historically, vaccination has been the most effective way of controlling BT (Maclachlan, 2011). Until the last decade, BT vaccines contained live attenuated virus that in some cases caused deleterious effects such as fetal malformations or even the re-emergence of BT (Schultz and Delay, 1955; Young and Cordy, 1964; Roy *et al.*, 2009). Since 2007, vaccines used in Europe have contained an inactivated virus and Al hydroxide as an adjuvant (Savini *et al.*, 2008; Eschbaumer *et al.*, 2009; Wäckerlin *et al.*, 2010).

Over the years, several BT outbreaks have appeared in latitudes where the vector (midges of the genus Culicoides) is present, including countries in southern Europe (Mellor *et al.*, 2008). In 2006, the BT virus serotype 8, which had not been previously reported in Europe, was identified in the Netherlands, and rapidly spread to other countries in central and northern Europe (Carpenter *et al.*, 2009). This event was totally unexpected, and brought about a state of emergency leading to the implementation of a compulsory vaccination campaign to protect against serotype 8 and other serotypes that were circulating at that moment in specific locations (European Commission, 2008).

In Spain, several serotypes were circulating concurrently and, typically, two serotypes predominated in a given geographical area (de Diego *et al.*, 2014). In

response, a dual vaccination program was implemented across the country, and the recommended protocol included two inoculations against two BT virus serotypes (i.e. serotypes 1 and 8 in the Aragón region and other parts of Spain) and a booster inoculation after 20-30 days, when two new doses were applied. As a result, animals received four vaccine doses within about a month, which culminated in an inoculation of 16 mg of Al per animal.

Indeed, this campaign was very effective at controlling viral circulation and thus avoiding the disease, but at the same time, a series of VAEs were reported concurrently to the use of these vaccines all over Europe. In France, for instance, several VAEs such as abortion, weakness, and anorexia were reported between 2008 and 2009 in cattle and sheep (Agence Française de Sécurité Sanitaire, 2009); and even a decrease in the fertility of dairy cows was demonstrated (Nusinovici *et al.*, 2011).

These effects gave rise to deep concern in France, and two questions aiming to clarify the issue were raised in the French National Assembly, both yielding ambiguous and only partially explanatory answers (Assemblée Nationale, 2015a, 2015b). In Germany and Switzerland, farmers complained about vaccination side-effects, but questionnaire-based epidemiological studies did not establish a clear relationship between vaccinations and VAEs that were observed (Tschuor *et al.*, 2010; Probst *et al.*, 2011). In the case of the United Kingdom, reports of suspected VAEs in cattle following vaccination against BT virus serotype 8 during the mass vaccination campaign in 2008 contributed largely to the increase in the overall total reports in that year (Dyer *et al.*, 2009).

In Spain, these reactions have been widely reported in sheep, affecting all kind of ovine breeds, production systems, and geographical locations. These reactions include a wide array of symptoms such as neurological signs, abortion, and inexplicable cachexia (González *et al.*, 2010; Luján *et al.*, 2013). Massive social alarm spread across the Spanish ovine industry in 2008 and 2009, leading to multiple allusions in the national press (Heraldo de Aragón, 2009; El Periódico de Aragón, 2010). In the following years, some cases have been adjudicated in courts with variable results, some demonstrating a cause-and-effect link between the vaccination events and the

pernicious process, while others remaining unelucidated (Dictamen del Consejo Consultivo de Aragón 67/2012, de 17 de abril de 2012; Dictamen de la Comissió Jurídica Assessora Generalitat de Catalunya 137/2012, de 3 de maig de 2012; Sentencia del TSJ AR 1/2014, de 7 de enero de 2014; Sentencia del TSJ AR 249/2015, de 28 de abril de 2015).

However, two official Spanish scientific reports failed to find a clear causal relationship between the vaccination procedures and the clinic-pathological picture observed and, after being unable to link the process with any known ovine disease, they finally categorized that problem as multifactorial (Pujols *et al*, 2009; Sánchez-Vizcaíno, 2009). In any case, the notion that a non-resolved problem had arisen with these vaccines persists among veterinarians and farmers in Spain and other parts of Europe, a fact that has been corroborated by a report issued by the European Medicines Agency that evaluated the VAEs linked to the 2008 BT vaccination campaigns (European Medicines Agency, 2009). Later on, a link was proposed between these massively observed VAEs and the repetitive use of vaccines containing Al adjuvants, and these VAEs were assigned to the *Autoimmune/inflammatory Syndrome Induced by Adjuvants* (ASIA syndrome) umbrella as a post-vaccine phenomenon (Shoenfeld and Agmon-Levin, 2011; Luján *et al.*, 2013; see 1.5.2).

BT is a disease with high and continuous re-emergence potential in the south of Europe. Recurrent outbreaks of this disease are always a possibility in Spain, and vaccination campaigns are needed to control them and avoid sanitary and economical losses. The unsolved episode of VAEs that occurred at the end of the first decade of the 21st century reduced the confidence that field veterinarians and farmers have towards vaccination strategies. There is still at atmosphere of skepticism towards these vaccines, which may hinder the correct application of BT vaccination campaigns (Diario del Campo, 2016; Oviespaña, 2016), and therefore have disastrous consequences on efforts to control the disease in the future.

Indeed, recent recirculation of BT virus serotypes has been detected in several locations in Europe (European Commission, 2019), and it is very likely that new vaccination campaigns will be implemented in the near future. The history of ovine

ASIA syndrome has to be considered when implementing such campaigns, and further research into this kind of processes is warranted to maintain the confidence of the ovine industry in future vaccination strategies.

1.5. ASIA syndrome

1.5.1. ASIA syndrome in human medicine

ASIA syndrome is an entity coined in 2011 by Shoenfeld and Agmon-Levin (Shoenfeld and Agmon-Levin, 2011; Perricone *et al.*, 2013). It includes several conditions that develop as a consequence of exposure to substances with adjuvant properties (i.e. substances that induce a continuous immune stimulation), leading to the development of autoimmunity. For a diagnosis of ASIA in human medicine, an autoimmune or immune-mediated condition needs to fulfil at least one major or two minor of the following criteria:

Major criteria: i) Exposure to an external stimulus (i.e. infection, vaccine, silicone, and adjuvant) prior to clinical manifestations; ii) the appearance of "typical" clinical manifestations: myalgia, myositis or muscle weakness, arthralgia and/or arthritis, chronic fatigue, un-refreshing sleep or sleep disturbances, and neurological manifestations (especially associated with demyelination), cognitive impairment, memory loss, pyrexia, and dry mouth; iii) removal of the inciting agent that induces improvement; an iv) typical biopsy of the involved organs.

Minor criteria: i) The appearance of autoantibodies or antibodies directed against the suspected adjuvant; ii) other clinical manifestations (i.e. irritable bowel syndrome); iii) specific human leukocyte antigen (HLA; i.e. HLA DRB1, HLA DQB1); iv) the evolvement of an autoimmune disease (i.e. multiple sclerosis).

After its presentation in 2011, a recording system of ASIA cases was established and as of 2017, a total of 4,479 cases have been identified, with the majority of these having developed following a vaccination (Jara *et al.*, 2017). However, due to the low number of cases reported, the variety of adjuvants implicated in the process, the lack of precise time-cause association in some cases, and the vague and broad criteria used

for its diagnosis, there is still an ongoing scientific debate on the condition (Hawkes *et al.*, 2015; Ameratunga *et al.*, 2017). Despite this occasionally inflamed debate (Bragazzi *et al.*, 2017), research on ASIA syndrome is currently ongoing and data on case series, associated syndromes, and related causes are released on a regular basis (Watad *et al.*, 2018, Watad *et al*., 2019). Furthermore, and despite the skepticism of some groups about the use of animals for the study of ASIA syndrome (Ameratunga *et al.*, 2018), several authors consider that establishing proper animal models is crucial to unravel the pathogenesis of this and related conditions (Cruz-Tapias *et al.*, 2013, Gherardi *et a*l., 2018)

Currently, five clinical conditions are considered to fulfil the criteria of inclusion, and are thus included under the umbrella of the ASIA syndrome (Perricone *et al.*, 2013):

I. Postvaccination phenomena: Vaccines are one of the main sources of exposure to adjuvants. Phenomena that occur after the use of an adjuvant-containing vaccine and fulfil the required criteria can be included as a facet of ASIA syndrome.

II. MMF: This is a specific postvaccination phenomenon that was first described by Gherardi *et al.* (1998, 2001). It was initially related to the use of human hepatitis B vaccines in France. Affected individuals presented a granuloma in the deltoid muscle composed of Al inclusions-containing macrophages. Time elapse from vaccine application and the beginning of the symptomatology can be as broad as 7-11 months. Symptoms include diffuse myalgias, chronic fatigue, cognitive perturbances (i.e. memory and attention alterations), and dyspnea (Gherardi and Authier, 2003; Gherardi and Authier, 2012; Aoun Sebaiti *et al.*, 2018; Gherardi *et al*., 2019). Muscle biopsy of the deltoid muscle has been used as routine diagnosis of MMF. Recently, a noninvasive approach has been established that identifies MMF-affected patients by their specific cerebral ^{18}F-FDG PET profiles (Blanc-Durand and Van Der Gucht, 2017). Research on this condition using rodent models has shown that Al particles are mobilized from the injection point to the spleen, liver, and brain by macrophages (Khan *et al.*, 2013; Crépeaux *et al.*, 2015; Eidi *et al.*, 2015; Crépeaux *et al*., 2017).

III. Siliconosis: Silicone is considered an inert material with various uses in medical and cosmetic devices. An adjuvant effect of silicone has been proposed, considering its capability to induce a focal inflammatory reaction around the implant. Furthermore, silicone particles can diffuse out of the implant into surrounding tissues via a process known as “bleeding.” In the 1990s, silicone capable of inducing an autoimmune condition was proposed as the cause of the “adjuvant disease” (Shoaib *et al.*, 1994). A causal relationship between a certain number of autoimmune conditions (i.e. multiple sclerosis, rheumatoid arthritis, Sjögren’s syndrome, systemic lupus erythematosus, and mixed connective tissue disease) and breast implants has been proposed (Bar-Meir *et al*., 2003).

IV. Gulf War syndrome (GWS): Some war veterans who were deployed in the Gulf War (1990-1991) developed an array of nonspecific symptoms such as fibromyalgia, chronic fatigue, numbness, arthralgia, and gastrointestinal disorders that were known as GWS or chronic multi-symptom illness. Exposure to several substances such as pyridostigmine bromide (used as a nerve gas prophylaxis), insect repellent, vaccinations, smoke from oil-well fires, depleted uranium from shells, and even mild traumatic brain injury have been proposed as a cause of the syndrome (Steele, 2000; Gronseth, 2005; Staines, 2005; Lucas *et al.*, 2007; McDiarmid *et al.*, 2011; Steele *et al*., 2012 Janulewicz *et al*. 2018). Indeed, Gulf War soldiers received mass-vaccinations against anthrax toxin, which contained adjuvants such as Al or squalene (Schumm *et al.*, 2002). Motor neuron disease has been induced in laboratory animals using these vaccines (Petrik *et al.*, 2007).

V. Sick building syndrome: A sick building is a place where at least 20 % of the occupants report health symptoms associated with their stay in the building. A definitive cause of the problem is yet to be identified, but there is usually a common history of symptom resolution among the occupants when they leave the building (Heinkel, 2016). The syndrome is observed mainly in offices where workers complain of an array of disabling symptoms such as headaches, fatigue, lethargy, eye and throat irritation, nasal congestion, shortness of breath, and a reduced ability to concentrate. Poor-quality air conditioners have been proposed to play a role in the development of the symptoms. Indeed, continuous exposure to several exogenous and

immunoreactive substances is very likely to be associated with the syndrome. Sick building syndrome was included, along with GWS and others, into the category of "functional somatic syndromes" in human medicine (Barsky and Borus, 1999). Although it was not included in the initial description of ASIA syndrome (Shoenfeld and Agmon-Levin, 2011), Perricone *et al.* (2013) proposed its inclusion as part of the syndrome in 2013, considering that it fulfilled several criteria of inclusion such as exposure to a foreign substance and the presence of antibodies against the suspected adjuvant in some individuals.

1.5.2. Ovine ASIA syndrome

The following description is based on studies performed by various research groups across Spain during the years 2008-2011, shortly after the beginning of the BT vaccination campaign (Luján *et al.*, 2013; see also 1.4). At this time, the sudden appearance of an unexplainable neurologic and cachectic process was noticed by farmers, veterinarians, and researchers; the consequences were widespread and sometimes severe, with thousands of animals lost, either directly or by culling for humanitarian reasons. The process was similar, irrespective of the geographical area or breeds, and included an array of clinical observations such as behavioral disturbances, acute and chronic neurological signs together with progressive neurodegenerative lesions and severe loss of fat deposits, leading to extreme cachexia.

As mentioned previously, the results of two official reports were unable to establish an explanation but at the same time, ruled out all major ovine diseases that could explain the process in affected sheep (Pujols *et al*, 2009; Sánchez-Vizcaíno, 2009). Two independent research studies were later published linking vaccination procedures and the observed pathological manifestations in sheep (González *et al.*, 2010; Luján *et al.*, 2013), and the syndrome was finally catalogued as an animal manifestation of ASIA syndrome (Luján *et al.*, 2013). The implementation of that vaccination protocol could have triggered the syndrome by a generalized over-immunization reaction caused by both the use of several vaccine doses at a specific point in time, and the repetitive use of such vaccines across time. Indeed, the role of Al-based adjuvants, their effectiveness in inducing a strong immune reaction, and their

in vivo persistence might have played a key role in the pathogenesis of the ovine ASIA syndrome. On top of this, at the time of the vaccination against BT, most animals had already been routinely vaccinated in the same year against other common ovine diseases, something that could have contributed to the global vaccine load in a short space of time.

Ovine ASIA syndrome presents in two phases or forms:

I. Acute phase: This phase is temporally associated with the vaccination event, with a range of two-six days between the vaccination and the development of clinical signs. It usually produces a morbidity of less than 1 % of the animals in a flock, while mortality can be even lower. Affected animals present severe nervous clinical signs such as disorientation, apathy, lethargy, bruxism, transient blindness, convulsions, and prostration. Most animals apparently recover after two-three days, while a small proportion may die. At postmortem examination, no significant gross changes are observed, but at the histopathological level, severe meningoencephalitis can be detected, consisting of mixed inflammatory infiltrates in the meninges and blood vessels of the brain and spinal cord. Other features include gliosis, subpial glial foci, scattered neuronal death in encephalon and spinal cord, choroiditis, multifocal white matter demyelination, and severe hyperemia of blood vessels with multifocal intraparenchymal hemorrhages.

II. Chronic phase: This is the most severe and pernicious consequence, in that it can be observed in a large proportion of animals in a given flock, sometimes reaching almost all sheep. The temporal relationship with the vaccination procedure is sometimes difficult to establish because this form can appear even months after the vaccination, especially under certain stresses. Even more interestingly, the chronic phase does not always follow the acute phase in the same animal, meaning that sheep that do not develop an acute reaction to the vaccine can be affected by the chronic phase at a later stage. This phase is triggered by an external stimuli or a combination of them, such as low temperatures, lambing in non-natural periods of time such as winter, poor nutrition, or any other stressful situation. At postmortem examination, animals show extreme cachexia, with a depletion of fat deposits, ascites, hydrothorax,

hydropericardium, and skeletal muscle atrophy. Peripheral nerves show a considerably thickened aspect and microscopically, the main lesions consist of multifocal neuronal necrosis and neuronal loss in the grey matter of the spinal cord, perineural myxedema with occasional multifocal lymphoplasmacytic perineuritis in peripheral nerves, as well as an array of changes in the brain, such as gliosis, satellitosis, meningitis, and perivascular cuffing. A similar chronic reaction has been seen in a small group of experimental lambs repeatedly vaccinated with several commercial ovine vaccines, including those against BT. Interestingly, it has been observed that animals affected by the chronic phase are prone to develop the acute phase following subsequent vaccinations. Furthermore, chronically affected animals that are repetitively inoculated with an Al-containing vaccine have shown a M1 macrophagic activation, which increases with the number of inoculations applied (Insausti, 2013).

The role of Al in the pathogenesis of the ovine ASIA syndrome is likely twofold: i) directly causing neurotoxicity; and ii) indirectly triggering a strong, unspecific immune reaction that can eventually affect the CNS through autoimmune reactions. Indeed, the neurotoxicity of Al is well known (Caito and Aschner, 2015; Li *et al.*, 2009), and the transportation of Al from the point of inoculation to the CNS has been experimentally demonstrated in mice (Khan *et al.*, 2013; Crépeaux *et al.*, 2015; Eidi *et al.*, 2015; Crépeaux *et al*., 2017). This incoming of Al to the CNS may promote not only acute toxicity, but also long-term neurological damage, because the mobilization of Al from the inoculum/a can increase the concentration of this metal in the nervous tissue over time, promoting neurodegenerative alterations such as those observed in the chronic phase of ovine ASIA syndrome.

Furthermore, the robust immune stimulation provoked by the repetitive inoculation of Al may induce not only antibodies or cellular reactions to the target antigens (a desirable effect of vaccination), but also antibodies to self-antigens, and a strong but unspecific and generalized immune stimulation may lower the threshold for recognition of the body's own antigens (Tsumiyama *et al.*, 2009). Moreover, it is generally accepted that Al-containing adjuvants induce the release of potent proinflammatory cytokines via the activation of the NLRP3 inflammasome route, which

is likely to play a crucial role in the generalized immune response and promotion of adverse reactions in ovine ASIA syndrome (Eisenbarth *et al.*, 2008).

In this way, ovine ASIA syndrome can be regarded as a severe VAE, and not only against BT vaccines. Indeed, the syndrome had already been observed by our group before the vaccination campaigns against BT in specific ovine flocks affected by an untreatable cachectic disease, in which all diagnostic efforts were unsuccessful (Luján *et al.*, 2012). The vaccination campaigns against BT only brought to the surface a syndrome that was already occurring at a low level, thus making the disease more clinically visible and identifiable. Previous vaccinations in most flocks against other common ovine diseases could have contributed to the massive appearance of the syndrome after 2008. The appearance of the syndrome continues to date, returning slowly towards the prevalence observed before the BT vaccination campaigns.

1.6. Ethological changes and Al inoculation in animal models

1.6.1. State-of-the-art and published work

There is a large number of studies that have focused on the neurotoxic and behavioral effects of Al administration by the oral route (i.e. drinking water) performed on experimental animals (Farhat *et al.*, 2017; Martínez *et al.*, 2017a, 2017b, Martínez *et al*., 2018), but few research groups have examined the effects of injected Al using reliable experimental animal models.

Some toxicity studies using experimental rabbits have encountered high level of Al neurotoxicity when inoculated subcutaneously. Yokel (1989) demonstrated that aged rabbits are more susceptible to toxicity and behavioral alterations than younger animals, when inoculated with a solution of Al and lactate. A similar study compared Al lactate solutions injected subcutaneously or applied directly to the cerebral ventricle, with similar patterns of neurotoxicity and behavioral symptoms obtained by the two routes (Forrester and Yokel, 1985).

Even fewer papers have been published on the likely link between Al-containing vaccine adjuvants and behavioral and/or cognitive altered profiles in experimental

animals. This issue has been addressed a limited number of times using murine models only.

Behavioral tests such as the wire-mesh test have demonstrated motor deficits in mice inoculated with Al hydroxide (Petrik *et al.*, 2007). Furthermore, in a subsequent experiment of the same research group, mice injected with six doses of Al hydroxide showed spatial and memory deficits (Shaw and Petrik, 2009). Furthermore, mice in both experimental works displayed neuronal death and neuroinflammation in different parts of the CNS, such as the lumbar spinal cord.

Mice intramuscularly inoculated with Al hydroxide or a vaccine containing Al hydroxide did not show changes in behavioral tests such as the elevated o-maze, the open field test (OFT), the novel object recognition test (NOT), the wire mesh hang test, and others, in comparison with a placebo-inoculated control group. Consistently, inoculated mice in this experiment did not demonstrate neither Al particles in the brain nor higher levels of Al (Crépeaux *et al.*, 2015). The same authors showed neurobehavioral changes such as anxious behavior by similar validated tests in mice inoculated with 200 µg Al/kg. Interestingly, these changes were not found in mice inoculated with two- and four-fold Al doses, likely pointing to a low-dose selective neurotoxicity of intramuscularly-injected Al hydroxide. Indeed, the lowest-dose-injected mice also showed higher brain Al levels and microglial activation (Crépeaux *et al.*, 2017).

A study by Inbar *et al.* (2017) highlighted behavioral abnormalities in female C57BL/6 mice inoculated with an Al hydroxide containing the vaccine or Al hydroxide only. The inoculated mice showed differences in a forced swimming test even six months after inoculation, likely indicating a long-term behavioral impairment due to the treatment. However, locomotor dysfunction was not demonstrated, and therefore the authors proposed that the observed changes were likely due to an inoculum-induced depressive behavior, rather than locomotor deficits. Indeed, mice inoculated with the Al-containing vaccines showed microglial proliferation in the hippocampus and their sera cross-reacted with a mouse brain extract.

1.6.2. Behavior and cognition evaluation in sheep

Sheep models have been widely used in behavioral and cognitive analyses by a number of research groups. Sheep have demonstrated excellent performance in modelling behavioral and cognitive abnormalities related to nutritional and other stressors through a number of approaches previously applied to other species such as the T-maze test, OFT, NOT, and home pen observations.

The T-maze test has been used to study learning capacity and memory as indicators of brain function in rodents (Zimmerberg *et al.*, 1991; Wan *et al.*, 1994). In ovine, the T-maze test has been validated for the study of different aspects of spatial learning and working memory (Hosoi *et al.*, 1995; Camm *et al.*, 2000; Peirce *et al.*, 2000; Kendrick *et al.*, 2001; Johnson *et al.*, 2012; Abecia *et al.*, 2014).

The OFT was first established in rats (Asano, 1986) and later validated for other species such as pigs or chicks as a test to evaluate emotionally and socially motivated behaviors (Marín *et al.*, 1997; Donald *et al.*, 2011). In the case of sheep, it is used to assess the response to the exposure of a novel environment and separation from flock mates (Forkman *et al.*, 2007; Caroprese *et al.*, 2010; Abecia *et al.*, 2014). Individual differences may be explained by a variable degree of fear among animals, which is quantifiable by counting the time animals spend exploring or escape attempts, among others parameters (Forkman *et al.*, 2007; Pedernera-Romano *et al.*, 2010).

In the NOT, animals are exposed several times to a new object in order to evaluate different types of memory and fear responses (Antunes and Biala, 2012). In sheep, the test has been used in different studies mainly to assess the fear reactions of animals (Romeyer and Bouissou, 1992; Vandenheede and Bouissou, 1993; Viérin and Bouissou, 2002, 2003; Desire *et al.*, 2004).

Home pen observations have been widely used in laboratory animals to establish behavioral patterns in different species and studies (Ferguson *et al.*, 1993; Reisbick *et al.*, 1994; Lijam *et al.*, 1997; Crawley, 1999). In sheep, they have been used to assess social and individual behaviors with different purposes such as appetence for different types of soil in the pen, responses to human stroking, the effects of the diet

in oral stereotypic behaviors, aggression and wool biting, and the effects of isolation in the development of abnormal behaviors (Cooper and Jackson, 1996; Yurtman *et al.,* 2002; Lauber *et al*., 2012; Coulon *et al*., 2015; Pascual-Alonso *et al*., 2015).

1.7. Granulomas at the injection site due to Al adjuvants

Injection-site reactions are among the most commonly reported VAEs overall (see 1.3.1a). The same applies to vaccines containing Al-based adjuvants, with numerous reports in several species.

In humans, persistent reactions in the form of itching nodules have been widely reported over the last 50 years after the use of vaccines containing Al salts, and they have invariably been identified as granulomas (Erdohazi and Newman, 1971; Fawcett and Smith, 1984; Chong *et al.*, 2006). The prevalence of such reactions may be underestimated (Bergfors *et al.*, 2014), while their role in systemic effects such as MMFs has been supported with different animal models (Khan *et al.*, 2013; Crépeaux *et al.*, 2017).

In companion animals, vaccine-associated injection site granulomatous panniculitis with Al-adjuvant particles is a relatively common phenomenon in canine and feline biopsies (Lee Gross *et al.*, 2005). Furthermore, in cats, the occurrence of these chronic inflammatory reactions was associated with the appearance of sarcomas at the injection site in the early 1990s, following the implementation of mandatory rabies vaccination campaigns (Hendrick and Goldschmidt, 1991; Kass *et al.*, 1993). Similar tumors have also been reported in dogs and ferrets occasionally (Munday *et al.*, 2003; Vascellari *et al.*, 2003). In the first reports of this matter in felines, Al was identified within macrophages at the borders of tumors (Hendrick *et al.*, 1992), and thus adjuvant-containing vaccines were considered to be the main factor implicated in the development of such sarcomas, therefore the condition was named “Feline Vaccine Associated Sarcoma” (Hartmann *et al.*, 2015). Later on, several studies suggested that non-adjuvanted vaccines and other injectable products such as long-acting glucocorticoids and other drugs (Esplin *et al.*, 1999; Gagnon, 2000; Kass *et al.*, 2003), or even more inert materials such as microchips or sutures (Buracco *et al.*,

2002; Carminato *et al.*, 2011) are also associated with the appearance of injection-site sarcomas, thus the condition was renamed as “Feline Injection Site Sarcoma” (FISS). Nevertheless, and according to the first reports and the evidence that adjuvant-containing vaccines increase the risk of these tumors, non-adjuvanted, Al-free, vaccines have been introduced to certain countries. In Switzerland, a non-adjuvanted feline leukemia virus vaccine was introduced in 2007, and data have shown that there has since been a marked decrease in the occurrence of FISS from 2004 to 2009. Interestingly, vaccine sales in this period increased exponentially (Graf *et al.*, 2018). Moreover, a direct effect in tumor promotion and initiation triggered by Al has recently been proposed via the use of feline fibroblasts or Chinese hamster ovary hybrid cell cultures, although this link needs to be further elucidated (AbdelMageed *et al.*, 2018).

In livestock, these Al-associated injection-site granulomas have been reported in pigs in which Al has been invariably identified by energy dispersive X-ray spectroscopy (Valtulini *et al.*, 2005), bovines (Marcato, 1990), and sheep, which some authors consider predisposed to these reactions (Spickler and Roth, 2003; Woodward, 2009). In all these species, granulomas are considered an anecdotal and casual side-effect, with no more implications than a detrimental effect in the commercial value of meat derived from the injection areas. They are also reported in other animals such as non-human primates, although it is considered a background lesion without clinical or pathological significance (Chamanza, 2011).

Overall, it is reasonable to suggest that the occurrence of a granuloma at the point of injection is a universal and invariable event in most animal species. Granulomas can be considered a desirable and necessary effect for the proper functioning of this kind of vaccines, but the long-term clinic-pathological effects of their presence has not yet been studied.

1.8. The presence of Al in distant tissues

1.8.1. Al in the CNS

Living organisms can be exposed to Al in different ways. People are exposed to Al through ambient air, diet, occupation, routinely used materials (i.e. Al foils, cooking utensils), cosmetic products (i.e. antiperspirants), drugs (i.e. antacid agents), vaccine adjuvants, and allergy immunotherapy (Exley, 2013; Klotz *et al.*, 2017). Its exposition in animals has been mainly studied in fish (Sparling *et al.*, 1997), and may be limited to environmental and dietary Al, with increasing interest in exposition via Al-containing adjuvants and other drugs.

As stated, Al is toxic to most biological systems, and specifically, has neurotoxic potential in different animal species (Li *et al.*, 2009; Caito and Aschner, 2015). Al presence in the CNS has been studied in a variety of human neurological and neurodegenerative conditions, and there seems to be an agreement among different research groups in reporting higher levels of Al in brains of people with Alzheimer's disease, thus pointing towards a putative role of the metal in the pathogenesis of the disease (Zanella and Roberti di Sarsina, 2013; Virk and Eslick, 2015; Mirza *et al.*, 2017; Mold *et al.*, 2018a, 2018b; McLachlan *et al.*, 2019). The accumulation of Al in the CNS has been studied using rodent and rabbit models experimentally subjected to Al exposure through different routes, mainly emulating environmental or dietary exposition (Rollin *et al.*, 1991; Oteiza *et al.*, 1993; Sahin *et al.*, 1994; Demirkaya *et al.*, 2017). Over the years, these animal experiments have provided information on the neurotropic potential and detrimental effects of Al exposure.

