VETBIONET

Veterinary Biocontained facility Network for excellence in animal infectiology research and experimentation

Deliverable D7.5

Optimised pathogenesis and immunity models in genetic context for BSL3 pathogens

Due date of deliverable: M60

Actual submission date: M72

Start date of the project: March 1st, 2017 **Duration:** 72 months

Organisation name of lead contractor: FLI

Revision: V1

Dissemination level	
Public	**x**
Confidential, only for members of the consortium (including