Several techniques have been used to determine Al presence in human and/or animal tissues. Analytical techniques provide quantitative data, generally expressed in μg Al/g dry matter. These techniques generally consist of an acid digestion followed by determination with an atomic spectrophotometer apparatus against a known calibration curve for Al. Among others, the most commonly used include: graphite furnace atomic absorption spectroscopy (GFAAS; House *et al.*, 2012), inductively coupled plasma mass spectrometry (ICP-MS; McDougall *et al.*, 2016), and

electrothermal atomic absorption spectrophotometry (McLachlan *et al.*, 2019). Several qualitative techniques have been used to visualize Al within tissue sections. By using these techniques, the precise location of the metal within the tissues (i.e. interaction with cells or other elements) can be established. Histochemical techniques such as lumogallion or Morin staining methods have been widely used for routine visualization (Platt *et al.*, 2001; Mirza *et al.*, 2016), while other more complex techniques such as fluorescent nanodiamonds have also been developed to track Al particles in experimental conditions (Eidi et al., 2015).

1.8.2. Translocation of injected Al in animal models

Al translocation to tissues distant to the injection point has been demonstrated in certain animal models. In a study performed with rabbits injected with Al hydroxide or Al phosphate, Al is absorbed and distributed to the kidney, spleen, liver, heart, lymph nodes, and brain, but it seems to be eliminated through the urine, although only mesenteric and not regional lymph nodes were studied, and therefore the total accumulation at that location should be underestimated (Flarend *et al.*, 1997). Interestingly, rabbits inoculated subcutaneously 20-28 times with 400 mμmol/kg injections of an Al lactate solution over one month developed similarly increased Al levels as those inoculated intracranially (Forrester and Yokel, 1985). Also in rabbits, the intravenous injection of Al demonstrated selective accumulation in neurons and other cells of the CNS (Wen and Wisniewski, 1985).

On the other hand, rats subjected to a protocol of subcutaneous allergen immunotherapy containing Al hydroxide did not show brain accumulation after 180 days, although there was persistent presence at the inoculation site, thus contributing to an increase in the global body burden of Al (McDougall *et al.*, 2016).

Redhead and collaborators (1992) established that the intraperitoneal injection of Al-adjuvant adsorbed vaccines leads to a transient increase in brain Al levels in mice. Using C57BL/6 *mdx* (leaky blood-brain barrier) mice, the appearance of biopersistent Al particles in draining lymph nodes, blood, and the brain was consistently observed after an intramuscular injection; Al particles accumulated in the brain in a linear way

up to six months after the inoculation and interestingly, Al biodistribution ceased after the regional lymph node was ablated (Khan *et al.*, 2013). The same research group demonstrated a selective low-dose accumulation of injected Al in the brain tissue of CD-1 mice (Crépeaux *et al.*, 2017). Indeed, in experiments undertaken with CD-1 mice by a different group, aggregates of macrophages with a blueish-grey intracellular material (i.e. consistent with Al), were observed in the lymph nodes adjacent to the site of injection, although the presence in distant tissues such as the brain was not studied (HogenEsch *et al.*, 2017). A large animal model has never been used to study these processes.

2. JUSTIFICATION AND OBJECTIVES

The appearance of ovine ASIA syndrome has been one of the major challenges facing the Spanish ovine industry over the last decades, and has caused significant economic losses and an important social alarm among the people involved in the ovine industry. The use of vaccines as the ideal protective tool has been questioned: on the one hand, specific parts of its composition – i.e. the Al adjuvant – were the most commonly blamed reasons for the process observed, while on the other, the repetitive use of this kind of vaccines, which likely led to over-immunization, was the other aspect said to be involved in the genesis of the syndrome.

Almost all commonly used ovine vaccines in our local management conditions contain an Al-based adjuvant, and therefore sheep are continuously exposed to this compound by the use of vaccines. In our local management conditions, a flock may be vaccinated between two and four times in a year, depending on factors such as the specific health problems or the cycle of management procedures. Moreover, compulsory vaccination campaigns against emerging or re-emerging diseases (i.e. BT) are recurrently applied in various areas, thus increasing the total vaccine load a single sheep may receive in a year. It is difficult to estimate the number of vaccines a sheep can receive in its productive life because of the high variability of the health management practices among flocks. However, it is reasonable to consider that a single sheep can receive about 14-28 vaccine shots in a mean of a seven-year lifespan.

Little work has been done on the repetitive inoculation of these compounds in sheep, and specifically, on ovine ASIA syndrome. Research on the effects of injected Al in sheep is needed due to its broad use, and in order to clarify its putative role in the development of ovine ASIA syndrome. New scientific data on these effects may benefit the ovine industry at large, either by strengthening the confidence of farmers and veterinarians in vaccine strategies that contain this adjuvant, or by laying a cornerstone for the development of more effective and safer vaccine adjuvants in the future.

In this work, we propose using a sheep experimental model where a first group of animals will receive the same number of Al-containing vaccines they would receive over six-eight years in normal productive conditions, but in a shorter timeframe. To

determine the specific role of Al in the changes observed in the over-vaccinated group, a second group will receive the equivalent Al dose, only. A third group will receive placebo injections (i.e. phosphate buffered saline [PBS]) to differentiate the putative effects of the other two groups from the effects caused by the procedure of repeated injections.

The action of the vaccine and its adjuvant begins at the inoculation point, with the stimulation of the innate response, cellular activation, generation of a cytokine storm, presentation of the antigen in the local lymph node, and eventual induction of an adaptive response with the generation of antigen-specific antibodies and immunologic memory. In this way, studying the morphological changes that occur at the injection point may be a necessary first step in the establishment of the model. Indeed, injection-site long-term intramuscular reactions with the presence of Al are associated with a specific syndrome in humans, MMF (Gherardi and Authier, 2012). Murine models of this condition have been established, and studies of the injection site reactions, the role of the Al in the genesis of local changes, and the mobilization of the metal to distant tissues have been performed, demonstrating the different roles of these processes in the development of MMF (Khan *et al.*, 2013; Crépeaux *et al.*, 2017). Furthermore, the mobilization of injected Al to other tissues has been also demonstrated in laboratory rabbits by a number of groups (Forrester and Yokel, 1985; Flarend *et al.*, 1997).

The array of symptoms described in ovine ASIA syndrome is broad and sometimes unspecific. Among them, the epidemiological observation of behavioral changes in flocks affected by the chronic phase is constant, and includes symptoms such as continuous movement, restlessness, and compulsive wool biting (Luján *et al.*, 2013). However, these observations have not been fully characterized and quantified, and its association with the number of vaccines applied remains non-elucidated. This work proposes a behavioral and cognitive characterization of experimental animals subjected to a protocol of over-immunization with Al hydroxide-containing products using previously established and validated methods for the study of sheep ethology.

This study will be the first large-scale experiment on ovine ASIA syndrome ever performed. Different aspects have been studied using laboratory animals modelling similar syndromes and inoculation schedules, but a large animal model is currently lacking. To unravel the various aspects of the syndrome, a suitable specific animal model would be of great interest; indeed, this experiment can generate a suitable *in vivo* model of over-immunization, and the results obtained could be very useful for our understanding of ovine ASIA syndrome. The occurrence of adverse reactions before and after the massive episode linked to the BT vaccination campaigns (2008-2010), together with the current broad use of this kind of vaccine adjuvants in sheep, justifies the present experimental work.

This work has been developed in the framework of a multi-institutional collaborative research project funded by the Spanish Ministry of Economy and Industry, whose main objective has been to determine the effects of the repeated inoculation of Al hydroxide-containing adjuvants in sheep. This project aims to establish a model of ovine ASIA syndrome in order to study its clinical, analytical, genetic, immunological, and pathological aspects. The present PhD thesis will mainly focus on: i) clinical aspects: a characterization of the effects on behavior and cognition of repeated inoculation procedures; and ii) pathological and analytical: a characterization of injection-site reactions and the mobilization of Al from the injection point to distant tissues.

The group at the Department of Animal Pathology of the University of Zaragoza that leads this research has been working on ovine ASIA syndrome and other sheep diseases for more than ten years. Multiple resources and facilities for ovine experimentation and pathologic studies are available at the Department. Studies on sheep ethology have been performed in collaboration with the animal ethology group at the Department of Animal Production and Food Science of the University of Zaragoza. This group has been performing studies on the behavior of farm animals for more than 20 years, and possesses broad international recognition in the field. Their methods are deeply established, validated, and generate reproducible results. Some of the studies on Al in tissues have been performed in the frame of a research externship at the Birchall Centre, Lennard-Jones Laboratories, Keele University (Stoke-on-Trent,

UK). This Centre is fully equipped to study the content and *in situ* presence of Al in tissues. The transversal approach provided by the three different groups involved generates a unique combination of knowledge and resources that ensures a team with the technical capabilities, equipment, material, and background to develop the present research work with full guarantees.

The objectives of the present study are:

1) To determine the effects in sheep of an experimental repeated inoculation procedure of Al hydroxide, either alone or as adjuvant in commercial vaccines by: i) quantifying cognition and behavior; ii) studying morphological and ultrastructural changes induced at the inoculation point; and iii) studying the persistency of these changes and Al translocation to other body locations.

2) To establish a model for ovine ASIA syndrome and to determine the specific role of Al in the genesis of this syndrome by studying Al interactions with tissues in a sheep model

3. MATERIAL & METHODS

3.1. Global experimental design

The present work is based on a single experiment using lambs. They were subjected to a protocol of repetitive inoculation and several *in vivo* and *post mortem* studies were performed. The Ethical Committee of the University of Zaragoza approved and licensed all experimental procedures (ref. PI15/14). Requirements of the Spanish Policy for Animal Protection (RED53/2013) and the European Union Directive 2010/63 on protection of experimental animals were always fulfilled.

3.1.1 Experimental flocks and groups

The study was based on four flocks of 21, three-month-old, neutered male lambs (total n=84). Flock 1 was established at the experimental farm of the University of Zaragoza and it was constituted by Rasa Aragonesa pure-breed lambs that were selected from a pedigree flock ("Masía El Chantre", Teruel, Spain) free of the most important sheep diseases (Pinczowski *et al*., 2017). This flock was always maintained indoors, with ideal conditions of housing, management and diet. The animals constituting flocks 2, 3 and 4 were selected from three commercial livestock farms representing different management conditions and geographical areas (Table 1, page 60). Each flock of 21 lambs was divided into three treatment groups (Vaccine, Adjuvant-only and Control). Two animals from each treatment group died for unrelated reasons along the course of the experiment; therefore, each treatment group consisted of 26 animals at the end of the experiment and data derived from dead animals were not considered. The complete study lasted 15 months.

Table 1. Characteristics of the animals and flocks used in the study

Flock	Breed	Geographic location	Management
1	Purebred[a]	Experimental farm[c] (University of Zaragoza)	Fully indoor; treatment groups not in contact; free of ovine diseases
2	Crossbred[b]	River Valley[d]	Intensive[g]
3	Crossbred[b]	Pre-Pyrenees area[e]	Semi extensive[g]
4	Purebred[a]	River Valley[f]	Semi extensive[g]

a. Rasa Aragonesa breed
b. Commercial crosses of Rasa Aragonesa (Rasa Aragonesa x Romanov)
c. 41°41'N 0°52'W
d. 41°31'N 0°32´W
e. 42°9'N 0°12´W
f. 41°36'N 0°41´W
g. Treatment groups pooled together and mixed with the rest of the flock

3.1.2. Inoculation schedule

Experimental lambs underwent an accelerated vaccination schedule that tried to reproduce the secondary effects due to the repetitive inoculation of a mean of three vaccines per year in a seven-year life-span, thus mimicking, in an acceptable experimental time frame for a project, the local management field conditions (Lacasta *et al*., 2015). Animals in each treatment group received a total of 19 subcutaneous injections of the corresponding substance at 16 inoculation dates, as there were 3 days that required double inoculations. The four flocks were inoculated in parallel, although time between the 14th and the 16th injection varied considerably among the flocks to ensure that every animal received the last inoculation exactly 5 days prior to the sacrifice (Fig. 1, Table 2).

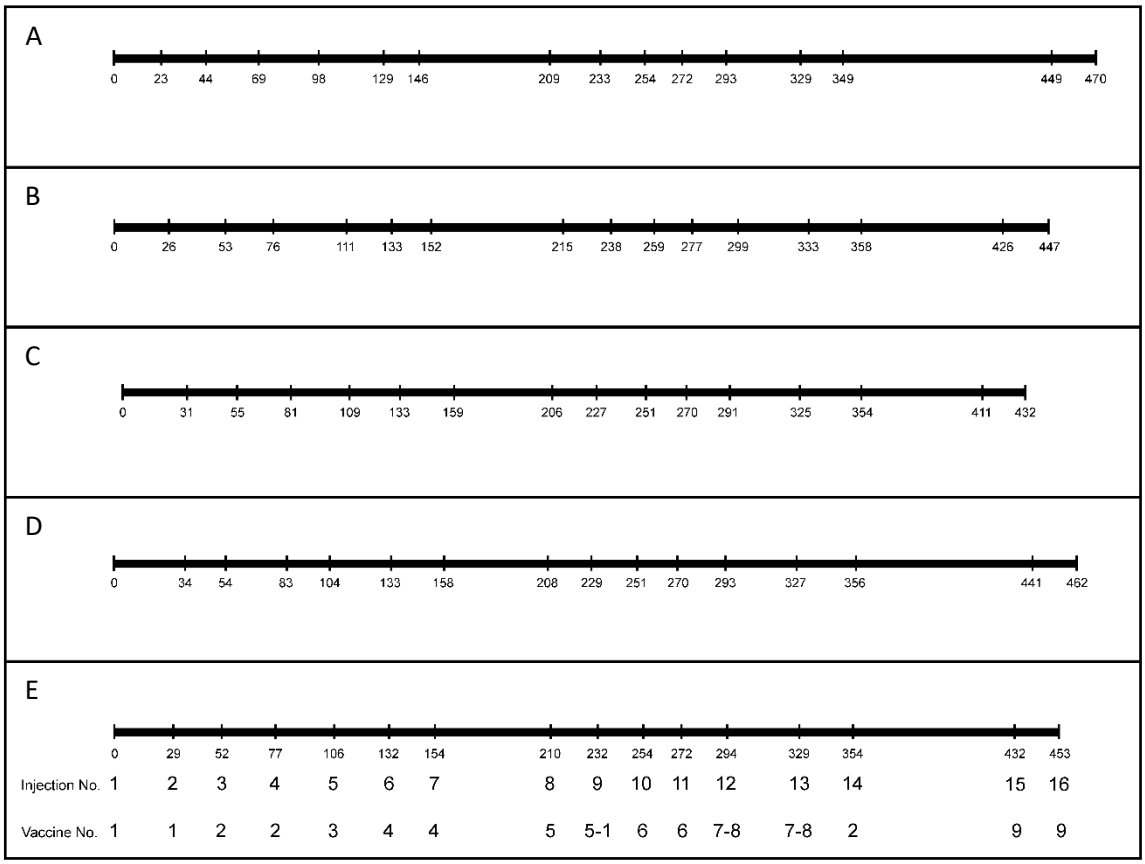


Figure 1. Inoculation schedule. Each mark represents an injection number; numbers indicate the days post first inoculation (dpi). See Table 2, page 63 **A**: Flock 1; **B:** Flock 2; **C:** Flock 3; **D:** Flock 4; **E:** Global, dpi are the mean of the 4 Flocks.

Table 2. Description of the vaccines used. Sixteen dates and 19 inoculations were stablished. Sixteen and three inoculations were performed in the right and left flank, respectively. Aluminum (Al) content was established by inductively coupled mass spectrometry (ICP-MS) and calculated per total dose.

Vaccine number (Fig. 1)	Commercial name	Manu-facturer*	Antigen/s	Inocu-lation date (Fig. 1)	Inoculation site		mg of Al per dose
					Right flank	Left flank	
1	Heptavac P Plus	MSD Animal Health S.L.	*Pasteurella multocida, Mannheimia haemolytica, Clostridium spp*.	1, 2, 9	2	1	7.5
2	Autogenous vaccine	Exopol	*Staphylococcus aureus* spp. *Anaerobius*	3, 4, 14	3	N/A[a]	1.644
3	Vanguard R	Zoetis	Rabies virus	5	1	N/A	1.025
4	Agalaxipra	Hipra	*Mycoplasma agalactiae*	6, 7	2	N/A	6.764
5	Ovivac CS	Hipra	*Chlamydophila abortus*, *Salmonella abortus ovis*	8, 9	2	N/A	5.6
6	Autogenous vaccine	Exopol	*Corynebacterium pseudotuberculosis*	10, 11	2	N/A	1.32
7	Bluevac-1	CZ Veterinaria S.A.	Bluetonge virus serotype 1	12, 13	2	N/A	4.18
8	Bluevac-4	CZ Veterinaria S.A.	Bluetongue virus serotype 4	12, 13	N/A	2	4.16
9	Bluevac BTV 8	CZ Veterinaria S.A.	Bluetongue virus Serotype 8	15, 16	2	N/A	4.4

*All vaccines manufactured in Spain

[a]:N/A: Not applicable

3.1.3. Inocula used

Vaccine group was inoculated with commercial vaccines against main ovine diseases (Table 2, page 62). The recommended period between vaccines and application procedure for each product was always fulfilled. Adjuvant-only group was injected with Al hydroxide only (Alhydrogel®, CZ Veterinaria, Spain). The concentration of Al for each inoculum was calculated to be identical to that of the corresponding vaccine by dilution with PBS. The Al content in the adjuvant and each vaccine was established by ICP-MS (Table 2 page 62). Vaccine and Adjuvant-only groups received a total of 81.29 mg of Al. Control group was inoculated with the same volume of PBS.

Lambs were injected subcutaneously in the back, within and area encompassing the ribs and scapula (Fig. 2). Sixteen injections were performed in the right flank, and those corresponding to double injections days were performed within the left flank.

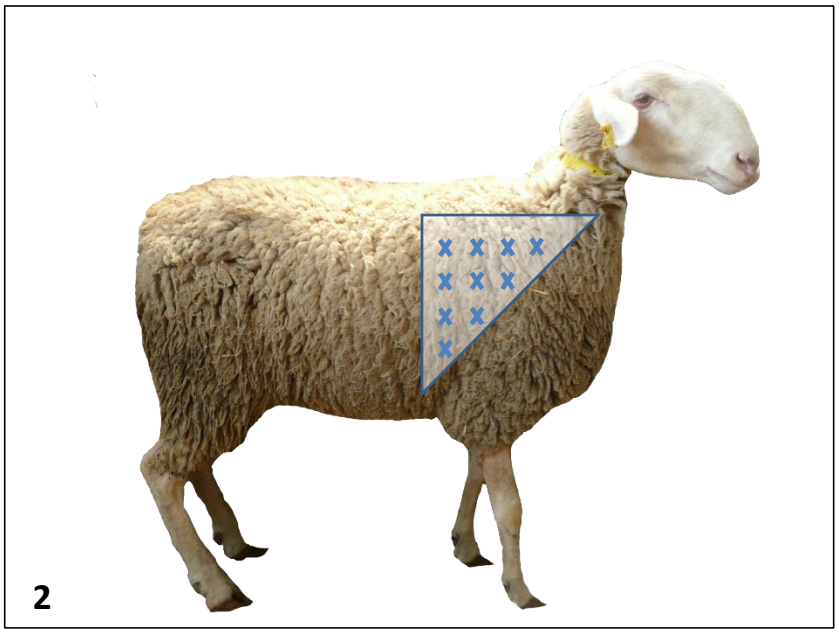


Figure 2. Area of inoculation. Injections were performed in different points within an area of encompassing 40x40 cm, between the ribs and scapula

3.2. Cognition and behavior studies

3.2.1. Design

These studies were carried out using only animals in flock 1 (n=7, Table 1, page 60), at the experimental research farm of the University of Zaragoza (41°41'N 0°52'W), northern Spain. The farm is in the Ebro River Valley, which has a dry Mediterranean climate, an average annual temperature of 15 °C, and an average annual rainfall of ~320 mm.

The first 349 days of experiment where considered for this study (Fig. 1A), thus including a full summer and a winter period between February 2015 and February 2016. Therefore, within the time frame of the present study, lambs received the first 16 inoculations (Table 2, page 62) including a total amount of 70.861 mg of Al in the Vaccine and Adjuvant-only groups.

3.2.2. Management of the animals

Housing, management conditions and diet were identical for all animals. The three treatment groups (Vaccine, Adjuvant-only and Control) were isolated from each other, but they occupied contiguous, identical pens within the same building. Pens were rectangular closed rooms, 7x3.5x6 m (length x width x height), open to the exterior of the building at one of the short walls through four closable, rectangular, 1x0.2 m (length x width) windows with bars (see Fig. S1 in Annex I for a detailed scheme of the home pens). The daily ration was 1 kg of concentrate per animal, and straw and water ad libitum. Each day at 0830 h, concentrate was offered from a 2-m-long hopper that had two openings, which permitted all animals in a group to eat simultaneously. Concentrate was a commercial mixture (Agroveco®, Zaragoza, Spain) in pellet form that had a nutritional composition of 15.7 % crude protein, 3.9 % crude fat, 10.4 % crude fiber, 9.5 % crude ash, 0.22 % sodium, and adequate proportions of mineral and vitamin supplements for growing lambs. Minimum, maximum, and average ambient daily temperatures in the area were obtained from the Spanish Agency of Meteorology (AEMET). The average daily temperature was 25.5 °C in

summer and 9.0 °C in winter. In both seasons, temperatures inside the pens (HOBO® data logger; Onset Computer Corporation®, USA) were slightly higher than the ambient air temperatures.

3.2.3. Cognitive and behavioral assessments

Animals were subjected to two rounds of each of the tests described below. The first round began at 196 dpi, in mid-September 2015 (summer). The second round began at 336 dpi, in late January / early February 2016 (winter; Table 3, page 66). In the summer round, all animals had been inoculated 7 times and, in the winter round, the animals had been inoculated 16 times.

Table 3. Timing of events performed in each test in the two rounds of the experiment.

	Summer round (September 2015)											Winter round (January/February 2016)									
Day	**8th**	**9th**	**10th**	**14th**	**15th**	**16th**	**17th**	**18th**	**19th**	**20th**	**21th**	**26th**	**27th**	**28th**	**1nd**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**
dpi	196	197	198	202-208							209	336	337	338	342-348							349
Test	TMd1	TMd2	OFT NOT	Recordings Home pen behavior observations							Blood tests	TMd1	TMd2	OFT NOT	Recordings Home pen behavior observations							Blood tests

Abbreviations: dpi: days post first inoculation; TMd1: T maze day 1; TMd2: T maze day 2; OFT: Open Field Test; NOT: Novel Object Test.

3.2.3a. Cognitive test: T-maze

Each animal was subjected to the T-maze test on two consecutive days in summer and in winter rounds (Table 3, page 66). The order in which lambs were tested alternated among the three groups, and the order was maintained in all tests. Tests were performed in a T-maze built of 1.4-m-high plastic panels, which had been validated for use with lambs (Abecia *et al*., 2014). The maze comprised an isolation chamber (2x2 m) that at one end was joined to a T-corridor that comprised a 2x0.80-m path connected to two perpendicular arms (1.65x1.65 m, each). A mirror (70x30 cm) clue and a loudspeaker were placed at floor level in the target zone in the left arm, which served as a social and a sound clue, respectively. To avoid influencing animal movements, an observation platform was placed 3 m above the ground, adjacent to the T-maze apparatus (Ortiz-Plata *et al*., 2012). The sound recordings were of ewes and lambs vocalizing during a short-term separation, which had been recorded 50 cm from the source using a H1 Handy Recorder (Zoom Corporation Tokyo, Japan). The recordings were imported into a computer at a sampling rate of 44.1 kHz, and saved in WAV format at 16-bit amplitude resolution (Briefer and McElligott, 2012). Audacity® audio software was used to prepare the sound sequences. Samples of the vocalizations were combined into a 5-min segment, and a randomly selected portion of the segment was played in each test. The noise level of the recordings was measured using a Bioblock Scientific Sound Level Meter type 50517, and the playback volume was set to ensure that lambs were exposed to 81 dB throughout most of the T-maze (see Fig S2 in Annex I for a detailed scheme of the T-maze test arena). After the lamb entered the maze, the recorded vocalizations were broadcast. Video recordings of each test were used to calculate latency (time spent in the first corridor), time taken to reach the target zone, time spent solving the maze, and the number of areas traversed. Each animal was given up to 5 min to solve the maze.

3.2.3b. Cognitive test: OFT

Each animal was subjected to the OFT once in each of the two rounds. The order in which lambs were tested alternated among the three groups, and the order was maintained in all tests.

The 4x4 m test arena, which was marked out in a grid of 0.50×0.50 m squares and built with 1.40 m high plastic panels, presented a novel environment for the animal that was completely isolated from other lambs. Water and food were offered from a bucket that was familiar to the lamb, which was placed against the wall of the arena opposite the entrance door (see Fig. S3 in Annex I for a detailed scheme of the OFT arena). Each animal was left in the test arena for 5 min, which was recorded with a videotape and a microphone. From the recordings, the observer calculated the amount of time (seconds) the animal spent walking, exploring, standing, and trying to escape, as well as the number of bleats, and the number of escape attempts (jumps).

3.2.3c. Cognitive test: NOT

The NOT was performed at the end of each round of OFT and in the same test arena. A blue plastic ball tied to a rope was lowered from the ceiling to the floor at the center of the arena. When it hit the floor, it was left there for 1 min before being lifted away. One minute later, the procedure was repeated. Each time the ball was lowered, after 30 s, an observer recorded the distance between the lamb and the novel object.

3.2.3d. Behavioral tests: home pen individual and social behavior observations

All lambs were individually identified by numbers painted on their sides and rump with a spray for animal marking. To record social and individual behaviors, a camera was placed at the top of each pen, and a videorecorder (model VDVR-9; Circontrol S.A., Spain) was set up in a room adjacent to the pens (see Fig. S1 in Annex I for a detailed scheme of the pens). In each of the two rounds, recordings were made for 12 h/day (0800 h – 2000 h) on seven consecutive days (Table 3, page 66). After the experiment had concluded, a single trained researcher analyzed consecutively the

videos of the two rounds in a blind manner, as this person was unaware of the treatment in each group.

The video data were quantified in two ways: Instantaneous sampling for individual behaviors, and continuous sampling for social behaviors. Instantaneous sampling involved quantifying 1 min from each 10 min of video, totaled 504 min per group and 1512 min per round. Continuous sampling involved quantifying three 2-h periods per day (0800 h – 1000 h, 1200 h – 1400 h and 1600 h – 1800 h) which totaled 42 h of observations per group and 126 h per round. The analysis of both individual and social behaviors involved documenting the number of events in which an animal displayed a specific behavior. Individual behaviors included feeding on concentrate, eating straw, resting, standing, and drinking (Table 4). Social behaviors were assigned to one of three categories: i) affiliative interactions; ii) agonistic (aggressive) interactions; and iii) stereotypies (Table 5, page 70). Affiliative interactions have a positive connotation and imply, in any case, a dual interaction component between two lambs. Agonistic (aggressive) interactions have a negative connotation and a component of intention from one animal to another. Stereotypies have also a negative connotation.

Table 4. Individual behaviors studied

Behavior	**Description**
Feeding on concentrate (FC)	Lamb searching for concentrate in the hopper and eating
Eating straw (ES)	Lamb searching for straw in the hopper and eating it
Resting (RT)	Lamb lying down
Standing (ST)	Lamb standing on all four legs or walking
Drinking (DK)	Lamb drinking water from the drinking trough

Table 5. Social behaviors studied.

Affiliative interactions	
Following	A lamb follows another lamb with the intention of keeping close to it
Licking	A lamb licks another lamb's body
Sniffing	A lamb sniffs another lamb's body
Sexual mounting	A lamb mounts another lamb in play or with sexual intent
Rubbing	A lamb is rubbed by the body of another lamb
Grooming	A lamb grooms another lamb
Resting together	Two or more lambs lie down together with an interaction component
Agonistic (aggressive) interactions	
Headbutting	A lamb uses the front of its head to make contact with another lamb
Body bumping	A lamb pushes another lamb with its body
Wool biting	A lamb bites another lamb´s wool
Pawing	A lamb uses its foreleg to kick to other lamb
Mounting	A lamb mounts another lamb with the intention of moving it away
Threatening	A lamb threatens another lamb with a head thrust without making contact
Chasing	A lamb actively moves towards another lamb, causing the latter to walk or runaway
Stereotypies	
Number of times that a lamb repeats an abnormal behavior, i.e.: Compulsive scratching against the wall, repetitive licking of the bars of the hopper etc.	

3.2.4. Hematology panel and welfare indicators

A standardized hematology panel was performed, and welfare and stress indicators including cortisol, creatine kinase (CK), lactate, glucose, non-esterified fatty acids (NEFA) and neutrophil/lymphocyte (N/L) ratio (Barnett *et al*., 1990; Mendl *et al*., 1991; Van de Water *et al*., 2003; Caroprese *et al*., 2008; Pascual-Alonso *et al*., 2014, 2015) were measured on the day after the recording period in each of the two rounds (Table 3, page 66), which was sufficiently removed from recent handling of the animals. Blood samples were taken at 0800 h by jugular venipuncture, which required <1 min per lamb. Plasma and serum were obtained by centrifugation at 3000 rpm for 10 min, and then stored at -20 C. The leukocyte formula was estimated from blood smears that had been stained with Diff-Quick (Microptic®). The N/L ratio was calculated based on 100 leukocytes per sample. A clinical chemistry system multi-analyzer (Alfa Wasserman ACE, USA) was used to measure the serum concentration of glucose (mg/dl), the activity of CK (IU/L), and the NEFA concentration (mmol/L). Cortisol concentration (nmol/L) in plasma was measured by enzyme immunoassay (Chacón-Pérez *et al*., 2004), and lactate concentration (mmol/L) was obtained using a Sigma Diagnostic kit (lactate no. 735-10) and a spectrophotometer (Lambda 5, PerkinElmer, USA).

3.3. Studies at injection sites and Al mobilization

3.3.1. *In vivo* studies

These studies were carried out considering animals from flocks 1 to 4 (Table 1, page 60). At each injection date, the injection points were assessed by palpation. A range of "degree of severity" from 0 to 3, was established (0: No reaction; 1: One nodule-forming reaction smaller than 0.5 cm; 2: One nodule-forming reaction bigger than 0.5 cm; 3: One nodule with central liquefaction and/or fistulation or more than one palpable nodule-forming reaction). This assessment was carried out up to and including the 14th injection (Fig. 1, page 61; Table 2, page 62).

3.3.2. Pathologic characterization

These studies were carried out considering animals from flocks 1 to 4 (Table 1, page 60). A complete necropsy including a systematic sampling of all tissues was performed but, for the purpose of this chapter, only features recorded in the right flank or in the right prescapular lymph node will be described. This sample collection procedure guaranteed the required samples for the study (see below) since the number of injections was higher in the right flank, and helped in collecting the samples in a minimal time frame. The subcutaneous tissue was exposed and the adipose panniculus dissected and removed. All grossly visible injection nodules were recovered and their number recorded. Presence of central necrosis within the nodules was also recorded. Four categories were established depending on the number of nodules found in each animal: 0, 1-2, 3-7 and 8 or more nodules.

For histopathologic purposes, only chronic, well-developed nodules were considered, avoiding acute tissue reactions corresponding to the injections performed five days before euthanasia. Nodules were fixed and embedded in paraffin but only one per animal was randomly selected for detailed histopathological studies. This procedure based upon the very homogeneous histopathologic characteristics of nodules (see Results). Samples were also obtained from the regional (right prescapular) lymph node. In total, 47 injection-site nodules (24 from Vaccine and 23 from Adjuvant-only animals), 26 Control injection-site areas and 76 lymph nodes (26 from Vaccine, 24 from Adjuvant-only and 26 from Control animals) were analyzed. Tissues were routinely processed and stained with hematoxylin-eosin (HE). The semi-quantitative histopathologic scoring system of the injection-site nodules and prescapular lymph nodes is detailed in Tables 6 and 7 (pages 73 and 74). Two pathologists performed a blind and individual microscopic evaluation of these parameters, reaching a final consensus.

Table 6. Histopathological evaluation of the granulomas

Granulomas	Scoring	Description
Voluminous macrophages	0 to 3	0: Absence 1: Less than 50 % of the cell population 2: 50 % to 75 % of the cell population 3: More than 75 % of the cell population
Multinucleated giant cells	P/A[a]	>3 nuclei giant cells, either foreign body or Langhans type
Lymphocytes	0 to 3	0: Absence 1: 1-2 inconspicuous groups 2: 3-4 readily visible groups 3: >4 voluminous groups
Tertiary lymphoid tissue	P/A	Group/s of densely packed lymphocytes with formation of germinal centers
Neutrophils	P/A	Group/s of >10 neutrophils
Necrosis	0 to 3	0: Absence 1: Slight multifocal eosinophilia 2: Central necrosis <50 % of the section 3: Central necrosis >50 % of the section
Mineralization	0 to 3	0: Absence 1: Rare, amorphous, multifocal <50 µm in diameter 2: Well-defined, crystalloid, coalescing areas 50-200 µm in diameter 3: Markedly crystalloid coalescing areas 200-500 µm in diameter
External fibrosis (capsule)	P/A	> 15 µm collagenous capsule
Internal fibrosis	P/A	Streams, bundles and trabeculae of fibrocytes, fibroblasts and collagen fibers
Internal hemorrhages	P/A	Aggregates of extravasated erythrocytes between macrophages
External hemorrhages	P/A	Aggregates of extravasated erythrocytes in the capsule and/or peripheral tissue
Internal blood vessels	P/A	Newly-formed blood vessels between macrophages
Eosinophilic crystalloid bodies	0 to 3	0: Absence 1: 1-4 bodies 2: 5-8 bodies 3: >9 bodies

[a]: P/A: Presence/Absence

Table 7. Histopathological evaluation of the right preescapular lymph nodes

Lymph nodes	Scoring	Description
Cortical hyperplasia	0 to 3	0: Absence. 1: Mild increase in cortical thickness. Non-coalescing lymphoid follicles 2: Moderate increase in cortical thickness. Increased lymphoid follicles 3: Marked and diffuse increase in cortical thickness. Coalescing lymphoid follicles.
Prominent germinal centers	0 to 3	0: Absence. 1: <50 % of follicles show germinal center 2: 50-75 % of follicles show germinal center 3: >75 % of follicles show a germinal center with increase in size
Aggregates of voluminous macrophages	P/A[a]	At least 4, 15-25 µm macrophages with foamy to granulated cytoplasm

[a]: P/A: Presence/Absence

3.3.3. Microbiology

These studies were carried out considering animals in flocks 1 and 4 (Table 1, page 60). Thus, a total of 40 animals were studied by microbiological means: Twenty-six injection site nodules (13 from Vaccine and 13 from Adjuvant-only animals), together with 14 areas of injection from Control animals were subjected to routine microbiological studies. Each sample was studied by direct Gram staining in smears and incubation of microbiologic cultures in aerobic conditions [Columbia blood agar and MacConkey agar (Oxoid, Basingstoke, Hampshire, UK), up to 48 hours at 37° C] and anaerobic conditions (Columbia blood agar, up to 5 days at 37° C). Basic phenotypic bacterial identification was based on colony and cell (Gram stain) morphology and by standard biochemical tests.

3.3.4. Al *in situ* studies

The presence of Al in granulomas and lymph nodes was studied by fluorescence microscopy with lumogallion staining and by electron microscopy. For fluorescence

microscopy, 4 randomly-selected injection site nodules from animals in flock 1 (Table 1, page 60; 2 from Vaccine and 2 from Adjuvant-only animals) were studied, along with two injection-site areas from Control lambs. Samples from the corresponding right prescapular lymph nodes of the same six above-mentioned animals were also analyzed. A protocol to identify Al in tissue sections using lumogallion staining was followed (Mold *et al*., 2014; Mirza *et al*., 2016). Briefly, 5 µm tissue sections were dewaxed, rehydrated and incubated with a 1 mM solution of lumogallion (Tokyo Chemical Industry, UK) prepared in 50 mM PIPES rinse solution. Serial control sections were in PIPES rinse solution only. Slides were washed 6 times with PIPES, rinsed in ultrapure water and mounted with an aqueous medium. Lumogallion and control autofluorescence analyses were performed using a bandpass excitation filter of 470-495 nm.

Eight randomly-selected injection site nodules from flock 1 (Table 1, page 60; 4 from Vaccine and 4 from Adjuvant-only animals, including those animals analyzed by fluorescence microscopy) were submitted to scanning transmission electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDS). Briefly, selected tissues were fixed in 2.5 % glutaraldehyde plus 2 % paraformaldehyde (0.1 M PBS) washed in PBS, post-fixed in 2 % osmium, dehydrated in increasingly-graded acetone and embedded in araldite. Selected ultra-thin sections were counterstained with 1 % uranyl acetate and Reynold´s lead citrate. STEM images were obtained in a Tecnai F30 microscope (FEI Company, USA) equipped with an EDS detector. Al determinations by EDS were always performed in intracytoplasmic aggregates, whereas determinations in nuclei were used as internal negative controls. Al particle size and Al aggregates area were measured using STEM images: the length of Al particles was determined in four STEM images per animal at 68,000x magnification by measuring 10 particles per image (40 particles per animal) and the area of Al aggregates was established in four STEM images per animal at x8,500 by measuring 5 well-delimited aggregates per image (20 aggregates per animal). Large, dense eosinophilic crystalloid bodies (made up of Al, see description in Results) were not included in the calculation of the area occupied by Al aggregates.

3.3.5. Al content in regional lymph nodes

Twelve lymph nodes from animals in flock 1 (Table 1, page 60; 4 from each group, including the animals analyzed by fluorescence microscopy, STEM and EDS) were analyzed by microwave digestion followed by TH GFAAS (House *et al*., 2012). Briefly, three replicate portions of 0.3-0.5 g from each lymph node were dried in a 37°C incubator until reaching a constant weight. They were then digested in a microwave (MARS Xpress CEM Microwave Technology Ltd.) in a mixture of 1 mL 15.8 M HNO3 and 1 mL of 30 % w/v H2O2. Upon cooling each digest was diluted to 5 mL with ultrapure water and total Al was measured using an atomic absorption spectrometer with a transversely heated graphite atomizer and longitudinal Zeeman-effect background corrector and an AS-80 autosampler with WinLab32 software (Perkin Elmer, UK). The Zeeman background corrected peak area of the atomic absorption signal was used for determinations. Results were expressed as µg Al/g tissue dry weight. Each determination was the arithmetic mean of three injections with a relative standard deviation of 10 %.

3.3.6. Al content in CNS

The content of Al in the brain (parietal lobe) and lumbar spinal cord was determined in a total of 21 animals (flock 1; Table 1, page 60) by microwave digestion followed by TH GFAAS. Three serial replicate portions of 0.3-0.5 g from each area were selected and submitted to the process described above for regional lymph nodes.

3.4. Statistical analyses

All statistical analyses were performed using IBM SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

3.4.1. Cognition and behavior studies

Comparisons within the different tests were performed as follows:

-T-maze test: Unpaired comparisons between groups for each of the two days of the test in each round, and paired comparisons within groups between the two days of the test in each round.

-OFT: Unpaired comparisons between groups within each round of tests and paired comparisons within groups between the two rounds.

-NOT: Unpaired comparisons between groups for each of the two distances to the novel object in each of the rounds, and, within each group, paired comparisons between the two distances in each round.

-Behavior: Unpaired comparisons between groups within each round of tests and paired comparisons within groups between the two rounds, which were restricted to social behaviors because of seasonal influences on individual behaviors due to normal physiological seasonal variations (e.g., all groups drank more water in summer and ate more straw in winter).

-Hematology panel & Welfare indicators: For most of the parameters, unpaired comparisons between groups were performed for each round. Additionally, within-group paired comparisons between rounds were performed for cortisol, only.

A Shapiro-Wilk´s Test was used to confirm whether the data of the quantitative variables met the assumptions of normality. A parametric test was used if the variable met the assumption, and a non-parametric test was used if it did not. Unpaired comparisons were performed using an ANOVA (A) Test (or Welch's t-test [We] if variances were not homogeneous based on a Levene's test) and a *post hoc* Duncan's test (parametric tests), or a Kruskal-Wallis (KW) test and a *post hoc* Dunn's test (non-parametric tests). Paired comparisons were performed using Student's t (t) test for dependent samples (parametric test) or a Wilcoxon (W) test (non-parametric test). General Linear Models (GLM) were developed to assess the influence of "Treatment Group" and "Round", and their paired interactions on the behavioral tests (Daniel and Cross, 2013).

3.4.2. Studies at injection sites and Al mobilization

Qualitative variables, as groups of treatment and histopathologic features, were described using absolute and relative frequencies. Assessment of the associations between two qualitative variables was carried out using Pearson´s Chi-square test (χ^2; or alternatively Likelihood ratio for n x m tables or Fisher´s exact test for 2 x 2 tables; Daniel and Cross, 2013).

Shapiro-Wilk´s test was used to check if the data of the quantitative variables (severity of the *in vivo* reactions, Al particle length/aggregates and Al content in regional lymph nodes and CNS) met the assumptions of normality. As any of them met the assumptions, they were considered non-normal, thus they were described using median and interquartile range (IQR) and graphically represented using box and whiskers plots. Association of a non-normal quantitative variable with a qualitative variable with two categories was assessed by Mann-Whitney U (U) test, i.e. comparison of the severity of the *in vivo* reactions, Al particle length and Al aggregates area between Vaccine and Adjuvant-only groups. Association of a non-normal quantitative variable with a qualitative variable with three or more categories was assessed by KW test followed by a *post hoc* Dunn´s test, i.e. comparisons of the content in Al in the regional lymph nodes and CNS among the three groups (Daniel and Cross, 2013).

4. RESULTS

4.1 Cognition and behavior studies

4.1.1 Cognitive tests

4.1.1a. T-maze Test

There were no significant differences between groups in any of the two rounds (summer or winter). Within groups, there were no significant differences between the two consecutive days of testing in summer. In winter, however, Vaccine group left the first area (latency) significantly (p_W=0.027) earlier on the second day of testing (see Annex II, Tables S1 – S8).

4.1.1b. OFT and NOT

Neither in summer nor in winter did the three groups differ significantly between them in either of the two tests. In the OFT in winter, Vaccine group lambs spent significantly (p_t=0.003) less time trying to escape (Fig. 3, page 82), and Adjuvant group animals spent significantly (p_W=0.043) more time exploring than they did in summer. In the NOT, in winter, Adjuvant group lambs were significantly (p_W=0.043) farther from the novel object in the second exposure than they were in the first exposure (see Annex II, Tables S9 – S16).

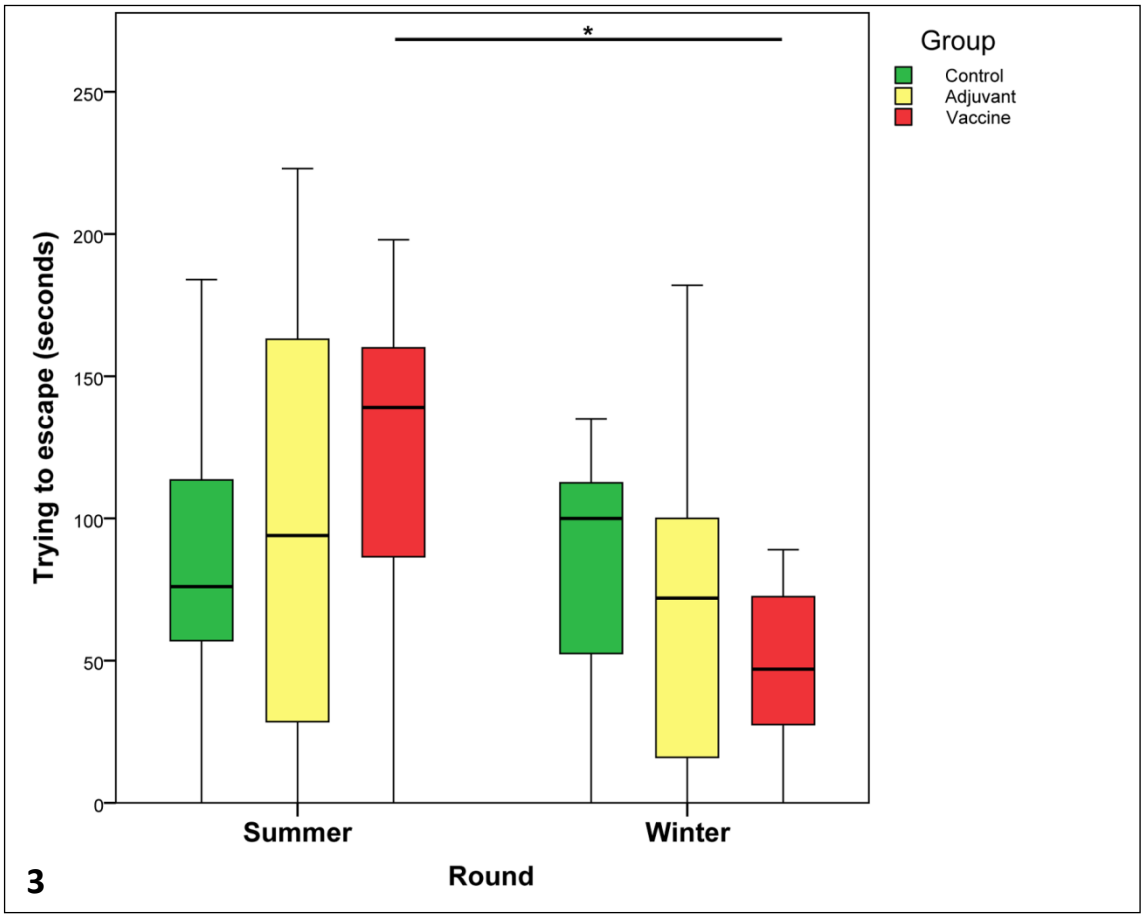


Figure 3. Open Field Test (OFT). Trying to escape. Vaccine group spent less time trying to escape from the test arena in the winter round of tests (*p_t=0.003). Statistical analysis was performed using Student's t-test for dependent samples. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are presented in Annex II, Table S11.

4.1.2. Behavioral tests

4.1.2a. Individual behavior

In summer, animals in Vaccine group ate straw significantly (p_{KW}=0.018) less frequently than did animals in Control group. Animals in Vaccine and Adjuvant-only groups spent significantly (p_A<0.001) more time standing than did animals in Control group. In winter, Vaccine and Adjuvant-only groups ate concentrate significantly (p_{KW}<0.001) fewer times than did Control group. Lambs in Vaccine and Adjuvant-only groups rested less often (p_{KW}=0.027) and stood significantly more often (p_A<0.001) than did lambs in Control group (Fig. 4; see Annex II, tables S17-S21). The GLM indicated that the interaction effect between "Treatment Group" and "Round" was significant (p=0.035) for standing (see Annex II, Table S22).

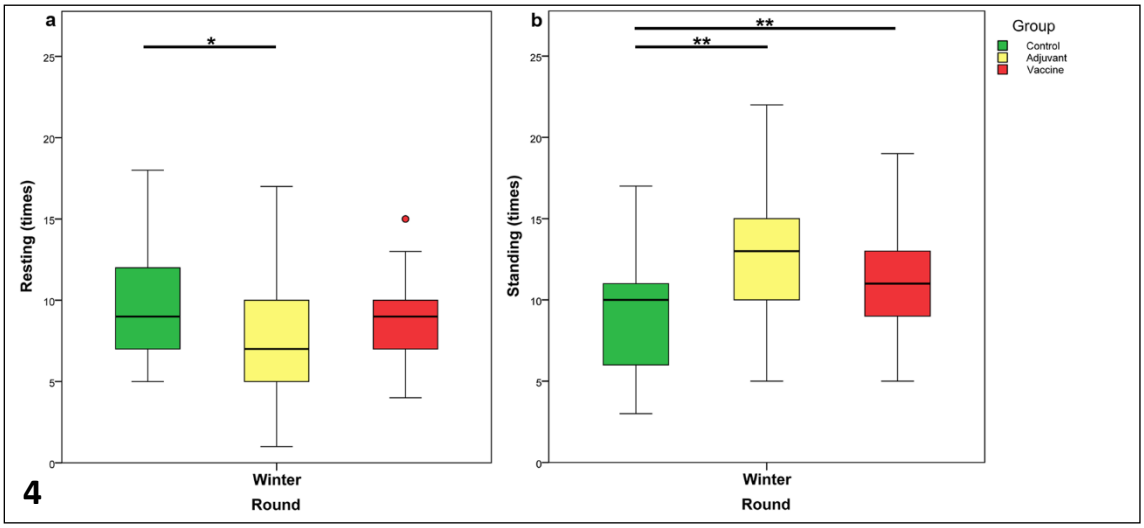


Figure 4. Individual behavior. Winter round of tests. **a.** Resting. Adjuvant-only group spent less time resting (*p_{KW}=0.027) than Control group. Statistical comparisons were based on Kruskal-Wallis (KW) and *post hoc* Dunn's test. **b.** Standing. Adjuvant-only and Vaccine groups spent more time standing (**p_A<0.001) than Control group. Statistical comparisons were based on ANOVA (A) test and *post hoc* Duncan's test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are presented in Annex II, Tables S18-S19.

4.1.2b. Social behavior

In summer, the level of affiliative interactions did not differ among groups (Fig. 5, page 85; see Annex II, Table S23) but lambs in Vaccine and Adjuvant-only groups exhibited significantly (p_{KW}<0.001) more aggressive interactions (Fig. 6, page 86; see Annex II, Table S24) and stereotypies (Fig. 7, page 87; see Annex II, Table S25) than did lambs in Control group, and Vaccine group lambs exhibited significantly (p_{KW}<0.001) more stereotypies than did lambs in Adjuvant-only group. In winter, Vaccine group animals exhibited significantly (p_{KW}<0.001) fewer affiliations than did animals in Adjuvant-only group (Fig. 5, page 85; see Annex II, Table S23). Furthermore, in winter, lambs in Vaccine and Adjuvant-only groups showed significantly (p_{KW}<0.001) fewer affiliations (Fig. 5, page 85; see Annex II, Table S23)., more aggressive interactions (Fig. 6, page 86; see Annex II, Table S24) and more stereotypies (Fig. 7, page 87; see Annex II, Table S25) than did lambs in Control group.

In Adjuvant-only and Control groups, the frequency of affiliations was significantly lower than Vaccine (Adjuvant-only: p_W=0.045; Vaccine: p_W<0.001; Fig. 5; see Annex II, Table S23) and the frequency of aggressive interactions was significantly higher (Ajuvant-only: p_W=0.018; Vaccine: p_W=0.003; Fig. 6, page 86; see Annex II, Table S24) in winter than it was in summer. In Vaccine group (p_W=0.002) animals exhibited significantly more stereotypies in winter than they did in summer (Fig. 7, page 87; see Annex II, Table S25). In Control group, those three types of behaviors did not differ significantly between summer and winter (Figs. 5, 6, 7). The GLM indicated that the interaction effect between “Treatment Group” and “Round” was significant for affiliative interactions (p=0.002) and aggressive interactions (p=0.024; see Annex II, Table S26). By the end of the winter round (February 2016), animals in Vaccine group exhibited wool biting, and five out of seven lambs had multifocal areas of wool loss and depilation, normally in the rumps and withers (Fig. 8, page 88).

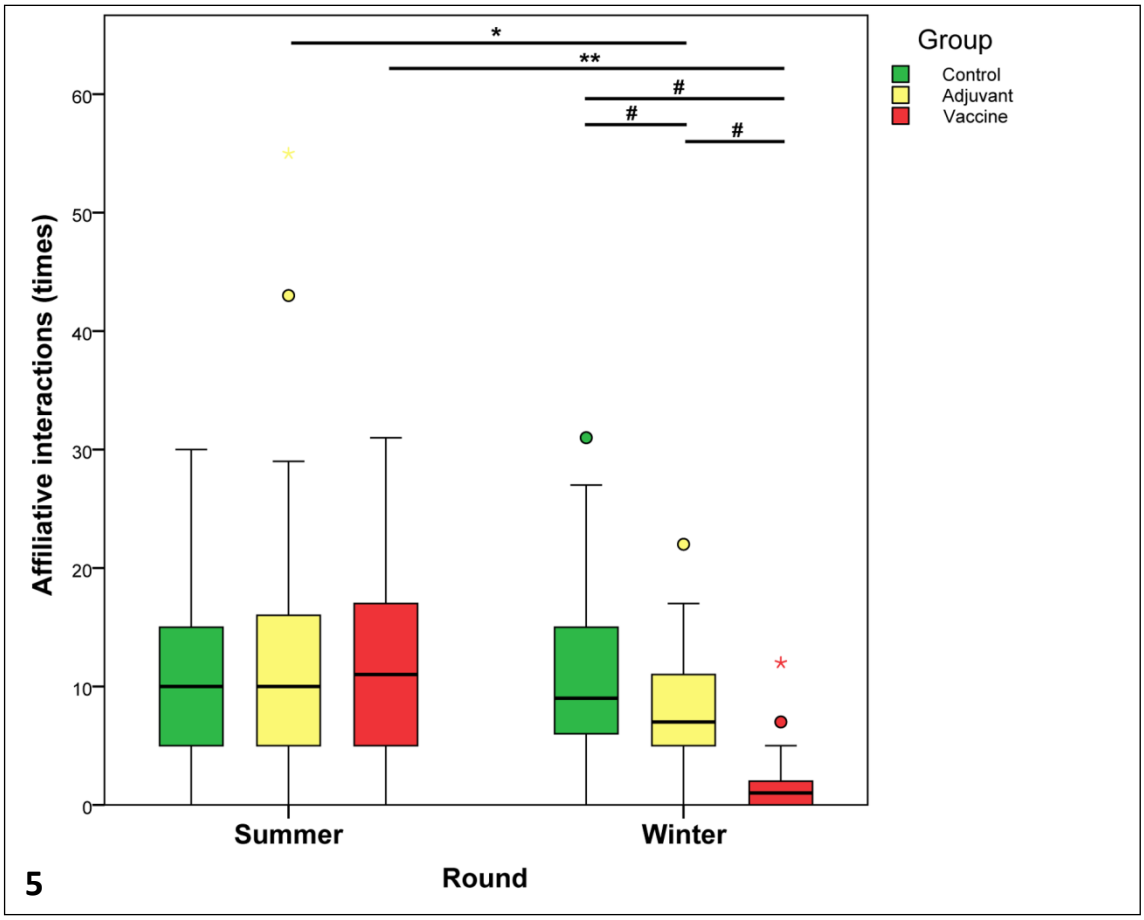


Figure 5. Social behavior: Affiliative interactions. Adjuvant-only group performs less affiliative interactions in winter (*p_W=0.045). Vaccine group performs less affiliative interactions in winter (**p_W<0.001). Adjuvant-only and Vaccine groups perform less affiliative interactions than Control group in the winter round ($^{\#}p_{KW}$<0.001). In addition, in the winter round, Vaccine group performs less affiliative interactions than Adjuvant-only group ($^{\#}p_{KW}$<0.001). Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn´s test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Annex II, Table S23.

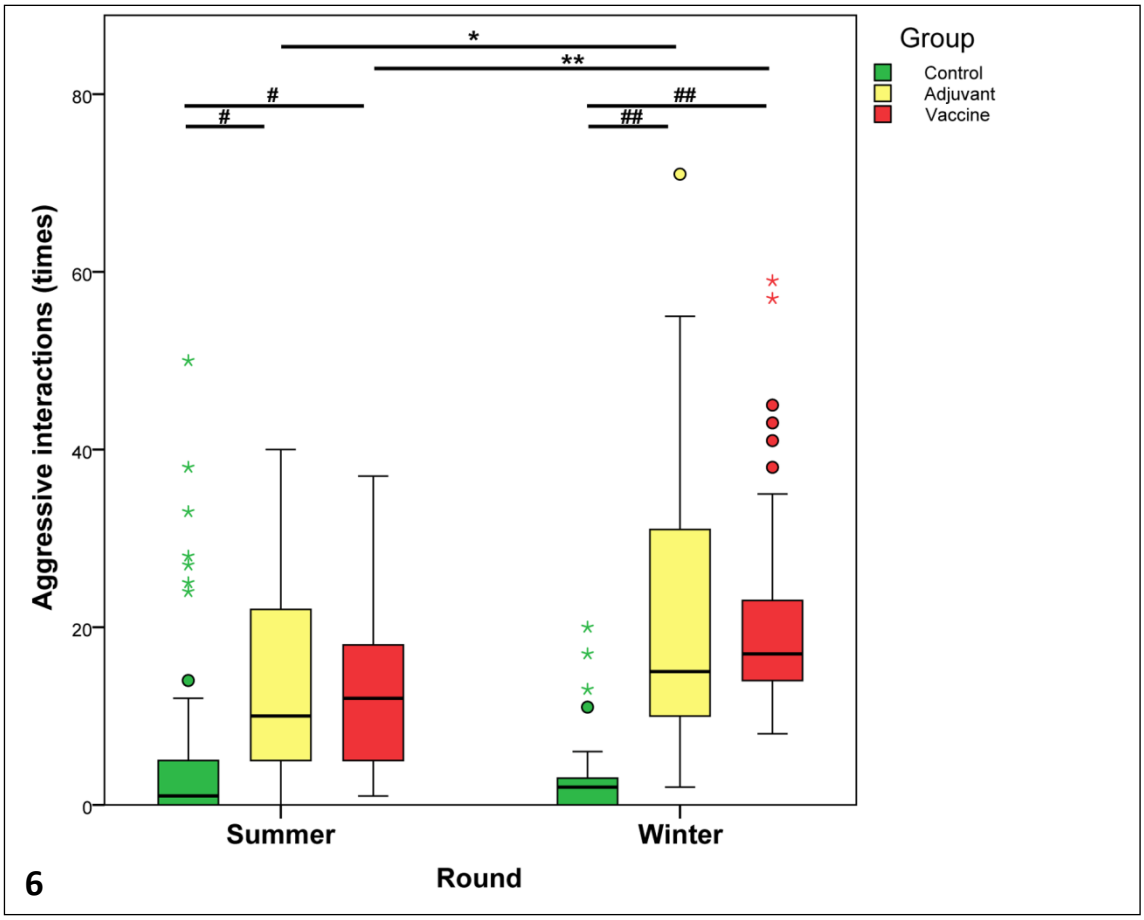


Figure 6. Social behavior: Agonistic (aggressive) interactions. Adjuvant-only group performs more aggressive interactions in winter (*p_W=0.018). Vaccine group performs more aggressive interactions in winter (**p_W=0.003). Adjuvant-only and Vaccine groups perform more aggressive interactions when comparing with Control group both in the summer ($^{\#}p_{KW}$<0.001) and in the winter ($^{\#\#}p_{KW}$<0.001) rounds. Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn´s test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Annex II, Table S24.

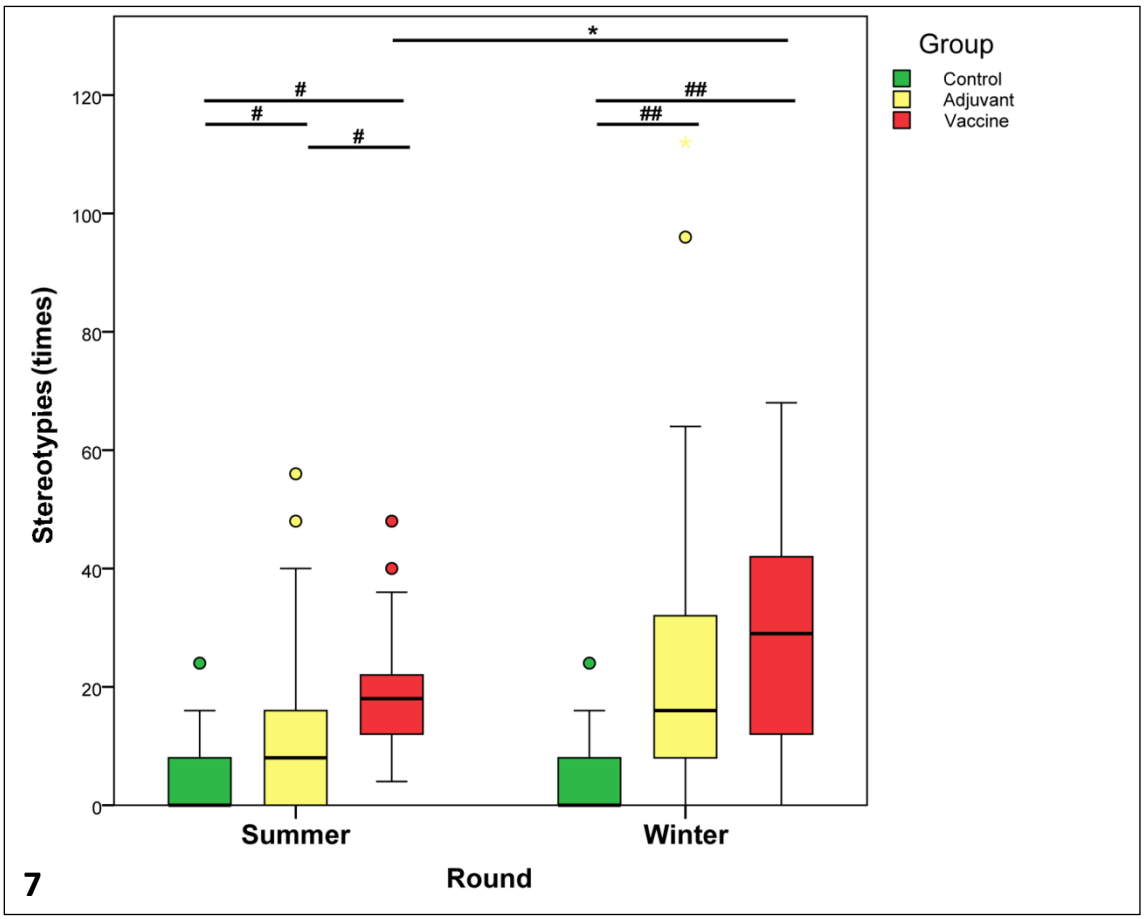


Figure 7. Social behavior: Stereotypies. Vaccine group performed more stereotypies in the winter round (*p_W=0.002). Adjuvant-only and Vaccine groups performed more stereotypies than did Control group in the summer ($^{\#}p_{KW}$<0.001) and the winter ($^{\#\#}p_{KW}$<0.001) rounds. Furthermore, Vaccine group performed more stereotypies than did Adjuvant-only group in the summer round ($^{\#}p_{KW}$<0.001). Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn's test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn´s test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Annex II, Table S25.

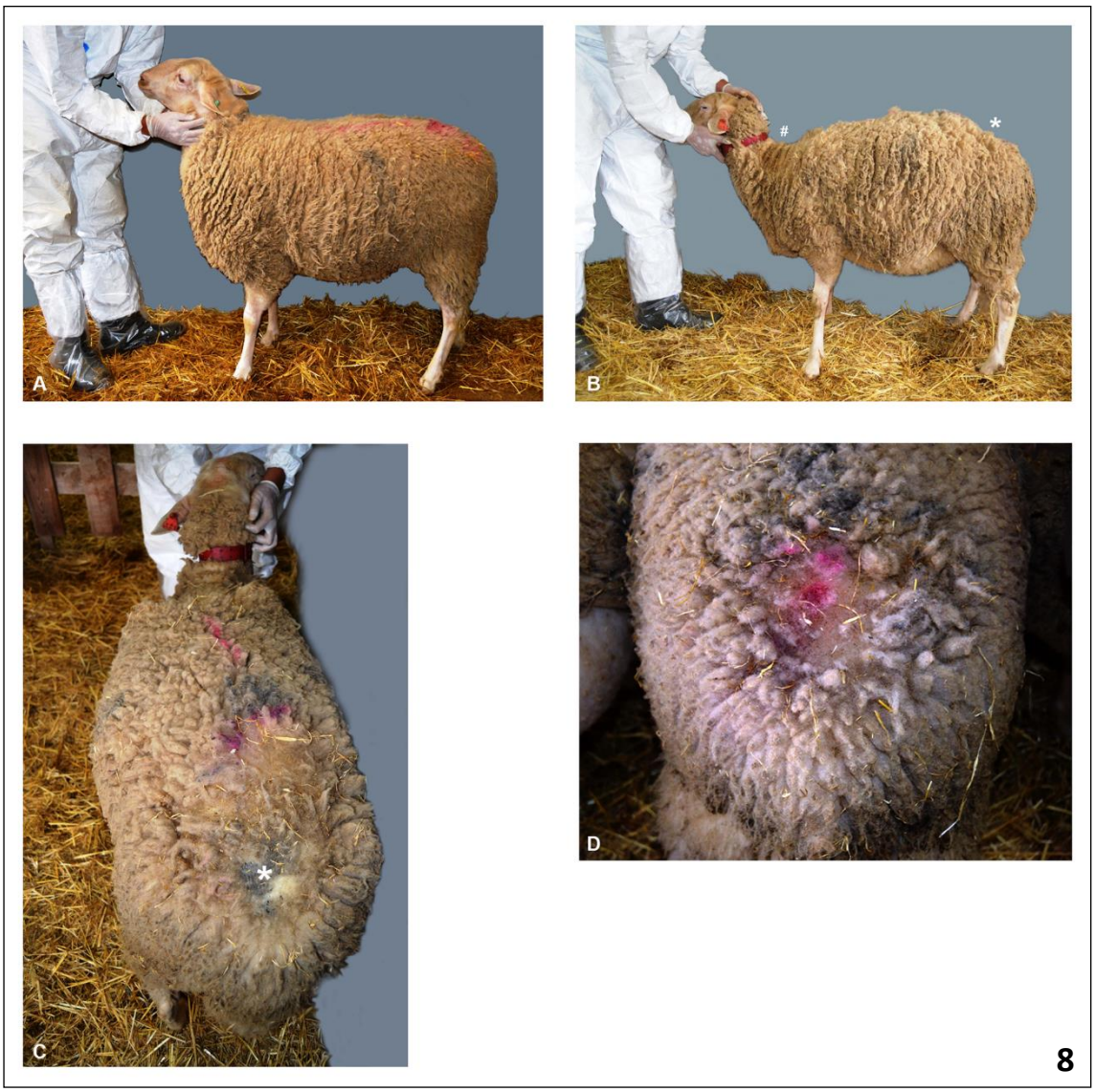


Figure 8. Wool biting in the Vaccine group. **A:** Control group (lamb No. 1). **B-D:** Vaccine group animals with multifocal areas of wool loss and depilation. **B-C:** Vaccine group (lamb No. 1). Poor external aspect with areas of wool loss in the neck (#) and rump (*). **D:** Vaccine group (lamb No. 6). Focal area of wool loss in the rump.

4.1.3. Hematology panel and welfare indicators

In summer, the hematology panels of the three groups did not differ significantly. In winter, the white blood cell counts (WBC; p_A=0.047) and eosinophil number (p_{KW}=0.016) were higher in Vaccine group than they were in the other two groups (see Annex II, tables S27-S28). In summer, cortisol levels did not differ significantly between groups, however, in winter, cortisol levels were significantly higher in Vaccine and Adjuvant-only groups than they were in Control group

(p_A=0.005). Furthermore, in Control group, but not in Vaccine and Adjuvant-only groups, cortisol levels were significantly (p_t=0.002) lower in winter than they were in summer (Fig 9)

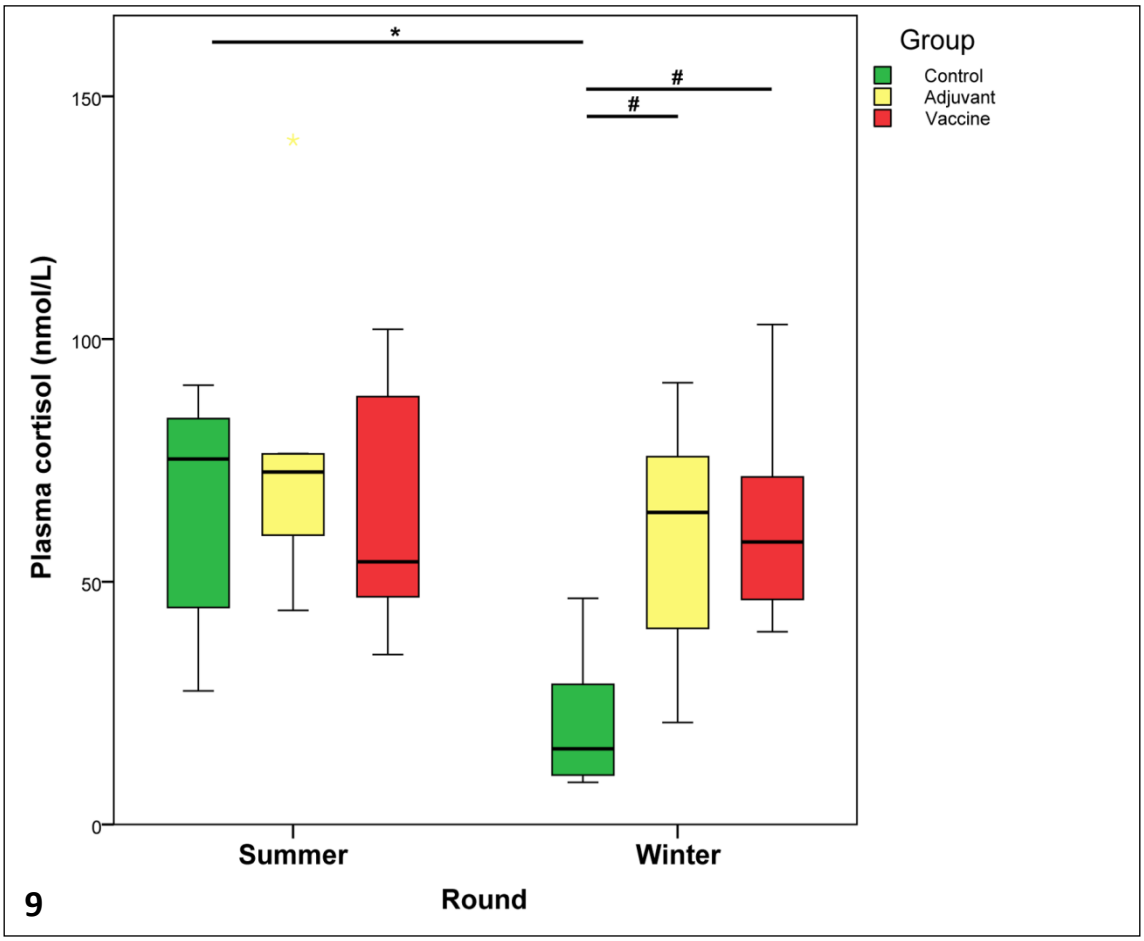


Figure 9. Plasma cortisol levels. Control group shows a winter decrease in the cortisol levels (*p_t=0.002) when compared with the summer round. Adjuvant-only and Vaccine groups show higher cortisol levels than Control group in the winter round ($^{\#}p_A$=0.005). Comparisons within groups were performed by Student's t test (t). Comparisons between groups were performed by ANOVA (A) test and *post hoc* Duncan's test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Annex II, Tables S27 and S28.

4.2. Studies at the injection site reactions and Al mobilization

4.2.1. *In vivo* studies

In vivo assessment of local reactions is shown in Figure 10. Injection-site nodules were only palpated in Vaccine and Adjuvant-only groups. Local reactions in the Vaccine group showed a significantly higher (P_U<0.001) degree of severity (cumulative median=0.79, IQR=0.59-9.04) when compared to Adjuvant-only animals (cumulative median=0.36, IQR=0.21-0.50). Peaks of severity were observed in both groups in parallel and were associated with a high Al dose in the immediately previous injection. In general, only one injection-site nodule was palpated per animal and most of the swollen, liquefactive and fistula-forming nodules were observed in the Vaccine group (Figs. 11-12).

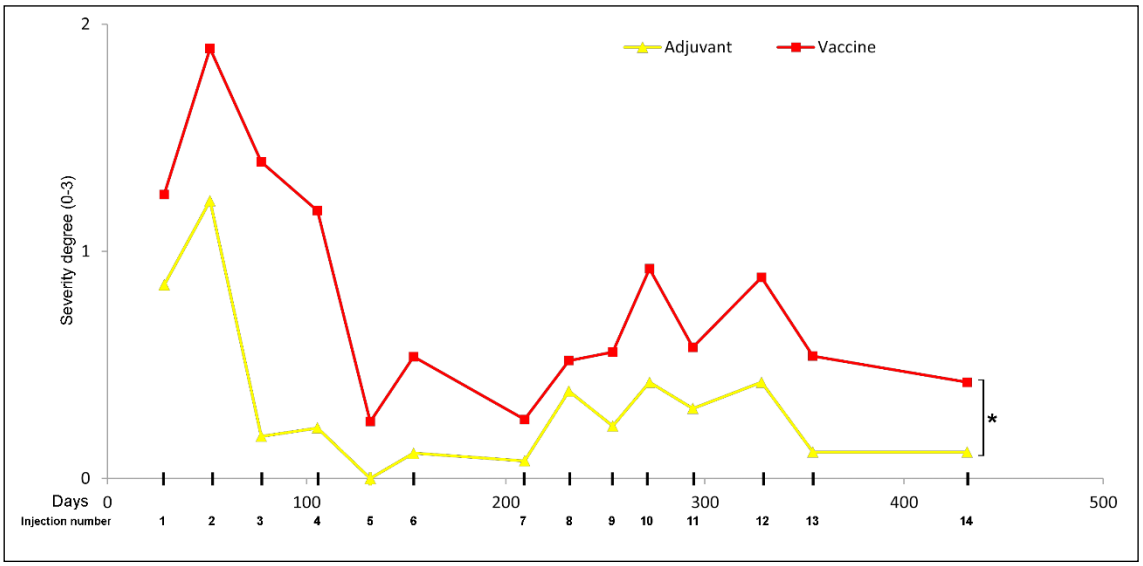


Figure 10. Degree of severity of *in vivo* post-injection local reactions evaluated by palpation. Vaccine group animals presented higher (p_U=0.001) cumulative degree of severity than the Adjuvant-only group animals. Comparison was performed using Mann Whitney´s U test (U). Each value is the mean of the degree of severity in the group at an evaluation date. Days: Days post first inoculation. Injection number: Sequential number of a given inoculation (see Fig. 1E, page 59).

Figures 11-12. *In vivo* injection-site local reactions in Vaccine group animals. **Fig. 11.** Vaccine group, animal No. 2, flock 3. Swollen and liquefactive injection-site nodule. **Fig. 12.** Vaccine group, animal No. 7, flock 2. Fistula-forming injection site reaction.

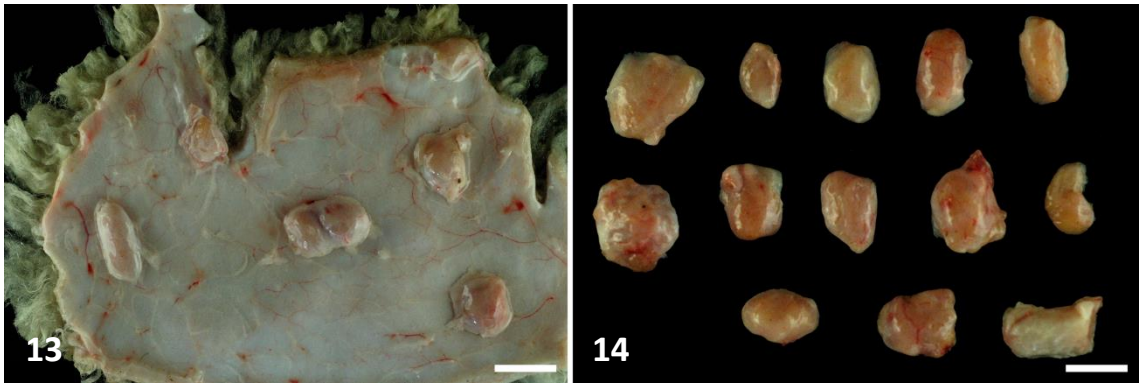
4.2.2. Gross and histopathology

Inspection of the subcutaneous tissue after the removal of the adipose panniculus revealed the presence of nodules, but only in Vaccine and Adjuvant-only groups (Fig. 13, page 92). All (26/26; 100 %) lambs of the Vaccine group and the majority (24/26, 92.3 %) of the Adjuvant-only animals presented nodules, whereas the Control group (0/26; 0 %) did not (Table 8). More than half of the Adjuvant-only lambs showed 1 or 2 nodules in total, whereas more than 75 % of the Vaccine group animals exhibited 8 nodules or more; the minimum number of nodules recovered in Vaccine group animals was 3 (Table 8). Remarkably, seven Vaccine group lambs (7/26, 26.9 %) showed between 13 and 16 nodules in the right flank (Fig. 14, page 92).

Table 8. Number (%) of granulomas collected postmortem

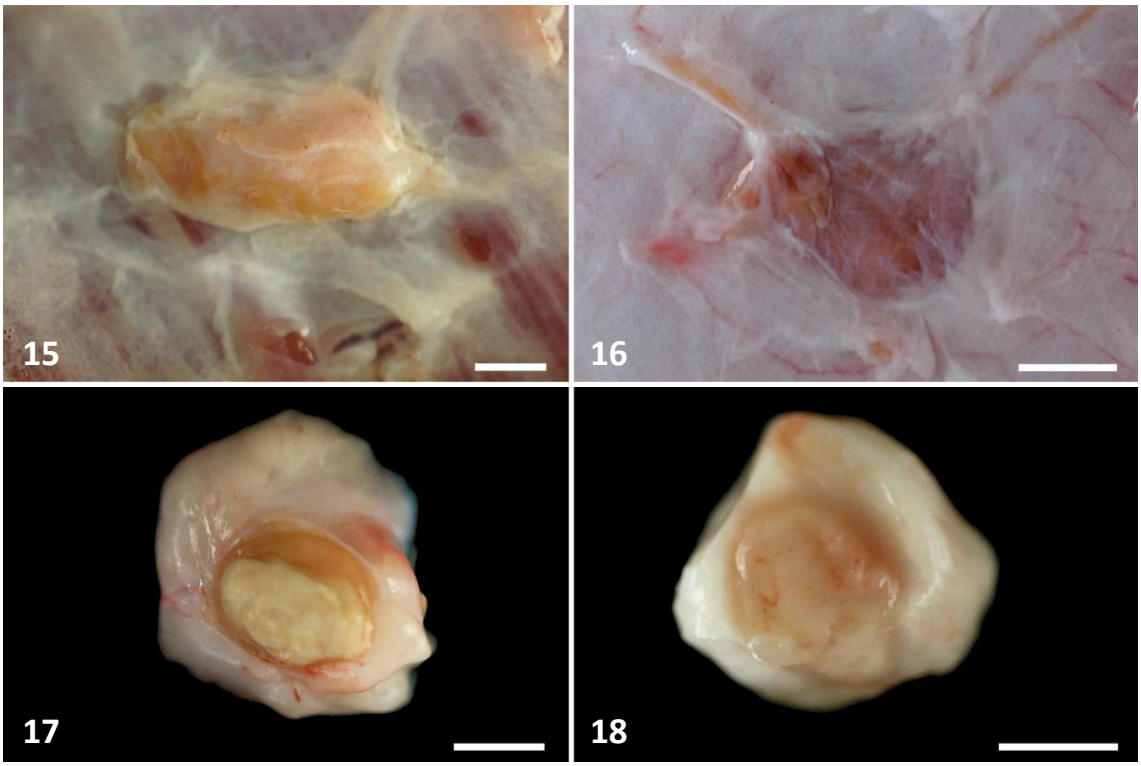
	Number of nodules*			
	0	1-2	3-7	≥8
Control	26 (100)	0 (0)	0 (0)	0 (0)
Adjuvant	2 (7.7)	14 (53.9)	7 (26.9)	3 (11.5)
Vaccine	0 (0)	0 (0)	6 (23.1)	20 (76.9)

*Association was assessed using likelihood ratio (LR; $p_{LR}<0.001$).



Figures 13-14. Injection-site granulomas in Vaccine group animals. **Fig. 13**. Vaccine group, animal No. 2, flock 2. Subcutaneous tissue of the right flank, fat has been removed. Multiple well-defined injection-site granulomas. Bar = 2 cm **Fig. 14.** Vaccine group, animal No. 6, flock 1. Thirteen subcutaneous injection-site granulomas isolated from the same animal. Nodules used for microbiology and electron microscopy studies are not present. Bar = 1 cm.

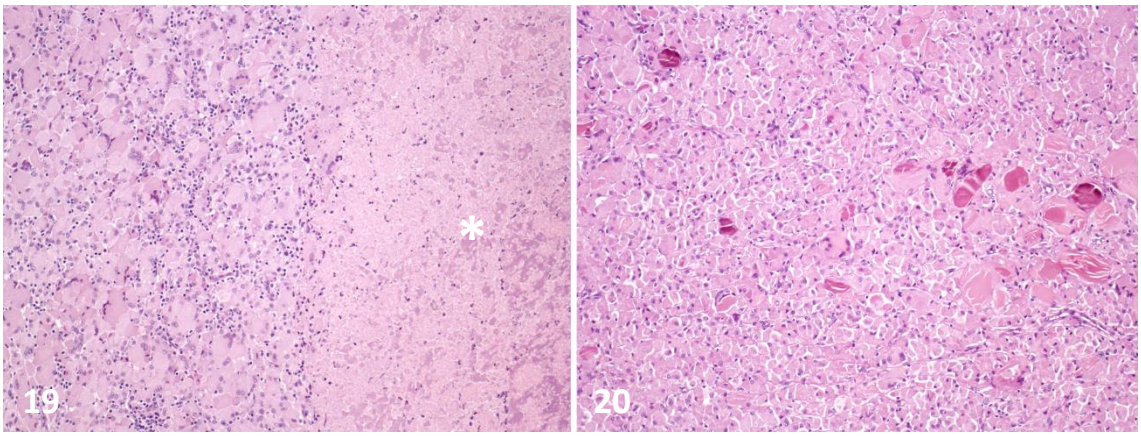
Vaccine-induced nodules were round and conspicuous (Fig. 15) whereas Adjuvant-only-induced nodules tended to be plaque-like or, at least, not as round (Fig. 16). In both groups, nodule size was generally within a range of 0.5 and 2 cm. However, especially in the Adjuvant-only group, some nodules were difficult to locate due to their small size; sometimes less than 2 mm. Central caseous necrosis of nodules was grossly observed in 84.6 % and in 13.6 % of the Vaccine and Adjuvant-only lambs, respectively (Figs. 17 and 18). Nodules were firmly attached to the subcutaneous fascia; there was occasionally visible vascularization surrounding nodules. The surface of the nodules was tan to pale brown, with smooth and firm texture. By pressuring some of them, necrotic centers oozed a soft whitish material leaving an empty cavity surrounded by a 1-2 mm firm capsule. Some nodules were hard at cut section, displaying partially mineralized centers.



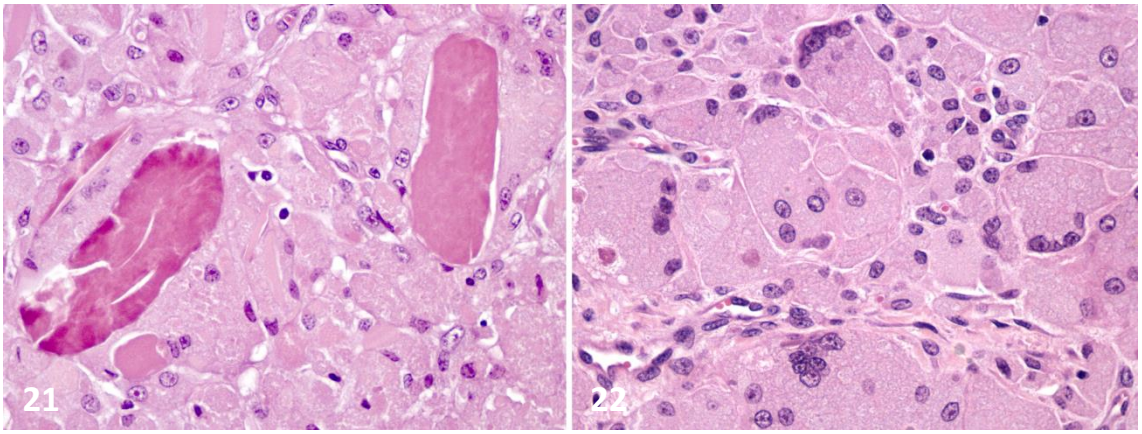
Figures 15-18. Injection-site granulomas in Vaccine and Adjuvant-only groups animals. Bars = 0.5 cm **Figs. 15-16**. Subcutaneous tissue of the right flank, fat has been removed. **Fig. 15**. Vaccine group, animal No. 6, flock 3. Well-defined, round and prominent granulomas. **Fig. 16.** Adjuvant-only group, animal No. 3, flock 1. Ill-defined, flat and inconspicuous granulomas. **Figs. 17-18**. Cut sections of isolated granulomas. **Fig. 17.** Vaccine group, animal No. 3, flock 3. Central caseous necrosis. **Fig. 18**. Adjuvant-only group, animal No. 4, flock 2. Homogenous and solid aspect without grossly visible necrosis.

The basic histological features of the injection site nodules and regional lymph nodes were comparable within and between the Vaccine and Adjuvant-only groups, just varying in their intensities (Table 9, page 96), while no Control animal presented with injection-site reactions. In the former two groups, nodules consisted of well-demarcated granulomas mostly composed of voluminous, activated macrophages that showed a vacuolated to coarsely-granulated cytoplasm (Figs. 19 and 20, page 94). In both groups, scattered within the granulomas, well-defined, straight bordered, intra- and extra-cellular, round to elongated (cigarette-shaped) eosinophilic crystalloid bodies of approximately 100-200 µm were observed. These bodies were significantly overrepresented in Adjuvant-only granulomas ($p_{LR}<0.001$; Figs. 20 and 21, pages 94 and 95). Multinucleated giant cells, either Langhans or foreign body type, were

observed significantly more often in granulomas from Vaccine animals (p_F=0.010; Fig. 22). Lymphocyte aggregates were observed at the periphery of granulomas in half of the animals of both groups (Fig. 23, page 97). Significantly higher degrees of central necrosis (p_{LR}=0.021; Fig. 19), and mineralization (p_{LR}=0.001, Fig. 24, page 97) were observed in granulomas of Vaccine group lambs, together with a significant presence of neutrophils (p_F=0.009, Fig. 25, page 97). Granulomas showed a conspicuous fibrous capsule (Fig. 26, page 97). The prescapular lymph nodes of Vaccine and Adjuvant-only lambs showed significantly higher severity of cortical hyperplasia (p_{LR}<0.001, Fig. 27, page 98) in comparison with control animals (Fig. 28, page 98) and significant presence of clusters of voluminous, foamy to granulated macrophages (p_{χ^2}<0.001; Figs. 29 and 30, page 98).



Figures 19-20. Injection-site granulomas in Vaccine and Adjuvant-only groups animals. HE, 10x. **Fig. 19.** Vaccine group, animal No. 3, flock 3. Dense aggregate of epithelioid macrophages, multinucleated giant cells delimiting a broad area of central necrosis (asterisk). **Fig. 20**. Adjuvant-only group, animal No. 1, flock 1. Dense aggregates of epithelioid macrophages with few multinucleated giant cells intermingled with intra- and extra-cellular eosinophilic crystalloid bodies.

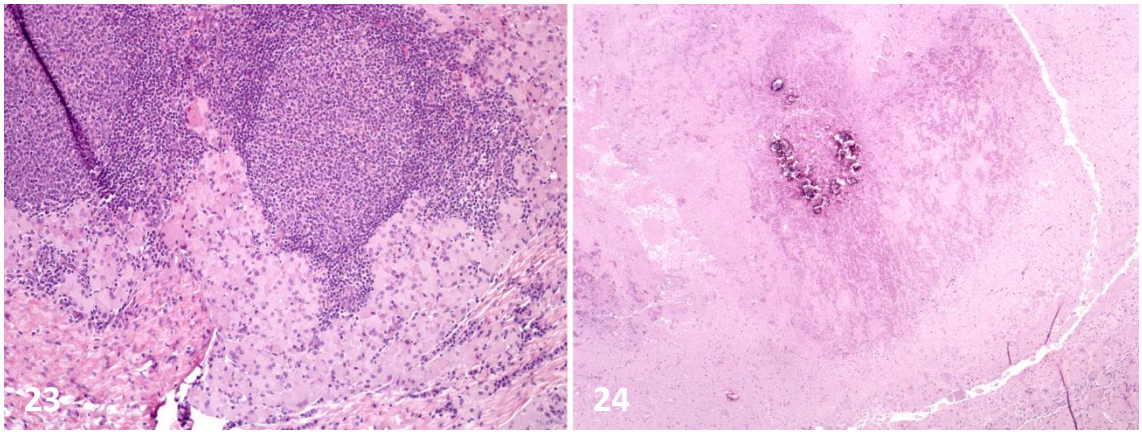


Figures 21-22. Injection-site granulomas in Vaccine and Adjuvant-only groups animals. HE, 40X. **Fig. 21.** Adjuvant-only group, animal No. 1, flock 1. Approximately up to 180 X 60 µm, straight, eosinophilic crystalloid bodies surrounded by macrophages and multinucleated giant cells. **Fig. 22.** Vaccine group, animal No. 4, flock 3. Abundant up to 150 µm in diameter multinucleated giant cells with peripheralized nuclei and pale eosinophilic to amphophilic granular cytoplasms. Some conspicuous vesicular-like aggregates are seen in some cytoplasms.

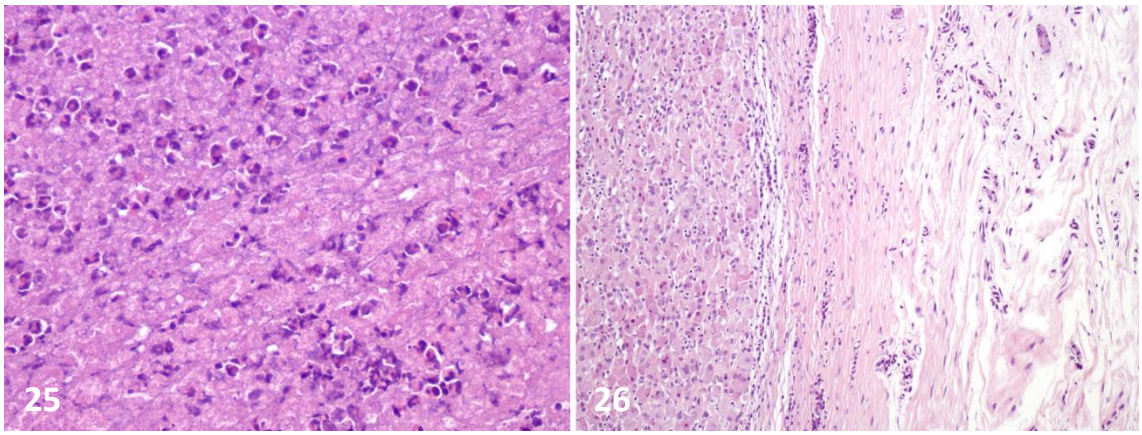
Table 9. Histologic features in subcutaneous granulomas and lymph nodes. Data show the number (percentage in brackets) of animals with each lesion within a group.

GRANULOMAS		**Control**	**Adjuvant (n=23)**	**Vaccine (n=24)**	***p***
Voluminous macrophages	**0**	NA	0 (0)	0 (0)	0.232^{LR}
	1		0 (0)	1 (4.2)	
	2		5 (21.7)	2 (8.3)	
	3		18 (78.3)	21 (87.5)	
Multinucleated giant cells	**+**	NA	15 (65.2)	23 (95.8)	**0.010**^{F}
	-		8 (34.8)	1 (4.2)	
Lymphocytes	**+**	NA	12 (52.2)	13 (54.2)	>0.999^{F}
	-		11 (47.8)	11 (45.8)	
Tertiary lymphoid tissue	**+**	NA	2 (8.7)	3 (12.5)	>0.999^{F}
	-		21 (91.3)	21 (87.5)	
Neutrophils	**+**	NA	7 (30.4)	17 (70.8)	**0.009**^{F}
	-		16 (69.6)	7 (29.2)	
Necrosis	**0**	NA	14 (60.9)	7 (29.2)	**0.021**^{LR}
	1		4 (17.4)	2 (8.3)	
	2		2 (8.7)	2 (8.3)	
	3		3 (13.0)	13 (54.2)	
Mineralization	**0**	NA	21 (91.3)	11 (45.8)	**0.001**^{LR}
	1		2 (8.7)	3 (12.5)	
	2		0 (0)	5 (20.8)	
	3		0 (0)	5 (20.8)	
External fibrosis (capsule)	**+**	NA	20 (87.0)	24 (100)	0.109^{F}
	-		3 (13.0)	0 (0)	
Internal fibrosis	**+**	NA	21 (91.3)	17 (70.8)	0.137^{F}
	-		2 (8.7)	7 (29.2)	
Internal hemorrhages	**+**	NA	4 (17.4)	5 (20.8)	>0.999^{F}
	-		19 (82.6)	19 (79.2)	
External hemorrhages	**+**	NA	1 (4.3)	2 (8.3)	>0.999^{F}
	-		22 (95.7)	22 (91.7)	
Internal blood vessels	**+**	NA	23 (100)	24 (100)	-
	-		0 (0)	0 (0)	
Eosinophilic crystalloid inclusions	**0**	NA	4 (17.4)	19 (79.2)	**<0.001**^{LR}
	1		4 (17.4)	2 (8.3)	
	2		8 (34.8)	2 (8.3)	
	3		7 (30.4)	1 (4.2)	
LYMPH NODES		**Control (n=26)**	**Adjuvant (n=24)**	**Vaccine (n=26)**	***p***
Cortical hyperplasia[a]	**0**	1 (3.8)	0 (0)	0 (0)	**<0.001**^{LR}
	1	9 (34.6)	2 (8.3)	1 (3.8)	
	2	15 (57.7)	10 (41.7)	10 (38.5)	
	3	1 (3.8)	12 (50.0)	15 (57.7)	
Prominent germinal centers	**0**	0 (0)	0 (0)	0 (0)	0.060$^{\chi^2}$
	1	9 (34.6)	2 (8.3)	4 (15.4)	
	2	12 (46.2)	11 (45.8)	9 (34.6)	
	3	5 (19.2)	11 (45.8)	13 (50.0)	
Aggregates of volumi-nous macrophages[a]	**+**	3 (11.5)	22 (91.7)	22 (84.6)	**<0.001**$^{\chi^2}$
	-	23 (88.5)	2 (8.3)	4 (15.4)	

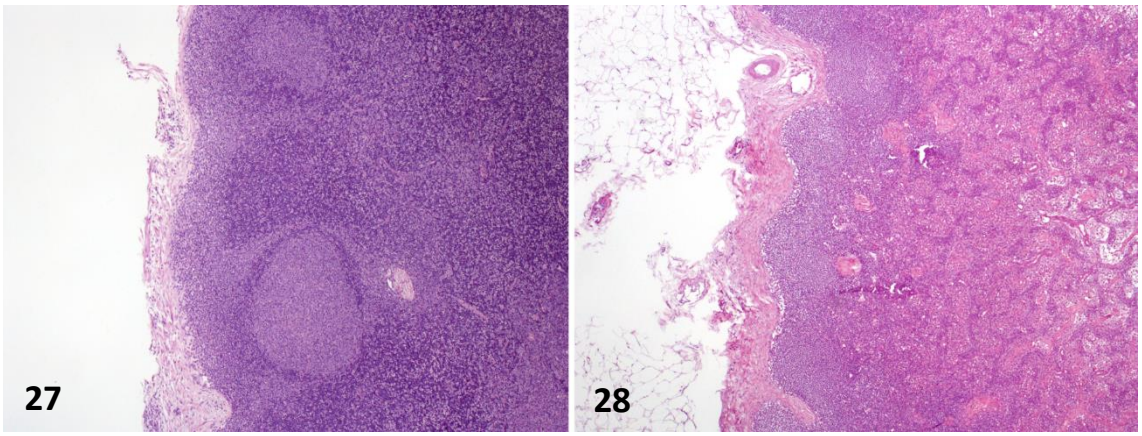
Abbreviations: N/A, Not applicable; LR, likelihood ration; F, Fisher exact test; χ^2, Pearson´s chi square test; +, presence; -, absence;
[a]Significant difference refers to comparing Vaccine/Adjuvant-only groups with Control group



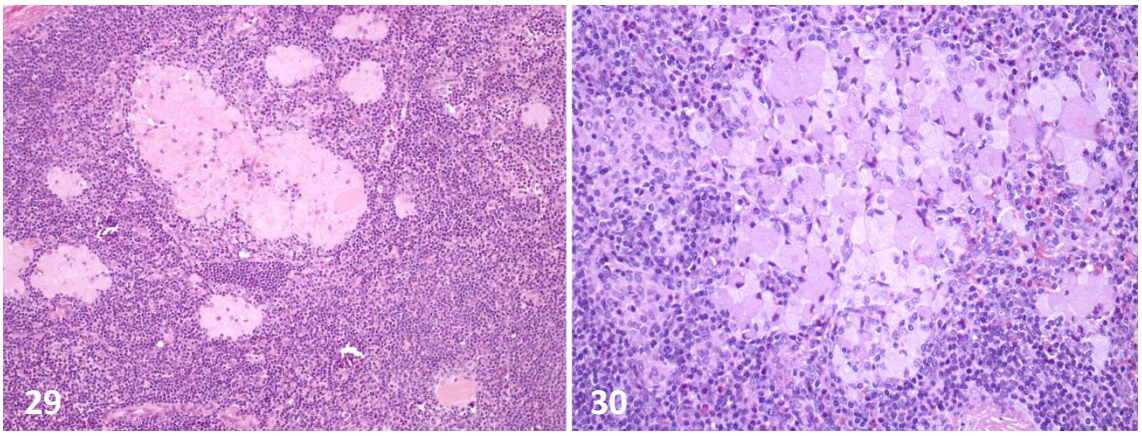
Figures 23-24. Injection-site granulomas in Vaccine and Adjuvant-only groups animals. HE. **Fig. 23.** Vaccine group, animal No. 2, flock 4. Prominent aggregates of lymphocytes with a well-defined germinal center (i.e. tertiary lymphoid tissue) in the periphery of a granuloma. 10x**. Fig. 24**. Vaccine group, animal No. 3, flock 3. Deep purple and crystalloid mineralized tissue within a necrotic center of a granuloma. 4x.



Figures 25-26. Injection-site granulomas in Vaccine group animals. HE. **Fig. 25.** Vaccine group, animal No. 6, flock 4. Abundant degenerated neutrophils and karyorrhectic debris embedded in necrotic tissue. 40x**. Fig. 26**. Vaccine group, animal No. 2, flock 2. Up to 500 µm wide fibrous capsule enclosing macrophagic aggregates.

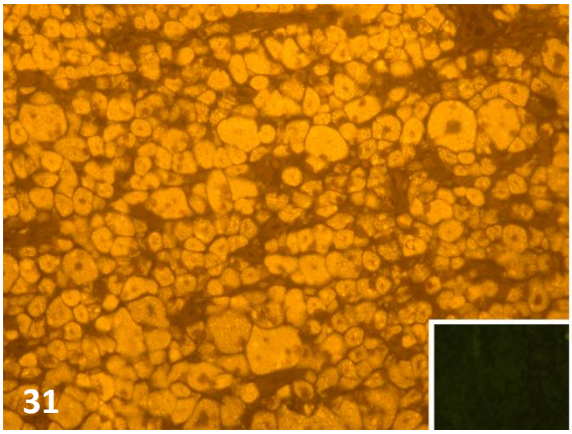


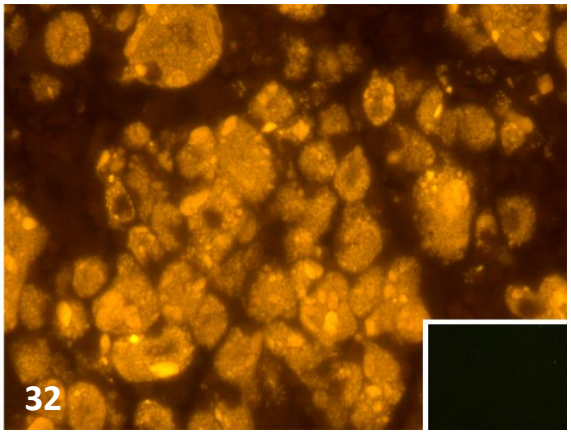
Figures 27-28. Regional (right prescapular) lymph nodes. HE, 4x. **Fig. 27.** Adjuvant-only group, animal No. 5, flock 1. Cortical hyperplasia. Prominent lymphoid follicles with broad germinal centers. **Fig. 28.** Control group, animal No. 5, flock 1. Normal lymph node. Resting lymphoid follicles with small to non-visible germinal centers.



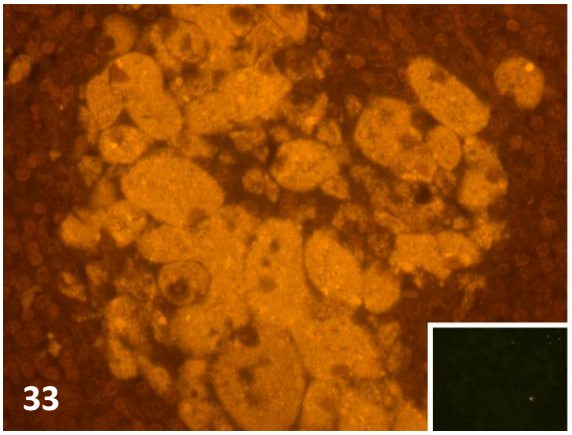
Figures 29-30. Regional (right prescapular) lymph nodes in Adjuvant-only and Vaccine groups animals. HE. **Fig. 29.** Adjuvant-only group, animal No. 3, flock 2. Multifocal aggregates of voluminous macrophages with granular cytoplasm. Some macrophages contain intracytoplasmic eosinophilic bodies. 10x. **Fig. 30**. Vaccine group, animal No. 6, flock 2. Aggregate of voluminous macrophages with granular amphophilic cytoplams. 20x.

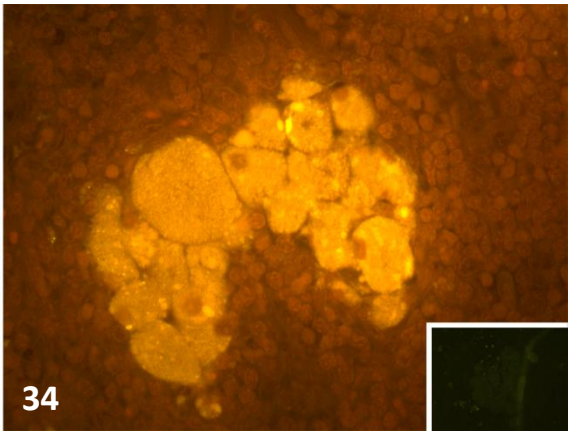
Using lumogallion staining, a granular, intense cytoplasmic orange fluorescence was observed in macrophages from granulomas and lymph nodes from both, Vaccine and Adjuvant-only animals (Figs. 31 to 34, page 99). Such fluorescence was not seen in the unstained autofluorescence serial sections (Figs. 31 to 34 insets). Remarkably, the eosinophilic crystalloid bodies observed with HE showed the most intense orange fluorescence (Figs. 35-36, page 100). Autofluorescence was not seen in the unstained serial sections (Figs. 35 to 36 insets). Fluorescence of similar characteristics was not observed in stained tissues from control lambs (Figs. 37-38, page 100).



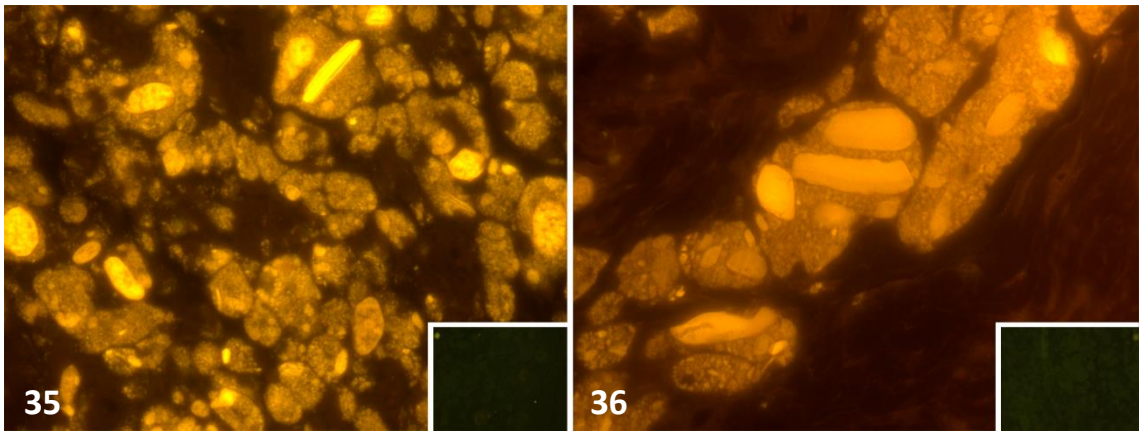


Figures 31-32. Injection-site granulomas in Vaccine and Adjuvant groups animals. Lumogallion. **Fig. 31.** Vaccine group, animal No. 7, flock 1. Dense aggregates of macrophages showing an intracytoplasmic, orangey, finely granular, fluorescent signal. Dark spaces in the center of some cells are the nuclei. 20x**.** *Inset*: Adjacent unstained serial section showing minimal autofluorescence. **Fig. 32**. Adjuvant-only group, animal No. 3, flock 1. Aggregates of macrophages showing an intracytoplasmic, orangey, densely granular, fluorescent signal. Dark spaces in the center of some cells are the nuclei. 40x. *Inset*: Adjacent unstained serial section

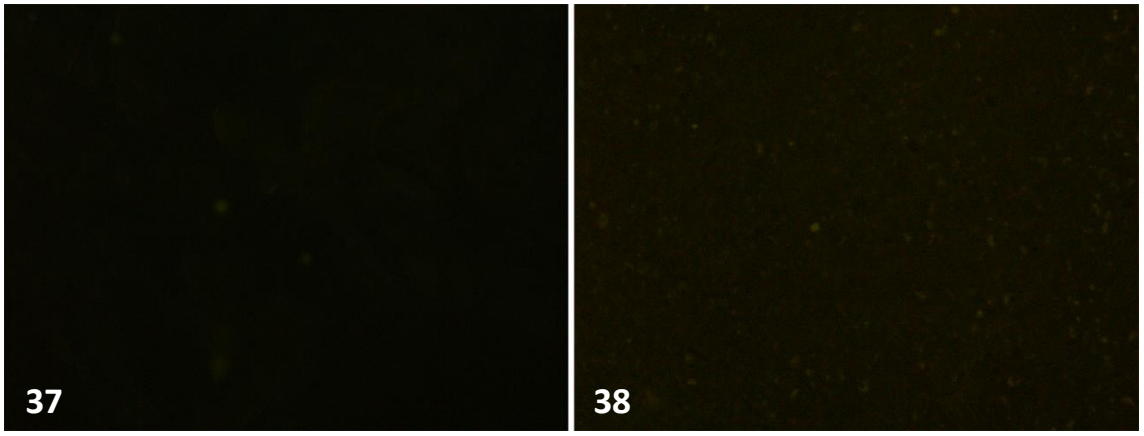




Figures 33-34. Regional (right prescapular) lymph nodes in Vaccine and Adjuvant groups animals. Lumogallion (see Figs 29-30 for comparison with similar areas in sections stained with HE). **Fig. 33.** Vaccine group, animal No. 7, flock 1. Macrophages forming aggregates and showing a granular orangey fluorescence. Dark spaces in the center of some cells are the nuclei. 40x**.** *Inset*: Adjacent unstained serial section showing minimal autofluorescence. **Fig. 34**. Adjuvant-only group, animal No. 6, flock 1. One macrophagic aggregate showing an intracytoplasmic, orangey, granular, fluorescent signal. There are very intense, fluorescent 1-2 µm in diameter, particles. Dark spaces in the center of some cells are the nuclei. 40x. *Inset*: Adjacent unstained serial section showing minimal autofluorescence.



Figures 35-36. Granulomas in Adjuvant-only group animals. Lumogallion. **Fig. 35.** Adjuvant-only group, animal No. 3, flock 1. Intracytoplasmic straight intensely fluorescence bodies within a general Al-positive orangey fluorescence. 40x**.** *Inset*: Adjacent unstained serial section showing minimal autofluorescence. **Fig. 36**. Adjuvant-only group, animal No. 3, flock 1. One macrophagic aggregate showing various intracytoplasmic, orangey, granular, fluorescent signal. Intracytoplasmic up to 150 x 200 µm fluorescent bodies. 40x. *Inset*: Adjacent unstained serial section showing minimal autofluorescence.

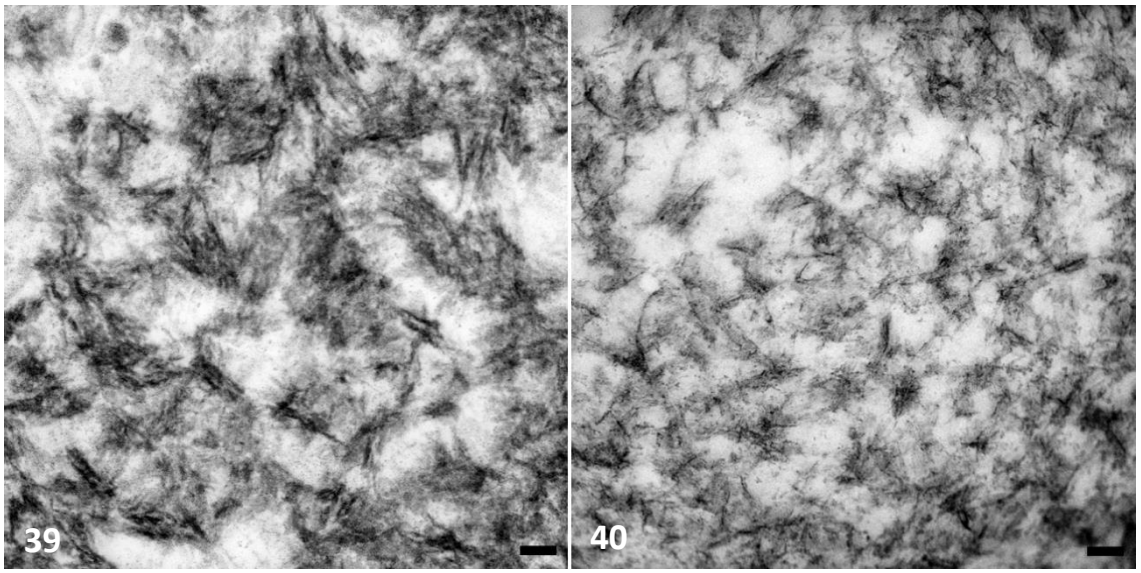


Figures 37-38. Stained sections of Control group animals. Lumogallion. **Fig. 37.** Area of injection. Animal No 1, flock 1. No fluorescence is seen. **Fig. 38**. Regional (right prescapular) lymph node. Animal No. 2, flock 1. No fluorescence is seen.

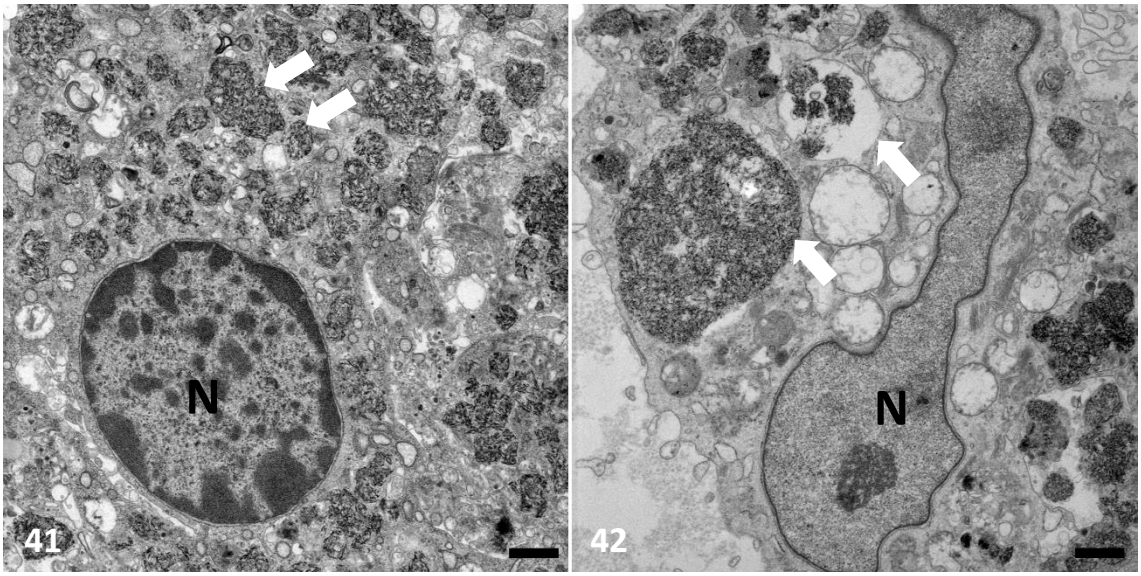
4.2.3. Electron microscopy studies

Macrophages within granulomas of both Vaccine and Adjuvant-only groups contained needle-shaped, electron-dense material (Figs. 39-40, page 101) that formed multiple intracytoplasmic aggregates, often surrounded by a subcellular membrane (phagolysosome; Figs. 41-42, page 102). This membrane showed occasional areas of discontinuity and loss leading to the presence of free intracytoplasmic spiculated material (Fig. 43, page 102). The eosinophilic, lumogallion-positive crystalloid bodies

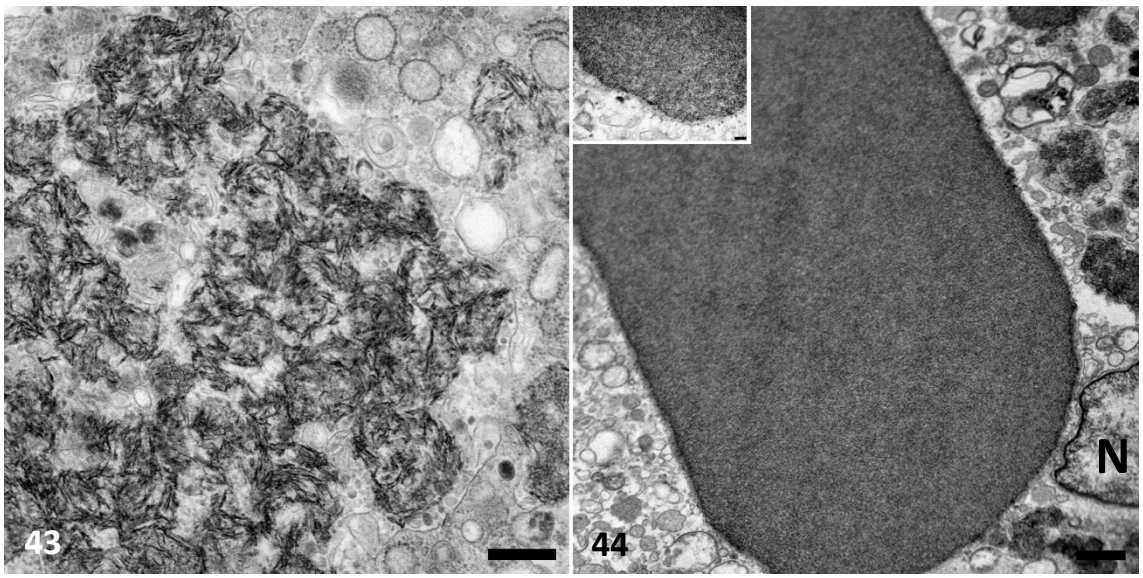
showed a dense and uniform aggregation of the same spiculated material (Fig. 44, page 102). Independently of the presentation and location, this needle-shaped material was identified as Al by EDS, and other frequently identified elements were carbon, oxygen, lead, copper and osmium (Fig. 45, page 103). EDS measurements performed in nuclei were always negative for Al (Fig. 46, page 103). Al particles in granulomas were significantly longer (P_U<0.001) in the Vaccine group (median=121.24 nm, IQR= 98.30-170.60) than particles in the Adjuvant-only group (median=69.47 nm, IQR=53.53-87.62; Fig. 47, page 104). The area of aggregates was similar in both groups (Vaccine: median=1.71 μm^2, IQR=0.84-3.10; Adjuvant-only: median=1.80 μm^2, IQR=1.19-2.80; Fig. 48, page 104). Finally, macrophages in both groups showed one or more of the following degenerative changes: swollen rough endoplasmic reticulum with prominent ribosomes, swollen mitochondria with disorganization of cristae, intracytoplasmic myelin figures, nuclear membrane blebs and indentations and margination of heterochromatin (Figs. 49-50, page 105).



Figures 39-40. Macrophages in granulomas in Vaccine and Adjuvant-only groups animals. High magnification within macrophages. STEM. **Fig. 39**. Vaccine group, animal No. 5, flock 1. Prominent, spiculated, needle-shaped electron-dense and branching Al particles. Bar = 100 nm **Fig. 40**. Adjuvant-only group, animal No. 6, flock 1. Less prominent, spiculated, needle-shaped, electrondense and branching Al particles. Bar = 100 nm.



Figures 41-42. Macrophages in granulomas in Vaccine and Adjuvant-only groups animals. STEM. **Fig. 41**. Vaccine group, animal No. 5, flock 1. Macrophage with cytoplasmic vesicles (i.e. phagolysosomes; arrows) containing the spiculated electron-dense material identified as Al. Nuclear condensation and peripheralization of heterochromatin. N: nucleus. Bar = 1 µm. **Fig. 42**. Adjuvant-only group, animal No. 3, flock 1. Aggregates of Al enclosed in different-sized phagolysosomes (arrows) and nuclear elongation. N: nucleus. Bar = 1 µm.



Figures 43-44. Macrophages in granulomas in Vaccine and Adjuvant-only groups animals. STEM. **Fig. 43**. Vaccine group, animal No. 5, flock 1. Intracytoplasmic aggregate of spiculated Al particles. The aggregate is surrounded by a discontinuous phagolysosomal membrane. Bar = 500nm. **Fig. 44**. Adjuvant-only group, animal No. 3, flock 1. Large and straight-bordered aggregate of densely packed material, which corresponds to the eosinophilic crystalloid bodies observed by hematoxylin-eosin. These aggregates show electron-dense borders. N: nucleus. Bar = 1 µm. *Inset*: Higher magnification of the aggregate border. Bar = 200 nm

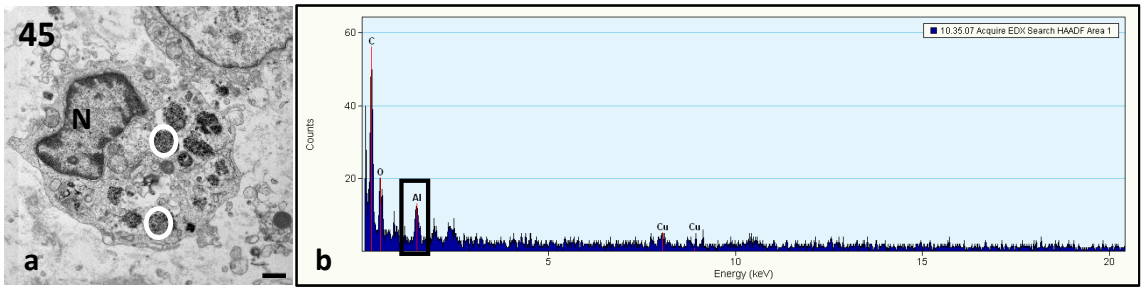


Figure 45. X-ray spectroscopy (EDS) measurements in intracytoplasmic aggregates of electron-dense spiculated material. **a:** Adjuvant-only group, animal No. 6, flock 1. Macrophage in granuloma. Measurements are performed in the 1-2 µm intracytoplasmic round aggregates (white circles). N=Nucleus. Bar = 1 µm. **b:** EDS profile graphic. The electron-dense material is identified as Al (see peak, black rectangle). Copper (Cu) is present as it is part of the grid.

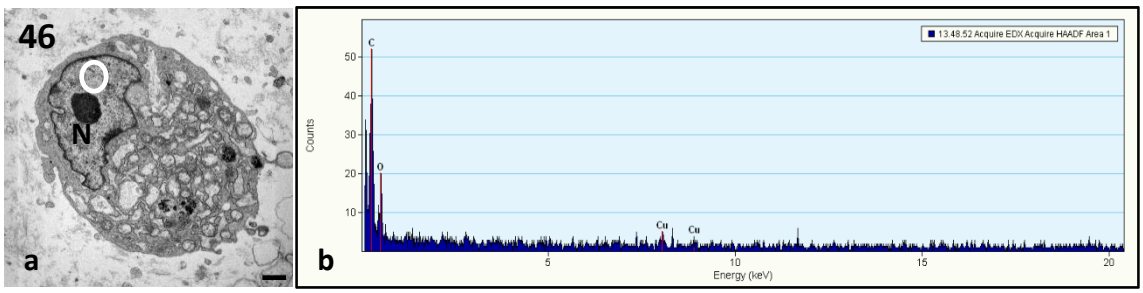


Figure 46. X-ray spectroscopy (EDS) measurements in the nucleus. **a:** Adjuvant-only group, animal No. 6, flock 1. Macrophages in granuloma. Measurements are performed in the nucleus (i.e. negative control; white circle). N=Nucleus. Bar = 1 µm. **b:** EDS profile graphic. There is not a positive Al peak. Copper (Cu) is present as it is part of the grid.

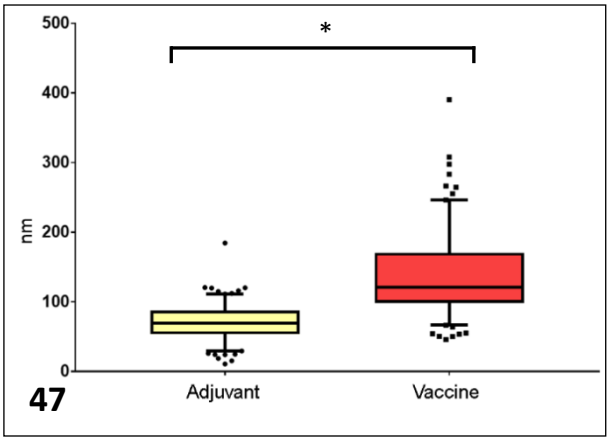


Figure 47. Length of the Al particles in granuloma macrophages. Vaccine granulomas have significantly longer Al particles than Adjuvant-only granulomas (*p_U<0.001). Comparisons were performed using Mann-Whitney U test (U). Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Points and squares represent the outlier values.

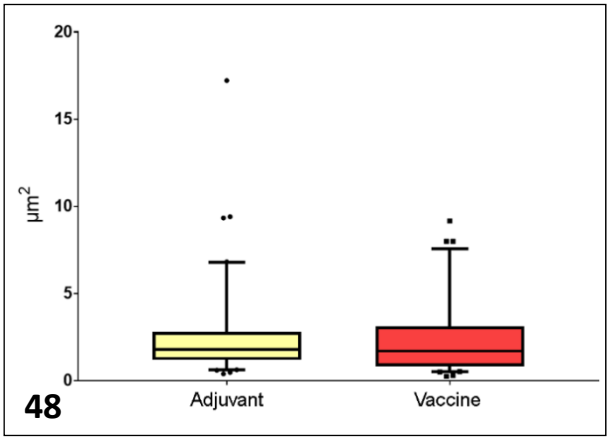
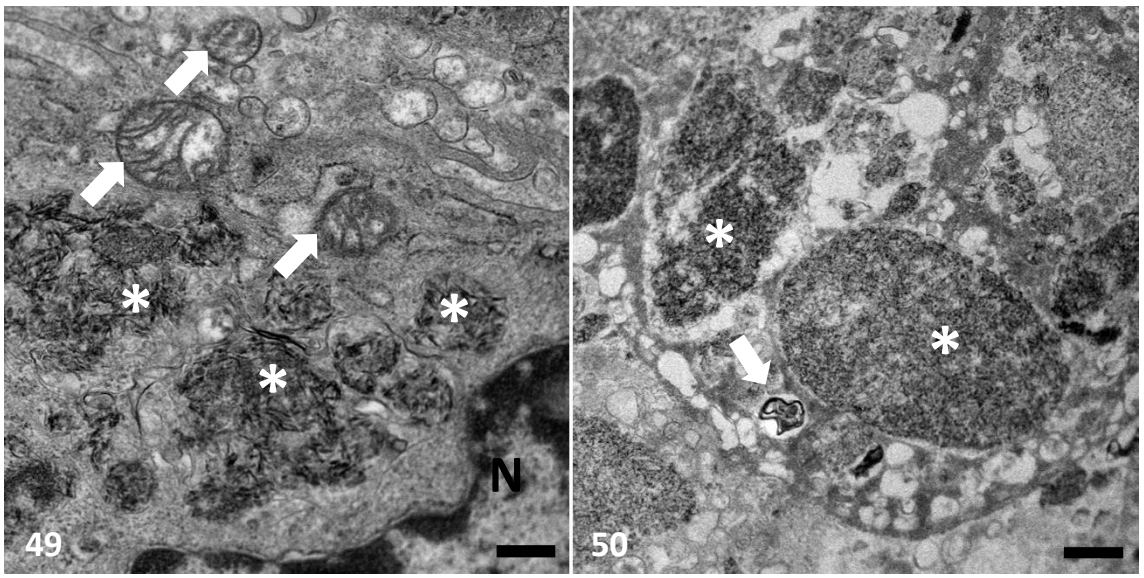


Figure 48. Area of Al aggregates in granuloma macrophages. Vaccine and Adjuvant-only granulomas show a similar aggregate area (P_U=0.33). Comparisons were performed using Mann-Whitney U test (U). Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Points and squares represent the outlier values.



Figures 49-50. Macrophages in granulomas in Vaccine and Adjuvant-only groups animals. Characteristics of cellular degeneration. STEM. **Fig. 49.** Vaccine group, animal No 5, flock 1. Swollen mitochondria with disorganized cristae (arrows) adjacent to Al aggregates (asterisks). N = Nucleus. Bar = 500 nm. **Fig. 50**. Adjuvant-only group, animal No 6, flock 1. Intracytoplasmic conspicuous myelin figure (arrow) adjacent to multiple Al aggregates (asterisks). Bar = 1 µm

4.2.4. Microbiological studies

All 26 granulomas and the 14 injection site areas from Control lambs were negative for bacteriological cultures and no bacterial forms were observed with Gram staining

4.2.5. Al content in regional lymph nodes

The content of Al in lymph nodes is detailed in Table 10. The Vaccine group contained significantly higher values than the other two groups (p_{KW}<0.001) and the content of Al in the Adjuvant-only animals was also significantly higher than the Control (p_{KW}<0.001).

Table 10. Median aluminum (Al) content (µg/g) in the right preescapular lymph node [1]

	Control	Adjuvant	Vaccine	p_{KW}*
Al (µg/g)	0.96[a] (0.68-1.39)	2.53[b] (1.99-7.19)	82.65[c] (20.62-682.27)	<0.001

[1]The data show the median and range in brackets. Different superscripts (a,b,c) indicate statistically significant differences between the groups. *Statistical analysis was performed using Kruskal-Wallis (KW) test with *post hoc* Dunn´s test

4.2.6. Al content in CNS

The content of Al within all tissue replicates measured in the parietal lobe is detailed in Table 11 (page 107). Replicates in the three groups were homogeneous, with most of the values below 1 µg/g. Within the Adjuvant-only group there were three replicates over this value. Al content in the Adjuvant-only group (median=0.2 µg/g, IQR=0.11-0.73) tended (p_{KW}=0.074) to be higher when compared with the Control group (median=0.1 µg/g, IQR=0.01-0.26) whereas none of these two groups differed with the Vaccine group (median=0.1 µg/g, IQR=0.01-0.35). Comparison is shown in Figure 51 (page 107).

The content of Al within all tissue replicates measured in the lumbar spinal cord is detailed in Table 12 (page 108). Only in the Control group, more than half of the replicates (11/21, 52 %) were below 0.01 µg/g. Two animals (111 and 113) within the Adjuvant-only group presented 2 high (over 2 µg/g) replicates each. Adjuvant-only and Vaccine groups (Adjuvant-only: median=0.49 µg/g, IQR=0.31-1.04; Vaccine: median=0.39 µg/g, IQR=0.33-0.62) showed significant (p_{kw}=0.001) higher content than Control group (median=0.08 µg/g, IQR=0.01-0.44). Comparison is shown in Figure 52 (page 108).

Table 11. Aluminum (Al) content (µg/g) in the parietal lobe measured by transversely heated graphite furnace atomic absorption spectroscopy (TH GFAAS).

Control			Adjuvant			Vaccine		
Animal	Replicate No.	Al (µg/g)	Animal	Replicate No.	Al (µg/g)	Animal	Replicate No.	Al (µg/g)
	1	0.01		*1*	0.13		*1*	0.79
131	*2*	0.29	111	*2*	0.21	121	*2*	0.34
	3	0.36		*3*	0.88		*3*	0.40
	1	0.17		*1*	0.32		*1*	0.84
132	*2*	0.23	112	*2*	0.01	122	*2*	0.33
	3	0.16		*3*	0.08		*3*	0.01
	1	0.01		*1*	0.20		*1*	0.71
133	*2*	0.22	113	*2*	0.28	123	*2*	0.35
	3	0.48		*3*	1.01		*3*	0.23
	1	0.01		*1*	0.02		*1*	0.01
134	*2*	0.01	114	*2*	0.18	124	*2*	0.01
	3	0.35		*3*	0.08		*3*	0.08
	1	0.01		*1*	0.74		*1*	0.08
135	*2*	0.12	115	*2*	0.13	125	*2*	0.10
	3	0.39		*3*	0.72		*3*	0.33
	1	0.01		*1*	1.29		*1*	0.01
136	*2*	0.08	116	*2*	0.17	126	*2*	0.29
	3	0.01		*3*	0.14		*3*	0.01
	1	0.09		*1*	0.37		*1*	0.01
137	*2*	0.04	117	*2*	0.08	127	*2*	0.01
	3	0.01		*3*	1.36		*3*	0.10

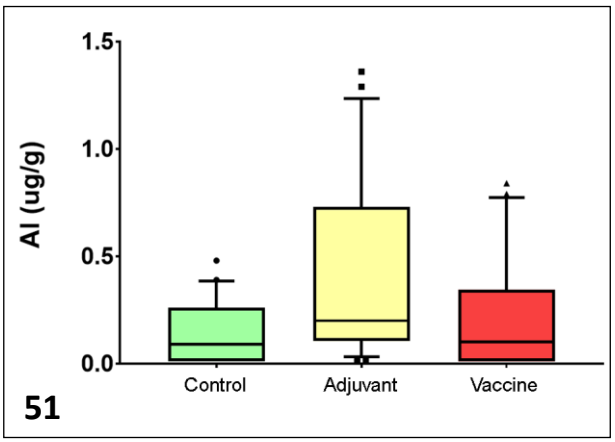


Figure 51. Aluminum (Al) content in the parietal lobe. Al concentration in Adjuvant-only group tended to be higher than in Control group (p_{KW}=0.074). Comparisons were performed using Kruskall-Wallis (KW) test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Points, squares and triangles represent the outlier measurements in each group.

Table 12. Aluminum (Al) content (µg/g) in the lumbar spinal cord measured by transversely heated graphite furnace atomic absorption spectroscopy (TH GFAAS).

Control			Adjuvant			Vaccine		
Animal	Replicate No.	Al (µg/g)	Animal	Replicate No.	Al (µg/g)	Animal	Replicate No.	Al (µg/g)
	1	0.19		*1*	1.24		*1*	0.93
131	*2*	0.41	111	*2*	2.14	121	*2*	0.31
	3	0.05		*3*	14.36		*3*	0.39
	1	0.03		*1*	0.29		*1*	0.36
132	*2*	0.08	112	*2*	0.37	122	*2*	0.91
	3	0.38		*3*	0.56		*3*	0.82
	1	0.01		*1*	7.83		*1*	0.58
133	*2*	0.02	113	*2*	0.20	123	*2*	0.47
	3	0.46		*3*	7.83		*3*	0.32
	1	0.58		*1*	0.49		*1*	0.47
134	*2*	0.28	114	*2*	0.71	124	*2*	1.23
	3	0.01		*3*	0.35		*3*	0.33
	1	0.52		*1*	0.84		*1*	0.35
135	*2*	0.21	115	*2*	0.79	125	*2*	0.19
	3	0.00		*3*	0.24		*3*	0.45
	1	0.01		*1*	0.29		*1*	0.39
136	*2*	0.72	116	*2*	0.33	126	*2*	0.21
	3	0.01		*3*	0.15		*3*	0.35
	1	0.01		*1*	0.48		*1*	0.65
137	*2*	0.82	117	*2*	0.56	127	*2*	0.46
	3	0.06		*3*	0.48		*3*	0.32

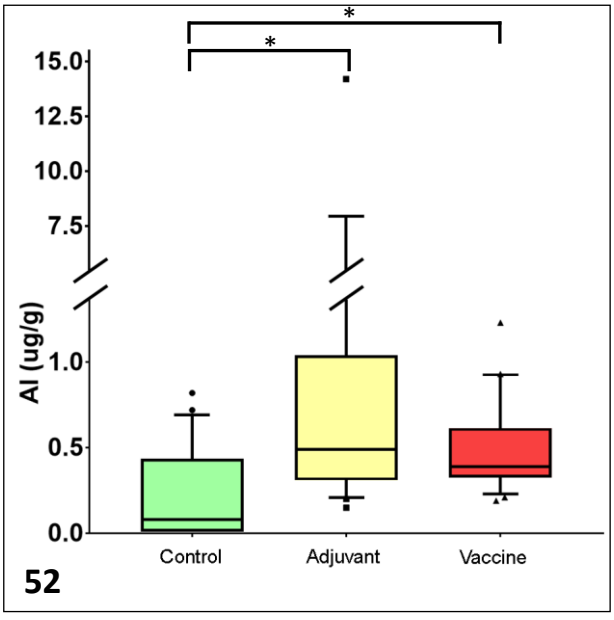


Figure 52. Aluminum (Al) content in the lumbar spinal cord. Al concentration in the Adjuvant-only and Vaccine groups is higher than in Control group ($p_{KW}<0.001$). Comparisons were performed using Kruskall-Wallis (KW) test followed by *post hoc* Dunn´s test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ=Q3-Q1), which indicates 50 % of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Points, squares and triangles represent the outlier measurements in each group.

5. DISCUSSION

The present PhD thesis is part of a research project funded by the Spanish Ministry of Economy and Industry (ref. AGL2013-49137-C3-2-R), whose main objective was to determine the effects of the repetitive inoculation of Al-hydroxide containing adjuvants, either alone or combined into commercial vaccines, in experimental sheep. Through this procedure, the project aimed to establish a suitable model of the ovine ASIA syndrome in order to study its clinical, analytical, genetic, immunological, and pathological aspects. The present work focuses mainly on two aspects: i) Clinical: the characterization of the effects on behavior and cognition of this repetitive inoculation schedule; and ii) Pathological: the characterisation of the injection site reactions and mobilisation of Al from the injection point to distant tissues, such as the regional lymph node, the brain, and the lumbar spinal cord.

The global project included an array of different studies using the animals in this experiment. Other aspects of the clinical changes have been studied, including periodic evaluation of vital signs (i.e. heart and respiratory rates and body temperature), weight evolution and hematological panels with the basic parameters (i.e. hematocrit, hemoglobin, or erythrocyte, and WBC). Although these results have not been presented in this PhD thesis, Vaccine and Adjuvant-only groups throughout the flocks presented few differences compared to their respective Control groups within these parameters. In some specific flocks, there were punctual differences along the schedule, although they were generally within the limits of normality at the end of the experiment. A comprehensive histopathological study of all organ systems was also performed. In addition, an evaluation of the astroglial activation by immunohistochemistry using animals in flock 1 (Table 1, page 60) was carried out. There were limited differences between the treatment groups on the histological characterization, but immunohistochemical studies pointed towards a degree of astroglial activation in certain parts of the CNS in the Adjuvant-only group (Rodríguez, 2018). This collection of results will be further developed and presented by the research group at another time in the future.

In addition, a subset of animals within flock 1 (Table 1, page 60) were used for genetic studies by a collaborative research group (University of the Basque country). Transcriptomic sequentiation of total RNA and miRNA on peripheral blood

mononuclear cells (PBMCs) was performed and a molecular signature activation profile was characterized. Studied animals in Vaccine and Adjuvant-only groups showed an enrichment of the *NF-κB* signalling pathway. Furthermore, Vaccine group animals presented an upregulation of cytokines and cytokine receptors implicated in danger signals compared to the Adjuvant-only group (Varela-Martínez *et al.*, 2018).

The two sets of studies presented in this PhD thesis are complementary to the above-described work and entail novelty on the effects and functioning of Al hydroxide-containing adjuvants in sheep. Overall, this project provides insight on the putative role of the repeated inoculation of Al hydroxide in the development of ovine ASIA syndrome. Some aspects of the syndrome, such as behavioral changes, were reproduced, while others, such as weight loss or hematological changes, were not, and, therefore, their etiopathogenesis remains unclarified and should be further studied in the future.

The ethological study presented in this memory is the first scientific work to investigate cognitive and behavioral effects observed in sheep after inoculation with Al hydroxide-containing products. Following compulsory vaccination campaigns against BT in the last decade, several behavioral changes were observed, which prompted research into the putative relationship between subcutaneously injected Al-containing vaccines and those changes. These changes were described and partially reproduced in a small subset of animals, but never fully characterized or quantified (González *et al.*, 2010; Luján *et al.*, 2013).

This work has used an experimental model to demonstrate that sheep repeatedly inoculated with vaccines containing Al hydroxide or with Al hydroxide only exhibit behavioral changes similar to those reported previously in the ovine ASIA syndrome. Only animals in flock 1 (Table 1, page 60) were subjected to these behavioral studies. Flocks 2 to 4 were integrated in real (i.e. non-experimental) farms, with different management and feeding conditions. The materials and systematic needed to perform the ethologic studies could only be established in an experimental research farm due to space and other limitations. Furthermore, in flocks 2 to 4 (Table 1, page 60), animals in the three treatment groups were mixed among them and/or with the rest of the animals in the farm. Thus, it was impossible to guarantee

exactly the same management conditions in the treatment groups. Only by maintaining each treatment group isolated from the others is possible to establish that the changes observed are occurring in that specific group.

The cognitive and behavioral tests applied have been used previously for studies in sheep and have been extensively validated (Romeyer and Bouissou, 1992; Hosoi *et al.*, 1995; Cooper and Jackson, 1996; Peirce *et al.*, 2000; Kendrick *et al.*, 2001; Yurtman *et al.,* 2002; Desire *et al.*, 2004; Forkman *et al.*, 2007; Caroprese *et al.*, 2010; Lauber *et al.*, 2012; Teixeira *et al.*, 2012; Abecia *et al.*, 2014; Pascual-Alonso *et al.*, 2014). To better understand the development of cognitive and behavioral changes, two rounds of tests were performed: one in late summer, and the other in mid-winter. As previously indicated, the spontaneous behavioral changes observed in affected flocks are always most apparent under cold temperatures (i.e., winter conditions in the Northern hemisphere; Luján *et al*., 2013), which is, so far, an observation difficult to explain. Furthermore, with that protocol, it was possible to quantify the cumulative effect of vaccine doses in the development of behavioral changes. The results of this experiment indicated that behavioral changes were most pronounced in winter; however, the changes might have been a response to reduced air temperatures, a by-product of the difference between seasons in the number of inoculations applied (i.e. seven inoculations in winter versus 16 in summer), or a combination of both. Likely, low temperatures act together with the cumulative impacts of multiple stressors, such as vaccines and/or Al adjuvant inoculations, playing a role in the pathogenesis of ovine ASIA syndrome.

The cognitive tests used in this work (the T-maze test, OFT, and NOT) did not detect significant differences between groups in neither of the two rounds. Within the lambs from the Vaccine and Adjuvant-only groups, a few significant differences existed between the two rounds of tests. Among the Vaccine lambs, the most significant change was the reduction in time spent trying to escape the test arena in the OFT in winter, which might reflect a reduction in social tendencies in the vaccinated lambs because, in this test, the animal tries to escape the isolation of the arena and return to the group (Price and Thos, 1980). In addition, Vaccine animals left the first area of the T-maze test (latency) earlier on the second day than they did on the first day of the

winter round, although the time taken to solve the maze did not differ. The significant difference in latency between days might have been a product of the exceptionally long latency of the animals in the Vaccine group on the first day of the test. In winter, lambs in the Adjuvant-only group spent more time exploring (OFT) than they did in summer and they kept farther away from the novel object in the second exposure (NOT). Those changes might reflect a level of agitation or fear (Wemelsfelder and Farish, 2004; Forkman *et al.*, 2007). Collectively, the cognitive tests presented to the animals indicated some changes in the Vaccine and Adjuvant-only groups; however, in general, vaccination or inoculation with the adjuvant only seemed not having a marked effect on the cognitive parameters evaluated by the different tests applied in the study.

Behavior has been assessed by home pen observations of individual and social behaviors. Two sampling techniques have been applied to the videos: instantaneous and continuous sampling. These techniques are broadly established in animal ethology and they have been widely used to assess behavioral changes in response to a given stressor (i.e. Vaccines containing Al-hydroxide, Al-hydroxide-only, or PBS), irrespectively of species, breed, age, or sex (Martin and Bateson, 2007). Instantaneous sampling is used to measure behavioral states that require some time (e.g. eating, drinking, walking, standing) and continuous sampling is used to measure relatively short behavioral events (affiliative and aggressive interactions or stereotypies). Indeed, home pen observations of affiliations, aggressions, and stereotypies have been successfully applied to *Rasa Aragonesa* sheep to study the effects of straw on their welfare (Teixeira *et al.*, 2012).

In the present work, home pen observations of individual behaviors identified several significant behavioral changes, which were most pronounced in winter. Lambs in the Vaccine and Adjuvant-only groups spent more time standing or walking and lambs in the Adjuvant-only group spent less time lying down than the animals in the Control group in the winter round. The differences between groups in time spent standing were already apparent in the summer round, which only entailed seven inoculations. The changes in the treatment groups reflect restless or excitatory behavior (Broom and Fraser, 2016) because resting patterns can be used to identify

social stress in animal husbandry (Fraser, 1997). Sheep exhibit a consistent and synchronous pattern of activity and resting (Rook and Penning, 1991; Fraser, 1997), which the inoculations appeared to have altered. Those changes were similar to some symptoms reported after the application of BT vaccines during the period 2008–2010 (Luján *et al.*, 2013). In this experiment, in winter, Vaccine and Adjuvant-only groups were fed on concentrate fewer times than the Control group. Given that the amount of concentrate offered to the three groups in either summer or winter was the same, and was consumed shortly after it was presented, the reduced frequency probably reflects prolonged bouts of feeding at the hopper, which is symptomatic of polyphagia or compulsive eating. Polyphagia was a symptom observed in animals that exhibited the chronic phase of the ovine ASIA syndrome after receiving the BT vaccinations (Luján *et al.*, 2013). It is uncertain why the Vaccine group ate straw less frequently than the Control lambs in summer, but not in winter. That difference was probably not associated with vaccination procedures.

Home pen observations of social behaviors demonstrated several significant changes in the behavior of Vaccine and Adjuvant-only groups. In winter, affiliative interactions among the Adjuvant-only and the Vaccine groups were much less frequent than they were in summer. In winter, but not in summer, Vaccine and Adjuvant-only groups exhibited significantly fewer affiliative interactions than the Control group. In winter, Vaccine animals engaged in very few affiliative interactions, which was even lower than Adjuvant-only lambs. In general, sheep are gregarious, and have a strong drive to be in the company of flock mates (Fischer and Matthews, 2001). *Rasa Aragonesa* is an autochthonous breed that is particularly gregarious, and a reduction in affiliative interactions by an individual is uncommon and readily detected by an observer. A reduction in affiliative interactions might indicate a deleterious effect on animal welfare (Mellor, 2015).

In this study, in summer and winter, the frequencies of aggressive interactions and stereotypies were significantly higher in Vaccine and Adjuvant-only group animals than they were in the Control group. Furthermore, in the former two groups, the frequency of those behaviors was significantly higher in winter than it was in summer. Apparently, these types of behavioral changes had occurred at a very early stage of the

inoculation protocol because they were already significantly higher in the Vaccine and Adjuvant-only groups than they were in the Control group during the summer round, after only seven inoculations had been administered. Aggressive behaviors are often associated with hierarchical interactions and the dominance of some individuals over others, and stereotypies, are repetitive and useless behaviors, associated with a worsened environmental condition (Mason, 1991). Increases in those behaviors have been correlated with poor welfare status (Barnett and Hemsworth, 1990), which might have also been reflected by the reduction in affiliative interactions. Wool loss and depilation, which were caused by wool biting, occurred in the Vaccine group, but only at the end of the winter round.

Aggression (including wool biting) and other stereotypies in sheep can be associated with housing, isolation (Lauber *et al.*, 2012), and other management factors, including diet (Cooper and Jackson, 1996; Yurtman *et al.,* 2002; Vasseur *et al.*, 2006). In flock 1, the three treatment groups were maintained in the same manner, which included long-term confinement to a limited space. However, in the Control group, the frequencies of aggressive interactions and stereotypies were always very low and did not differ between summer and winter; these are interpreted as the normal basal levels for the study. These results in the Control group rule out other known causes for these behavioral changes and link them to the treatments applied in the other groups. The presence of this Control group validates the model and system presented, as it is a fully-matched group designed for comparison with the other two treatments.

The reduction in the cortisol levels in the Control group reflected a previously-described seasonal variation in sheep between September and February (Snoj *et al.*, 2014). The Vaccine and Adjuvant-only lambs did not exhibit a similar reduction, therefore differing significantly to the Control group in the winter round. Cortisol is a good indicator of stress in animals that are exposed to adverse situations (Broom *et al.*, 1996; Fisher *et al.*, 1997), and reflects the stimulation of the hypothalamic-pituitary-adrenal gland axis (Hewagalamulage *et al.*, 2016). This study suggests that stress levels were higher in the Vaccine and Adjuvant-only animals than they were in the Control group in winter. The increase in the WBC and eosinophils in the vaccinated

animals might indicate an increase in stress. In humans, stress, either physical or emotional, is one of the main causes of an elevated WBC, which has also been demonstrated in mice (Heidt *et al.*, 2014). In ruminants, an elevated WBC has been associated with handling, which can be stressful (Ramos and Mormede, 1998). In this study, conditions were not stressful, which suggests that the vaccination was responsible for the increase in the WBC in the Vaccine group. Al-containing vaccines induce a pro-inflammatory effect through the activation of the inflammasome (Eisenbarth *et al.*, 2008), which could lead to leukocytosis among other effects (Grant and Dixit, 2013). Therefore, the repetitive inoculation of vaccines or Al adjuvant only could have induced a persistent proinflammatory status in the present animals, which contributed to the observed behavioral changes. Although the pathogenesis for this effect is still to be elucidated, autoimmune reactions generated along the course of a chronic-active inflammation may play an important role (Shoenfeld and Agmon-Levin, 2011; Terhune and Deth, 2013).

The characterization of the injection-site reactions and the translocation of Al to the regional lymph node presented in the second set of results is the first comprehensive description of the morphology of persistent granulomas in sheep following injection with Al-based adjuvant-containing products. Al was unequivocally identified by different methods both in granulomas and lymph nodes, with higher lesion severity in granulomas from the Vaccine group animals. The ultrastructure of the granulomas varied according to whether Al was administered as a vaccine preparation or simply as an adjuvant. Furthermore, the translocation of Al from the injection site to the lymph node is demonstrated for the first time in a large animal model.

This part of the experiment aimed to study the lesions caused by the administration of Al hydroxide-containing products, independently of the identification of individual injections, the exact age of the granuloma, or the role of specific vaccine antigens, as evaluated in other studies (Verdier *et al.*, 2005). These results showed that granuloma was the only type of local reaction produced by these injections, differing only in shape, persistency, and histopathologic aspects. Lambs were sourced from four different flocks (Table 1, page 60). However, *in vivo* measurements and observations of

gross and microscopic changes were similar, irrespective of flock, breed, or management conditions. Therefore, these observations are combined as three large treatment groups, each with 26 animals, at the end of the experiment.

Gross pathology data demonstrated that injection site granulomas were much more numerous than it was noticed by *in vivo* examination. In sheep, these reactions are reported to disappear with time, although it is not known exactly how long this takes (Ross and Titterington, 1984). The gross data underline that the development of granulomas in both treated groups was very common and was a universal fact in the Vaccine group animals. More than 75 % of vaccinated animals demonstrated at least eight granulomas (Table 8, page 91), indicating more frequent development or persistence of granulomas induced by vaccines. Assuming that each granuloma corresponded to a different single injection (injections were performed always in different points), the *post mortem* detection of granulomas in all injection sites would indicate that they may persist for at least 15 months, the duration of the present experiment. This persistence might reflect a low capacity, perhaps genetic, for clearing Al from the injection site in certain lambs, as has also been postulated in humans with specific HLA polymorphisms (Guis *et al.*, 2002). Interestingly, sheep are considered a species predisposed to develop vaccine-associated injection-site granulomas (Spickler and Roth, 2003; Woodward, 2009), which may support the hypothesis of the genetic predisposition. On the other hand, granulomas induced in Adjuvant-only group animals showed a lower persistency, which might indicate a quicker clearance of Al, perhaps due to a less severe immune reaction from the lack of antigen and/or different Al particle conformation.

Histologically, the reactions observed were interpreted as immune-mediated granulomas (i.e. they are induced by persistent immunostimulating agents versus inert materials, which cause foreign body granulomas; Kumar, 2016). In the Vaccine group, there were more frequent and extensive necrotic centers, which might be due to the presence of antigens, different Al particle conformations, or a combination of both. It is known that Al particle size can increase in the presence of antigens (Shardlow *et al.*, 2016), and indeed our results point to a higher particle size in Vaccine group granulomas. Al particles have been linked to phagolysosome membrane disruption and

the release of Al into the cytoplasm, leading to cell death by activating the cathepsin-mediated necrosis pathway (Jacobson *et al.*, 2013). Indeed, a relationship between particle size and the immunostimulation capability of different adjuvants has been postulated (Xiang *et al.*, 2006; Oyewumi *et al.*, 2010). Therefore, a different particle conformation due to the interaction with antigens may induce an increased immune stimulation, leading to a higher degree of tissue necrosis in vaccine granulomas. More accurate techniques, such as dynamic light scattering or the gravimetric/FTIR method, should be employed to determine the real particle size or surface area (Johnston *et al.*, 2002; Shardlow *et al.*, 2016), as our measurements are only two-dimensional estimations of particle length for comparison purposes. Nevertheless, these methods have been used to perform measurements in solutions of Al compounds prepared *in vitro*, as there is no established method to measure Al particle size within tissues.

In this PhD thesis, for the first time the presence of large dense Al aggregates in the form of pale crystalloid eosinophilic bodies with straight borders within the granulomas are described, the number of which was significantly increased in the Adjuvant-only group. The reason for the formation of these crystalloid bodies remains obscure, and further research is needed to clarify their role in the genesis of the granulomas and subsequent interactions with the surrounding tissues.

Lumogallion demonstrated excellent performance in sheep granulomas and lymph nodes, giving an Al-selective orangey fluorescence (Mold *et al.*, 2014; Mirza *et al.*, 2016). This fluorescence is easier to interpret than other stains such as morin, which has been classically used to identify Al in tissue sections (Guillard *et al.*, 2012). Lumogallion has recently been used to reliably identify Al in tissues from rats fed an Al-containing diet (Martínez *et al.*, 2017). EDS has been previously used to determine the presence of Al in postvaccine granulomas in pigs and humans (Valtulini *et al.*, 2005; Kalil *et al.*, 2007). The presence of other elements in EDS determinations are explained by the technical processing of the samples: lead and osmium are part of the staining, and copper is in the grid (Figures 45, 46, page 101; Valtulini *et al.*, 2005). TH GFAAS has been previously used to determine Al content in human and animal tissue (Crépeaux *et al.*, 2017; Martínez *et al.*, 2017; Mirza *et al.*, 2017). In the present work, this technique demonstrated a significant increase of Al in lymph nodes of Vaccine lambs when

compared with both Adjuvant-only and Control animals. Quantitative analyses using TH GFAAS and qualitative imaging using lumogallion demonstrate, for the first time in a large animal model, that Al is carried in macrophages from the injection site to the lymph nodes. In fact, egress of mycobacteria-infected macrophages from granulomas is a well-described mechanism (Davis and Ramakrishnan, 2009). Translocation of metals to lymph nodes has recently been reported in a case of a dog with hip-implant-associated metallosis (DiVincenzo *et al.*, 2017) and also in humans with subcutaneous tattoos that carry a variety of metals, including Al (Schreiver *et al.*, 2017). The Al translocation observed in this work might suggest a systemic distribution throughout the body, as demonstrated in mice or rabbits (Flarend *et al.*, 1997; Khan *et al.*, 2013; Crépeaux *et. al*, 2015; Eidi *et al*., 2015). In our animals, Al-containing macrophages tended to form aggregates in the lymph nodes, as it has been similarly observed in mice (Khan *et al.*, 2013; HogenEsch *et al.*, 2017). Some Control lambs showed rare, similar, but smaller macrophage aggregates, negative to lumogallion staining, in the draining lymph node (Table 9, page 96). This finding might simply indicate the drainage of other, non-related, lipidic phagocytic debris to the lymph node (Elmore, 2006).

Injection-site granulomas may be considered an acceptable and even desirable effect of a vaccine application. Indeed, the antibody titers of vaccinated calves that developed injection-site reactions was higher than in calves that did not develop such reactions in a study (Troxel *et al.*, 2001). On the other hand, the increased presence of Al within these reactions, such as those observed in our experimental sheep, contributes to the global Al body burden as suggested in laboratory rats (McDougall *et al.*, 2016). Our study may indicate that, in sheep, most of the subcutaneously injected Al is retained at the injection sites and regional lymph nodes, which contradicts the previously-accepted idea that most of the injected Al is eliminated through urine (Flarend *et al.*, 1997).

In this study, TH GFAAS was also applied to the determination of Al content in certain parts of the CNS of animals in flock 1. This flock was selected for being the most experimentally controlled during the experiment. There were exactly the same management and diet conditions in the three treatment groups, as they were always at the University of Zaragoza experimental farm. These experimental conditions

guaranteed that Al results obtained in this work were not influenced by factors such as diet, water, or other environmental exposures. Two areas were studied in the CNS: the lumbar spinal cord and the parietal lobe. The lumbar spinal cord was selected as it is one of the points at which neurodegenerative lesions were observed during the chronic phase of the ovine ASIA syndrome (Luján *et al.*, 2013); the parietal lobe was selected as a representative area of the brain.

Most of the replicates measured in the parietal lobe were within the same limits in the three treatment groups. Just the Adjuvant-only group presented a few slightly higher replicates. This is similar to some findings observed in mice inoculated intramuscularly, which failed to demonstrate Al accumulation in the brain, although subcutaneous inoculation in a subsequent experiment demonstrated clear translocation (Crépeaux *et al.*, 2015; Crépeaux *et al.*, 2017). Just three replicates of 0.3–0.5 g per brain were measured in the present work, that is 0.9–1.5 g per brain, which may not be totally representative of the full brain mass. Indeed, in a study, sheep brain weighed 2.3 years after birth was of 104.4 ± 2.6g (Louey *et al.*, 2005). Thus, 0.9 – 1.5 g represent a 0.87 – 1.44 % of the total brain weight. Probably, more replicates that also included replicates from other lobes should be studied to establish the accurate Al levels and distribution. On the other hand, injected Al translocated to the brain in a very delayed fashion in a murine model (Crépeaux *et al.*, 2015). Maybe the 15-month experiment was not long enough to permit Al input into the CNS of a large animal such as a sheep. In the future, longer term studies are warranted to study Al accumulation in the sheep's brain.

The Al levels established in the lumbar spinal cord were considered very accurate because the replicates studied contained always the same proportion of grey and white matter; the spine presents a cylindrical and symmetric structure, which can be easily cut into slices. These proportions could not be precisely maintained in the brain, in which the cerebral convolutions hampered this procedure, i.e. each parietal lobe replicate contained similar but variable proportions of grey and white matter. The Adjuvant-only and Vaccine groups presented higher Al levels in the spinal cord compared to the Control group. The difference was more marked in the Adjuvant-only group and, indeed, the highest individual replicates were obtained in animals from this

group. In measurements performed in human brain tissue with this technique, replicates over 2 μg/g were considered high and potentially pathologic (Mirza *et al.*, 2016). One animal in the Adjuvant-only group presented two replicates of 2.14 μg/g and 14.36 μg/g each, while another presented two replicates of 7.83 μg/g each. These measurements could represent punctual Al aggregates in the tissue replicates measured, as demonstrated in human brain tissue. Indeed, this patchy distribution is characteristic of Al, which does not diffuse homogeneously within the tissues (House *et al.*, 2012; Mirza *et al.*, 2016; McLachlan *et al.*, 2019). The Vaccine group did not show high replicates in the lumbar spinal cord. The highest measurement in this group was of 1.23 μg/g. Nevertheless, the median Al levels were significantly higher than in the Control group, which showed similar low levels to the brain tissue. Interestingly, more than half of all replicates measured in the lumbar spinal cord of the Control group were below 0.1 μg/g. These results suggest that sheep selectively accumulate injected Al in the lumbar spinal cord. A likely explanation of this may be a weak blood brain barrier at this level, which might facilitate Al income as demonstrated in some mice models with a leaky blood brain barrier (Khan *et al.*, 2013).

As these are the first Al measurements performed in ovine nervous tissue (Tables 11 and 12, pages 107 and 108, respectively), the values obtained in the Control group will be considered as the normal Al levels for this species: this being so, a range of 0.01 – 0.26 μg/g with a median of 0.1 μg/g in the brain; and a range 0.01 – 0.44 μg/g with a median of 0.08 μg/g in the lumbar spinal cord. These values contribute to the set of levels established in other models, such as mice, rats, and rabbits (Oteiza *et al.*, 1993; Demirkaya *et al.*, 2017; Rollin *et al.*, 1991; Sahin *et al.*, 1994), and will be the starting point for further studies to be performed in sheep. Generally, analytical studies are accompanied by the qualitative determination of Al in tissue (Mirza *et al.*, 2016). In the present work, this has been performed in the regional lymph nodes, with both Al level determinations by TH GFAAS and Al visualization within macrophages by lumogallion staining and fluorescence microscopy. At some point, the Al levels determination in the CNS of these animals should be complemented by its visualization. This is necessary to determine the interaction of Al with the nervous tissue components, i.e. Al may be in the grey/white matter, meninges, blood vessels,

and/or intra/extracellular. Our research group will expose these results elsewhere in future works.

Some of the observations performed in the second set of studies of this PhD thesis have to be considered as likely contributors to the behavioral changes observed in the first set of studies. Animals in the Vaccine and Adjuvant-only groups showed surprisingly persistent injection site subcutaneous Al-containing granulomas. Sheep are exposed to many stress factors during their lives, and a specific behavioral response is indicative to a stressor that the animal cannot cope with. These granulomas could have acted as stressors themselves, which might have completely or partially caused some of the changes observed, either by their simple presence (i.e. by causing a degree of physical discomfort to the animals), the increase in the total body burden of Al, or a combination of both. Furthermore, the genetic activation of certain signalling pathways in PBMCs in the Adjuvant-only and Vaccine groups has to be considered as a likely participant in the ethological changes observed too (Varela-Martínez *et al.*, 2018). This activation may have acted as an endogenous stressor itself, which was caused by the repetitive applications of Vaccines or Al adjuvant only (i.e. exogenous stressors). Another factor that must be considered is the presence of Al in the CNS. Although the presence of Al in the spinal cord of Adjuvant-only and Vaccine groups has been demonstrated, the levels in the area of the brain studied (parietal lobe) remained similar in the three groups, with just a tendency towards the Adjuvant-only inoculated animals compared to the Control group. Thus, the simple presence of Al in the brain may not explain the profound differences observed in the behavior of Vaccine and Adjuvant-only groups lambs; this direct neurotoxic effect of Al is unlikely in this model. Contrarily, a systemic immune activation caused by the repetitive inoculation of Vaccines or Adjuvant-only, i.e. over-immunization, remains the most plausible explanation of the changes observed.

The results of the present work, and the global research project that encompasses it, provide new information on the effects of Al salts as vaccine adjuvants in sheep. These effects are much more relevant than previously described and they suggest limiting (or even banning) the use of these salts as vaccine adjuvants to immediately improve vaccine safety. Indeed, the removal of Al from cat vaccines has

drastically reduced the occurrence of FISS (Graf *et al*., 2018). It is likely that avoiding Al in vaccines will also be of benefit in sheep and many other species. This study justifies further research on alternative adjuvants, a field in need of extensive investigation for providing better and safer vaccines in the future.

6. CONCLUSIONS

1. In sheep, the injection of Al hydroxide, either alone or in vaccines, induces a wide-spectrum of behavioral changes and increased levels of stress. These changes are generally more important when Al acts as a vaccine adjuvant.

2. In sheep, the injection of Al hydroxide, either alone or in vaccines, induces persistent subcutaneous granuloma formation. These granulomas are more persistent and severe when Al acts as a vaccine adjuvant.

3. In sheep, subcutaneous granuloma formation is a universal post-vaccine event: a single injection of an Al-containing vaccine induces an individual granuloma.

4. Subcutaneously-injected Al in sheep is mobilized from the inoculation point to the regional lymph node, in which it accumulates. Such accumulation is more marked when Al acts as a vaccine adjuvant. This suggests that sheep handles Al differently depending upon its presentation at injection sites.

5. The repeated inoculation schedule performed in this work has induced an increase in the Al levels in the lumbar spinal cord. This increase is more marked when Al hydroxide is applied alone.

6. Data obtained in this work point to the central role of Al in the pathogenesis of post-vaccine secondary reactions in sheep. The ovine species can be a useful model to understand the complex syndrome that follows Al injection.

6. CONCLUSIONES

1. En ovino, la inyección de hidróxido de Al, bien solo o en vacunas, induce un amplio espectro de cambios comportamentales y aumento de los niveles de estrés. Estos cambios son generalmente más importantes cuando el Al actúa como adyuvante vacunal.

2. En ovino, la inyección de hidróxido de Al, bien solo o en vacunas, induce la formación de granulomas subcutáneos persistentes. Estos granulomas son más persistentes y severos cuando el Al actúa como adyuvante vacunal.

3. En ovino, la formación de granulomas subcutáneos es un evento post-vacunal universal: una única inyección de una vacuna con Al induce un granuloma individual.

4. El Al inyectado por vía subcutánea es movilizado desde el punto de inoculación al linfonodo regional, donde se acumula. Esta acumulación es más marcada cuando el Al actúa como adyuvante vacunal. Esto sugiere que las ovejas procesan el Al de forma distinta dependiendo de su presentación en el punto de inyección.

5. El programa de inoculación repetitiva llevado a cabo en este trabajo ha inducido un aumento en los niveles de Al en la médula lumbar. Este aumento es más marcado cuando el hidróxido de Al es aplicado solo.

6. Los datos obtenidos en este trabajo apuntan al papel central del Al en la patogenia de las reacciones post vacunales secundarias en ovino. La especie ovina puede ser un modelo útil para entender el síndrome complejo que se da tras la inyección de Al.

7. ABBREVIATIONS

Al-Aluminum

AMHA-Autoimmune hemolytic anemia

APCs-Antigen presenting cells

ASIA-Autoimmune/inflammatory syndrome induced by adjuvants

BHV-1-Bovine herpesvirus 1

BNP-Bovine neonatal pancytopenia

BT-Bluetongue

BVD-Bovine viral diarrhea

CDV-Canine distemper virus

CK-Creatine kinase

CNS-Central nervous system

DAMPs-Damage associated molecular patterns

DK-Drinking

EDS-X-ray spectroscopy

ES-Eating straw

FC-Feeding on concentrate

FISS-Feline injection site sarcoma

FMD-Foot-and-mouth disease

GFASS-Graphite furnance atomic absorption spectroscopy

GLM-General linear models

GWS-Gulf War syndrome

HLA-Human leukocyte antigen

ICP-MS-Inductively coupled mass spectrometry

IQR-Interquartile range

KW-Kruskal-Wallis

MLV-Modified live vaccines

MMF-Macrophagic myofasciitis

MSU-Monosodium urate

N/L-Neutrophil/lymphocyte ratio

NEFA-Non-esterified fatty acids

NOT-Novel object test

O/W-Oil-in-water

OFT-Open field test

PAMPs-Pathogen associated molecular patterns

PBS-Phosphate buffered saline

PPR-Peste des petits ruminants

PRRs-Pattern recognition receptors

RT-Resting

RVF-Rift Valley fever

STEM-Scanning transmission electron microscopy

ST-Standing

TLRs-Toll-like receptors

t-Student´s t

U-Mann-Whitney U

VAEs-Vaccine-associated adverse events

W/O/W-Water-in-oil-in-water

W/O-Water-in-oil

WBC-White blood cell counts

W-Wilcoxon

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ANNEXES

ANNEX I

Schemes of the home pens and test arenas used for the cognition and behavior tests

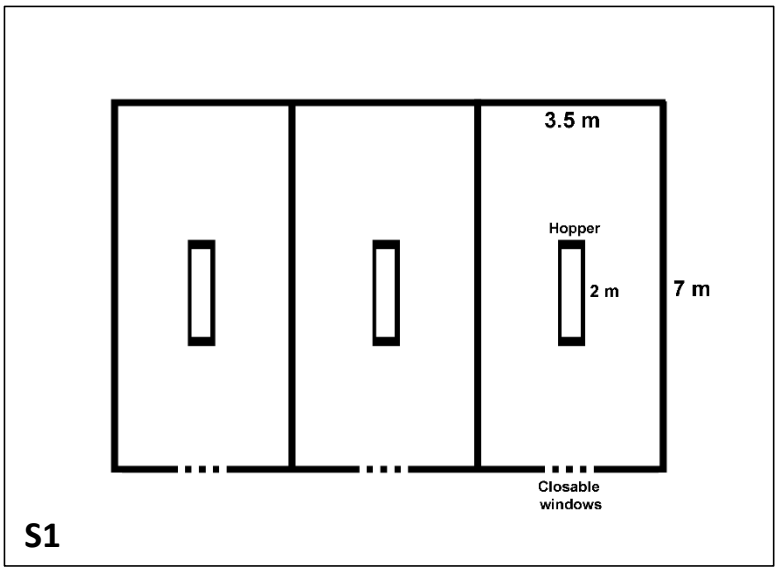

Figure S1. Home pens. Animals in flock 1 were maintained in these pens for 15 months. Recordings for evaluation of individual and social behaviors were performed in these pens.

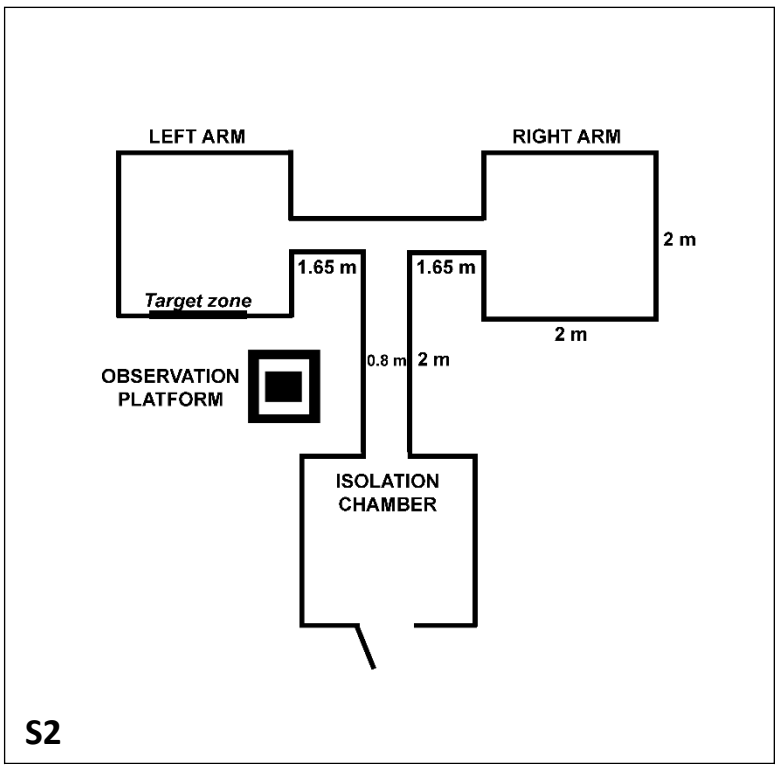

Figure S2. T-maze test arena. The target zone included a mirror and a loudspeaker which displayed ovine vocalizations. Adapted from Abecia *et al*., 2014

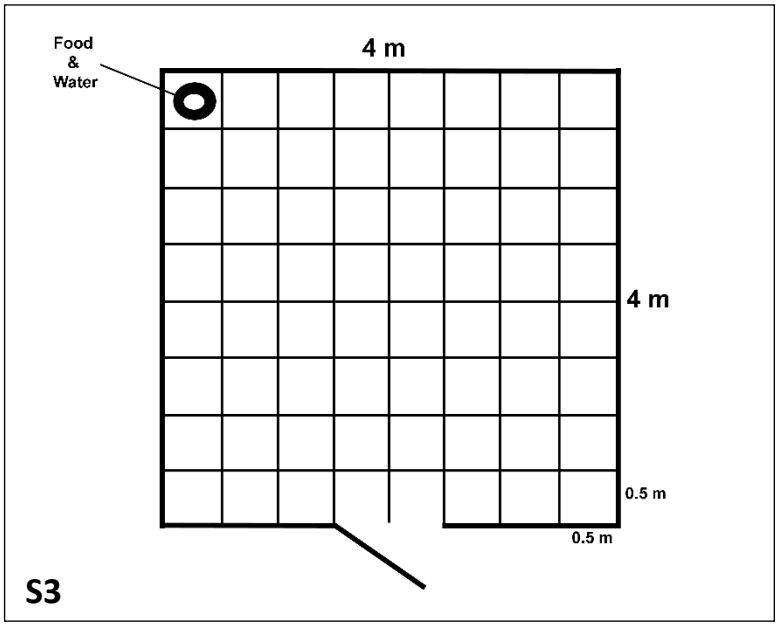

Figure S3. Open field test (OFT) arena. The novel object recognition test (NOT) was performed in the same test arena by placing a plastic ball tied with a rope in the center.

ANNEX II

Supplementary data of cognition and behavior tests

Table S1. Cognition. T-maze test. Time spent in leaving the first area (latency) in the summer round. Unpaired (columns) and paired (rows) comparisons for latency in the summer round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs in each group spent in leaving the first area of the T-maze ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; A: ANOVA; KW: Kruskal-Wallis.

	Day 1	**Day 2**	**p**
Control	2.29 ± 0.42 2 (1.5-3)	5.29 ± 1.69 4 (2.5-6.5)	0.089^{t}
Adjuvant	4.86 ± 1.67 2 (1.5-8)	9.71 ± 3.88 8 (4.5-9)	0.116^{W}
Vaccine	4.29 ± 1.54 2 (1-7)	54.57 ± 48.41 7 (5-9)	0.306^{W}
p	0.378^{A}	0.416^{KW}	

Table S2. Cognition. T-maze test. Time spent in leaving the first area (latency) in the winter round. Unpaired (columns) and paired (rows) comparisons for latency in the winter round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in leaving the first area of the T-maze ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; KW: Kruskal Wallis.

	Day 1	**Day 2**	**p**
Control	2.57 ± 0.65 2 (1.5-3)	2.57 ± 0.48 2 (2-3)	$>0.999^{t}$
Adjuvant	5.14 ± 2.05 3 (2-5)	4.29 ± 1.39 2 (2-6.5)	0.680^{W}
Vaccine	11.14 ± 6.87 5 (2.5-7)	1.71 ± 0.42 1 (1-2)	$\mathbf{0.027^{W}*}$
p	0.218^{KW}	0.152^{KW}	

Table S3. Cognition. T-maze test. Time taken to reach the target zone in the summer round. Unpaired (columns) and paired (rows) comparisons for the time taken to reach the target zone in the summer round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs in each group took to reach the target zone of the maze ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; KW: Kruskal Wallis.

	Day 1	Day 2	p
Control	33.86 ± 19.77 17 (13.5-18)	74.86 ± 47.20 13 (9.5-72)	0.735^{W}
Adjuvant	70.71 ± 24.34 47 (29.5-89)	19.57 ± 6.48 11 (7.5-29)	0.083^{t}
Vaccine	107.00 ± 47.20 73 (14-146)	80.14 ± 46.24 25 (13-77.5)	0.398^{W}
p	0.209^{KW}	0.329^{KW}	

Table S4. Cognition. T-maze test. Time taken to reach the target zone in the winter round. Unpaired (columns) and paired (rows) comparisons for the time taken to reach the target zone in the winter round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs took to reach the target zone of the maze ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; A: ANOVA; KW: Kruskal Wallis.

	Day 1	Day 2	p
Control	26.71 ± 9.64 18 (6-40.5)	18.43 ± 6.66 12 (6.5-24)	0.512^{t}
Adjuvant	43.00 ± 22.90 12 (10-44.5)	13.14 ± 3.01 15 (6-18)	0.310^{W}
Vaccine	26.43 ± 8.46 30 (7-34.5)	10.57 ± 2.68 7 (5-15.5)	0.061^{t}
p	0.865^{KW}	0.467^{A}	

Table S5. Cognition. T-maze test. Time spent in solving the maze in the summer round. Unpaired (columns) and paired (rows) comparisons for the time spent in solving the maze in the summer round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in placing themselves in front of the mirror and the loudspeaker ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; KW: Kruskal Wallis.

	Day 1	Day 2	p
Control	39.71 ± 19.29 20 (18-31)	76.71 ± 46.96 15 (11.5-74.5)	0.866^{W}
Adjuvant	73.71 ± 24.77 51 (31.5-91)	21.29 ± 6.46 13 (9-30.5)	0.081^{t}
Vaccine	111.00 ± 46.32 75 (21.5-149.5)	81.71 ± 45.94 27 (15.5-78.5)	0.398^{W}
p	0.200^{KW}	0.329^{KW}	

Table S6. Cognition. T-maze test. Time spent in solving the maze in the winter round. Unpaired (columns) and paired (rows) comparisons for the time spent in solving the maze in the winter round. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in placing themselves in front of the mirror and the loudspeaker ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; t: Student´s T test; KW: Kruskal Wallis; A: ANOVA.

	Day 1	Day 2	p
Control	34.29 ± 9.56 36 (13.5-49)	20.29 ± 6.63 14 (8-26.5)	0.308^{t}
Adjuvant	48.00 ± 22.33 29 (14-47.5)	15.00 ± 3.20 17 (7.5-20.5)	0.063^{W}
Vaccine	38.57 ± 9.86 36 (21.5-54)	16.57 ± 2.26 18 (14-20)	0.064^{t}
p	0.904^{KW}	0.694^{A}	

Table S7. Cognition. T-maze test. Number of areas traversed in the summer round. Unpaired (columns) and paired (rows) comparisons for the number of areas traversed in the summer round. For each group, numbers in the upper row indicate the mean number of areas of the maze traversed by lambs ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; KW: Kruskal Wallis.

	Day 1	**Day 2**	**p**
Control	8.57 ± 1.73 6 (6-11)	14.14 ± 7.07 6 (4-13)	0.674^{W}
Adjuvant	7.71 ± 1.41 6 (6-7)	6.57 ± 2.26 4 (4-5)	0.219^{W}
Vaccine	9.29 ± 1.94 8 (7-9.5)	10.14 ± 5.10 4 (4-9)	0.498^{W}
p	0.603^{KW}	0.594^{KW}	

Table S8. Cognition. T-maze test. Number of areas traversed in the winter round. Unpaired (columns) and paired (rows) comparisons for the number of areas traversed in the winter round. For each group, numbers in the upper row indicate the mean number of areas of the maze traversed by lambs ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Day 1: First day of the test; Day 2: Second day of the test; W: Wilcoxon´s test; KW: Kruskal Wallis.

	Day 1	**Day 2**	**p**
Control	13.71 ± 3.04 12 (8-20)	9.00 ± 2.63 6 (4-11)	0.496^{W}
Adjuvant	13.71 ± 5.83 6 (4-16)	6.86 ± 1.74 4 (4-8)	0.500^{W}
Vaccine	14.00 ± 5.86 10 (5-13)	8.57 ± 0.84 10 (8-10)	0.588^{W}
p	0.771^{KW}	0.490^{KW}	

Table S9. Cognition. Open Field Test (OFT). Walking. Unpaired (columns) and paired (rows) comparisons of the amount of time lambs spent walking in each of the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent walking ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; t: Student´s T test; A: ANOVA.

	Summer	Winter	p
Control	117.29 ± 18.37 113 (91-135)	170.29 ± 16.13 168 (139-208.5)	0.074[t]
Adjuvant	117.71 ± 15.34 114 (94.5-143)	170.43 ± 19.90 167 (138.5-207)	0.068[t]
Vaccine	127.57 ± 14.93 121 (117-139)	153.86 ± 17.22 168 (125-179.5)	0.330[t]
p	0.881[A]	0.755[A]	

Table S10. Cognition. Open Field Test (OFT). Exploring. Unpaired (columns) and paired (rows) comparisons of the amount of time lambs spent in exploring in each of the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in exploring ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; t: Student´s T test; W: Wilcoxon´s test A: ANOVA; KW: Kruskal Wallis.

	Summer	Winter	p
Control	46.43 ± 10.73 41 (27-52)	45.14 ± 6.06 49 (39-54.5)	0.896[t]
Adjuvant	35.57 ± 8.23 34 (22-37)	54.71 ± 8.27 54 (38.5-69)	**0.043[w]***
Vaccine	37.29 ± 7.09 40 (25.5-45.5)	46.86 ± 12.92 44 (17-67.5)	0.543[t]
p	0.527[KW]	0.754[A]	

Table S11. Cognition. Open Field Test (OFT). Trying to escape. Unpaired (columns) and paired (rows) comparisons of the amount of time lambs spent in trying to escape in each of the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in trying to escape from the test arena ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; t: Student´s T test; A: ANOVA.

	Summer	Winter	p
Control	85.86 ± 23.33 76 (57-113.5)	80.71 ± 18.37 100 (52.5-112.5)	0.766[t]
Adjuvant	100.00 ± 32.82 94 (28.5-163)	69.43 ± 24.60 72 (16-100)	0.201[t]
Vaccine	118.57 ± 25.71 139 (86.5-160)	48.00 ± 12.70 47 (27.5-72.5)	**0.003[t]***
p	0.707[A]	0.486[A]	

Table S12. Cognition. Open Field Test (OFT). Standing. Unpaired (columns) and paired (rows) comparisons of the amount of time lambs remained standing in each the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) time (s) that lambs spent in remaining standing ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; t: Student´s T test; A: ANOVA.

	Summer	Winter	p
Control	138.57 ± 30.22 130 (89-201)	142.57 ± 22.23 131 (127-163.5)	0.906[t]
Adjuvant	176.86 ± 38.58 161 (105-262)	144.14 ± 30.48 99 (86-193)	0.132[t]
Vaccine	134.57 ± 28.00 103 (86-165.5)	195.86 ± 32.07 193 (163-215.5)	0.127[t]
p	0.607[A]	0.346[A]	

Table S13. Cognition. Open Field Test (OFT). Escape attempts. Unpaired (columns) and paired (rows) comparisons for the number of escape attempts (jumps) that the animals performed in each of the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) number of escape attempts (jumps) that lambs performed ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; t: Student´s T test; A: ANOVA.

	Summer	Winter	p
Control	15.86 ± 4.86 12 (11.5-17)	22.57 ± 4.52 25 (19-29.5)	0.256[t]
Adjuvant	20.29 ± 6.51 19 (7-31.5)	19.86 ± 5.98 20 (9-29)	0.893[t]
Vaccine	22.00 ± 4.74 26 (17-29)	17.14 ± 5.46 11 (9-24.5)	0.357[t]
p	0.716[A]	0.776[A]	

Table S14. Cognition. Open Field Test (OFT). Bleats. Unpaired (columns) and paired (rows) comparisons for the number of bleats that the animals performed in the two rounds of the OFT. For each group, numbers in the upper row indicate the average (mean) number of bleats that lambs performed ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; W: Wilcoxon´s test; t: Student´s T test; A: ANOVA; KW: Kruskal Wallis.

	Summer	Winter	p
Control	39.00 ± 13.36 36 (23-39.5)	45.29 ± 8.48 43 (31-63)	0.619[t]
Adjuvant	38.43 ± 7.17 47 (31-50.5)	36.57 ± 6.91 38 (28-46.5)	0.691[t]
Vaccine	40.57 ± 10.03 41 (27.5-52)	33.57 ± 8.94 19 (14-55)	0.398[W]
p	0.989[A]	0.585[KW]	

Table S15. Cognition. Novel Object Test (NOT) in the summer round. Unpaired (columns) and paired (rows) comparisons of the distance between the lamb and the novel object in the summer round. For each group, numbers in the upper row indicate the average (mean) distance (cm) between the lamb and the novel object 30 s after each exposure to the novel object ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Dist. 1: Distance after the first exposure; Dist. 2: Distance after the second exposure; W: Wilcoxon´s test; t: Student´s T test; A: ANOVA; KW: Kruskal Wallis.

	Dist 1	Dist 2	p
Control	78.57 ± 21.43 50 (50-75)	67.86 ± 24.83 50 (37.5-75)	0.671^{W}
Adjuvant	92.86 ± 17.00 100 (50-125)	82.14 ± 22.96 50 (50-125)	0.629^{t}
Vaccine	75.00 ± 19.67 50 (50-112.5)	142.86 ± 24.83 125 (100-200)	0.089^{t}
p	0.699^{KW}	0.094^{A}	

Table S16. Cognition. Novel Object Test (NOT) in the winter round. Unpaired (columns) and paired (rows) comparisons of the distance between the lamb and the novel object in the winter round. For each group, numbers in the upper row indicate the average (mean) distance (cm) between the lamb and the novel object 30 s after each exposure to the novel object ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Dist. 1: Distance recorded after the first exposition; Dist. 2: Distance recorded after the second exposition; W: Wilcoxon´s test; t: Student´s T test; KW: Kruskal Wallis.

	Dist 1	Dist 2	p
Control	75.00 ± 23.78 50 (25-112.5)	114.29 ± 17.13 125 (87.5-150)	0.235^{W}
Adjuvant	75.00 ± 22.49 75 (37.5-100)	128.57 ± 12.71 125 (100-150)	**0.043^{W}***
Vaccine	89.29 ± 26.08 100 (37.5-137.5)	78.57 ± 21.43 75 (37.5-125)	0.751^{t}
p	0.917^{KW}	0.259^{KW}	

Table S17. Individual behavior. Feeding on concentrate (FC). Unpaired (columns) comparisons for FC in the two rounds of tests. For each group, numbers in the upper row indicate the average (mean) number of times that lambs ate concentrate from the concentrate hopper ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b) indicate statistically significant differences between the groups based on *post hoc* tests. KW: Kruskal Wallis test.

	Summer	**Winter**
Control	3.33 ± 0.28 3 (2-4)	3.45 ± 0.21 3^{a} (3-4)
Adjuvant	3.08 ± 0.31 3 (2-4)	2.63 ± 0.14 2^{b} (2-3)
Vaccine	2.94 ± 0.23 2 (2-4)	2.24 ± 0.14 2^{b} (2-3)
p	0.355^{KW}	$\mathbf{<0.001^{KW}*}$

Table S18. Individual behavior. Eating straw (ES). Unpaired (columns) comparisons for ES in the two rounds of tests. For each group, numbers in the upper row indicate the average (mean) number of times that lambs ate straw from the forage hopper ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b) indicate statistically significant differences between the groups based on *post hoc* tests. KW: Kruskal Wallis.

	Summer	**Winter**
Control	7.88 ± 0.55 7^{a} (5-11)	14.71 ± 0.49 15 (12-17)
Adjuvant	6.59 ± 0.49 $6^{a,b}$ (4-9)	14.90 ± 0.37 15 (14-16)
Vaccine	5.49 ± 0.38 5^{b} (5-7)	14.43 ± 0.34 14 (12-16)
p	$\mathbf{0.018^{KW}*}$	0.472^{KW}

Table S19. Individual behavior. Resting (RT). Unpaired (columns) comparisons for RT in the two rounds of tests. For each group, numbers in the upper row indicate the average (mean) number of times that lambs laid down ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b) indicate statistically significant differences between the groups based on *post hoc* tests. KW: Kruskal Wallis.

	Summer	**Winter**
Control	20.20 ± 0.56 21 (17-24)	9.94 ± 0.48 9^{a} (7-12)
Adjuvant	18.73 ± 0.60 18 (16-20)	8.00 ± 0.57 7^{b} (5-10)
Vaccine	19.37 ± 0.66 19 (16-24)	8.73 ± 0.35 $9^{a,b}$ (7-10)
p	0.156^{KW}	**0.027^{KW}***

Table S20. Individual behavior. Standing (ST). Unpaired (columns) comparisons for ST in the two rounds of tests. For each group, numbers in the upper row indicate the average (mean) number of times that lambs stood on all four legs or walked ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b) indicate statistically significant differences between the groups based on *post hoc* tests. A: ANOVA.

	Summer	**Winter**
Control	6.33^{a} ± 0.39 6 (4-8)	9.33^{a} ± 0.50 10 (6-11)
Adjuvant	9.31^{b} ± 0.48 9 (8-12)	12.43^{b} ± 0.57 13 (10-15)
Vaccine	10.00^{b} ± 0.49 10 (7-12)	11.06^{b} ± 0.47 11 (9-13)
p	**$<0.001^{A}$***	**$<0.001^{A}$***

Table S21. Individual behavior. Drinking (DK). Unpaired (columns) comparisons for DK in the two rounds of tests. For each group, numbers in the upper row indicate the average (mean) number of times that lambs drank water from the drinking trough ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; KW: Kruskal Wallis.

	Summer	Winter
Control	0.41 ± 0.10 0 (0-1)	0.14 ± 0.05 0 (0-0)
Adjuvant	0.43 ± 0.12 0 (0-1)	0.04 ± 0.03 0 (0-0)
Vaccine	0.27 ± 0.08 0 (0-0)	0.12 ± 0.06 0 (0-0)
p	0.576^{KW}	0.228^{KW}

Table S22. General Linear Model (GLM) for individual behaviors. Numbers indicate the statistical significance (p) of the factors evaluated in each behavior and their paired interactions. FC: Feeding on concentrate; ES: Eating straw; RT: Resting; ST: Standing; DK: Drinking.

	FC	ES	RT	ST	DK
Group	<0.001*	0.029*	0.007*	<0.001*	0.610
Round	0.016*	<0.001*	<0.001*	<0.001*	<0.001*
Group x Round	0.051	0.094	0.889	**0.035***	0.335

Table S23. Social behavior. Affiliative interactions. Unpaired (columns) and paired (rows) comparisons for affiliative interactions in the two rounds. For each group, numbers in the upper row indicate the average (mean) number of times that lambs performed an affiliative interaction ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a,b,c) indicate statistically significant differences between the groups. W: Wilcoxon; KW: Kruskal Wallis.

	Summer	Winter	p
Control	10.61 ± 1.00 10 (5-15)	11.16 ± 1.05 9^{a} (6-15)	0.674^{W}
Adjuvant	12.49 ± 1.54 10 (5-16)	8.18 ± 0.66 7^{b} (5-11)	$\mathbf{0.045^{W}*}$
Vaccine	11.82 ± 1.10 11 (5-17)	1.49 ± 0.33 1^{c} (0-2)	$\mathbf{<0.001^{W}*}$
p	0.742^{KW}	$\mathbf{<0.001^{KW}*}$	

Table S24. Social behavior. Agonistic (aggressive) interactions. Unpaired (columns) and paired (rows) comparisons for aggressive interactions in the two rounds. For each group, numbers in the upper row indicate the average (mean) number of times that lambs performed an aggressive interaction ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b) indicate statistically significant differences between the groups. W: Wilcoxon; KW: Kruskal Wallis.

	Summer	Winter	p
Control	6.61 ± 1.64 1^{a} (0-5)	2.67 ± 0.60 2^{a} (0-3)	0.062^{W}
Adjuvant	13.06 ± 1.43 10^{b} (5-22)	20.90 ± 2.28 15^{b} (10-31)	$\mathbf{0.018^{W}*}$
Vaccine	13.08 ± 1.31 12^{b} (5-18)	21.14 ± 1.67 17^{b} (14-23)	$\mathbf{0.003^{W}*}$
p	$\mathbf{<0.001^{KW}*}$	$\mathbf{<0.001^{KW}*}$	

Table S25. Social behavior. Stereotypies. Unpaired (columns) and paired (rows) comparisons for stereotypies in the two rounds. For each group, numbers in the upper row indicate the average (mean) number of times that lambs performed a stereotypy ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Summer: Summer round; Winter: Winter round; Superscripts (a, b, c) indicate statistically significant differences between groups. W: Wilcoxon; KW: Kruskal Wallis.

	Summer	**Winter**	**p**
Control	6.20 ± 1.17 0^{a} (0-8)	4.41 ± 0.99 0^{a} (0-8)	0.158^{W}
Adjuvant	13.27 ± 2.18 8^{b} (0-16)	22.29 ± 3.42 16^{b} (8-32)	0.055^{W}
Vaccine	18.78 ± 1.30 18^{c} (12-22)	29.02 ± 2.71 29^{b} (12-42)	**0.002^{W}***
p	**$<0.001^{KW}$***	**$<0.001^{KW}$***	

Table S26. General Linear Model (GLM) for social behaviors. Numbers indicate the statistical significance (p) of the factors evaluated for each behavior and their paired interactions. AFFIL: Affiliative interactions; AGG: Agonistic (aggressive) interactions; STY: Stereotypies.

	AFFIL	**AGG**	**STY**
Group	0.010*	<0.001*	<0.001*
Round	<0.001*	0.048*	0.013*
Group x Round	**0.002***	**0.024***	0.066

Table S27. Blood welfare indicators and hematology panel in the summer round. Unpaired (rows) comparisons for the blood parameters evaluated in the summer round. For each parameter, numbers in the upper row indicate the mean value for each group ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Superscripts (a,b) indicate statistically significant differences between groups based on *post hoc* tests; A: ANOVA; KW: Kruskal Wallis; We: Welch. CK: Creatine Kinase; NEFA: Non-Esterified Fatty Acid; ratio N/L: Neutrophil/Lymphocyte ratio; WBC: White Blood Cells; NE: Neutrophils; EO: Eosinophils; BA: Basophils; LY: Lymphocytes; MO: Monocytes; RBC: Red Blood Cells; HG: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

	Control	Adjuvant	Vaccine	p
Cortisol (nmol/L)	64.27 ± 9.51 75.30 (44.70-83.60)	75.66 ± 11.94 72.60 (59.60-76.35)	65.89 ± 9.94 54.10 (46.90-88.15)	0.953^{KW}
CK (UI/L)	156.14 ± 20.82 143.00 (131.50-173.50)	218.29 ± 79.79 133.00 (123.00-172.50)	132.29 ± 25.81 110.00 (98.00-125.50)	0.175^{KW}
Glucose (mg/dl)	47.29 ± 2.31 49.00 (45.00-51.00)	49.43 ± 4.06 50.00 (43.00-51.00)	46.57 ± 3.56 43.00 (40.50-51.50)	0.405^{A}
Lactate (mmol/L)	61.77 ± 4.60 65.40 (52.95-67.65)	50.29 ± 8.69 52.50 (40.70-68.10)	64.64 ± 7.85 63.50 (55.45-67.55)	0.356^{A}
NEFA (mmol/L)	0.27 ± 0.07 0.20 (0.10-0.40)	0.37 ± 0.07 0.30 (0.25-0.45)	0.51 ± 0.16 0.30 (0.30-0.55)	0.324^{KW}
Ratio N/L	0.39 ± 0.05 0.31 (0.30-0.46)	0.35 ± 0.02 0.33 (0.32-0.38)	0.48 ± 0.06 0.49 (0.35-0.61)	0.340^{KW}
WBC ($10^3/mm^3$)	6.49 ± 0.58 6.01 (5.61-6.83)	5.21± 0.27 5.32 (4.62-5.41)	6.56 ± 0.61 6.34 (6.23-6.81)	0.135^{A}
NE ($10^3/mm^3$)	1.67 ± 0.22 1.58 (1.38-1.70)	1.25 ± 0.06 1.21 (1.13-1.37)	1.94 ± 0.30 1.85 (1.38-2.19)	0.114^{A}
EO ($10^3/mm^3$)	0.29 ± 0.20 0.09 (0.07-0.15)	0.12 ± 0.02 0.12 (0.08-0.15)	0.27 ± 0.06 0.26 (0.15-0.33)	0.081^{KW}
BA ($10^3/mm^3$)	0.02 ± 0.00 0.02 (0.01-0.02)	0.02 ± 0.00 0.02 (0.02-0.03)	0.03 ± 0.00 0.03 (0.02-0.03)	0.296^{A}
LY ($10^3/mm^3$)	4.32 ± 0.26 4.34 (3.86-4.68)	3.67 ± 0.22 3.57 (3.24-3.78)	4.16 ± 0.38 3.82 (3.61-5.06)	0.190^{KW}

MO ($10^3/mm^3$)	0.18 ± 0.02 0.16 (0.15-0.18)	0.14 ± 0.02 0.10 (0.10-0.15)	0.17 ± 0.02 0.16 (0.16-0.18)	0.224^{KW}
RBC ($10^6/mm^3$)	12.85 ± 0.42 12.87 (12.13-13.23)	12.75 ± 0.37 13.26 (12.37-13.38)	12.51 ± 0.30 12.62 (12.15-13.12)	0.558^{KW}
HG (g/dl)	13.64 ± 0.36 13.90 (13.30-14.00)	13.11 ± 0.32 12.90 (12.50-13.80)	13.07 ± 0.18 13.00 (12.70-13.40)	0.336^{A}
HCT (%)	46.89 ± 1.31 47.30 (45.50-48.90)	45.00 ± 1.05 44.20 (43.60-45.65)	47.59 ± 1.42 47.20 (44.65-51.00)	0.351^{A}
MCV	36.59 ± 0.96 36.05 (35.25-36.46)	35.39 ± 0.91 34.78 (33.63-36.90)	38.09 ± 1.01 38.98 (36.72-39.70)	0.152^{KW}
MCH	10.64 ± 0.16 10.62 (10.35-10.88)	10.30 ± 0.18 10.46 (10.04-10.57)	10.48 ± 0.24 10.32 (10.10-10.81)	0.499^{A}
MCHC	29.15 ± 0.61 29.39 (28.80-30.10)	29.16 ± 0.52 28.96 (28.18-29.51)	27.64 ± 1.01 26.48 (25.49-29.67)	0.426^{We}

Table S28. Blood welfare indicators and hematology panel in the winter round. Unpaired (rows) comparisons for the blood parameters evaluated in the winter round. For each parameter, numbers in the upper row indicate the mean value for each group ± standard error and numbers in the bottom row indicate the median with the interquartile rank. Superscripts (a,b) indicate statistically significant differences between groups based on *post hoc* tests; A: ANOVA; KW: Kruskal Wallis; We: Welch. CK: Creatine Kinase; NEFA: Non-Esterified Fatty Acid; ratio N/L: Neutrophil/Lymphocyte ratio; WBC: White Blood Cells; NE: Neutrophils; EO: Eosinophils; BA: Basophils; LY: Lymphocytes; MO: Monocytes; RBC: Red Blood Cells; HG: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

	Control	**Adjuvant**	**Vaccine**	
Cortisol (nmol/L)	21.27^{a} ± 5.64 15.60 (10.15-28.85)	58.37^{b} ± 10.55 64.30 (40.40-75.75)	62.40^{b} ± 8.37 58.20 (46.35-71.60)	**0.005^{A}***
CK (UI/L)	490.43 ± 262.10 154.00 (147.00-424.00)	416.14 ± 148.47 158.00 (149.00-674.50)	326.00 ± 201.63 130.00 (117.50-145.50)	0.151^{KW}
Glucose (mg/dl)	62.29 ± 0.99 63.00 (60.50-63.00)	63.86 ± 2.85 61.00 (59.50-68.00)	66.00 ± 1.35 68.00 (63.50-68.50)	0.266^{KW}
Lactate (mmol/L)	24.81 ± 3.39 22.70 (18.30-30.70)	31.70 ± 3.51 30.00 (24.15-39.50)	35.13 ± 5.13 43.20 (26.55-45.45)	0.220^{A}
NEFA (mmol/L)	0.11 ± 0.01 0.10 (0.10-0.10)	0.19 ± 0.05 0.10 (0.10-0.25)	0.19 ± 0.04 0.20 (0.10-0.20)	0.251^{KW}
Ratio N/L	0.47 ± 0.05 0.44 (0.36-0.57)	0.39 ± 0.02 0.41 (0.37-0.44)	0.49 ± 0.08 0.45 (0.39-0.61)	0.449^{A}
WBC (10^3/mm^3)	5.96^{b} ± 0.30 6.00 (5.25-6.61)	5.68^{b} ± 0.24 5.65 (5.37-5.86)	7.01^{a}± 0.51 7.16 (6.26-7.84)	**0.047^{A}***
NE (10^3/mm^3)	1.78 ± 0.17 1.71 (1.38-2.16)	1.49 ± 0.06 1.47 (1.37-1.60)	2.06 ± 0.30 1.96 (1.60-2.71)	0.129^{wl}
EO (10^3/mm^3)	0.16 ± 0.06 0.09^{b} (0.08-0.16)	0.16 ± 0.03 0.11^{b} (0.11-0.21)	0.36± 0.05 0.37^{a} (0.28-0.44)	**0.016^{KW}***
BA (10^3/mm^3)	0.02 ± 0.00 0.02 (0.01-0.02)	0.03 ± 0.00 0.03 (0.02-0.04)	0.02 ± 0.00 0.02 (0.01-0.02)	0.218^{A}
LY (10^3/mm^3)	3.86 ± 0.19 3.94 (3.56-4.12)	3.88 ± 0.23 3.71 (3.59-3.95)	4.45 ± 0.42 4.27 (3.69-5.41)	0.474^{wl}

MO ($10^3/mm^3$)	0.13 ± 0.02 0.15 (0.09-0.15)	0.11 ± 0.02 0.13 (0.07-0.14)	0.12 ± 0.02 0.13 (0.09-0.14)	0.161^{A}
RBC ($10^6/mm^3$)	11.77 ± 0.47 12.24 (10.97-12.73)	11.73 ± 0.30 11.92 (11.10-12.34)	11.52 ± 0.42 11.53 (10.96-12.32)	0.891^{A}
HG (g/dl)	12.94 ± 0.42 13.20 (12.30-13.55)	12.56 ± 0.30 12.30 (11.90-13.20)	12.39 ± 0.37 12.10 (11.60-12.80)	0.557^{A}
HCT (%)	43.46 ± 1.11 44.30 (42.05-44.90)	41.84 ± 0.83 41.50 (41.05-43.55)	43.86 ± 1.33 43.80 (41.85-45.50)	0.414^{A}
MCV	37.12 ± 1.11 36.36 (35.68-37.43)	35.78 ± 1.05 35.16 (33.72-37.38)	38.27 ± 1.28 38.50 (36.66-40.46)	0.332^{A}
MCH	11.03 ± 0.21 11.06 (10.62-11.41)	10.72 ± 0.18 10.74 (10.56-10.99)	10.79 ± 0.27 10.71 (10.21-11.17)	0.601^{A}
MCHC	29.77 ± 0.51 30.21 (29.62-30.53)	30.03 ± 0.60 29.79 (28.99-30.80)	28.37 ± 1.09 27.82 (26.06-30.59)	0.447^{KW}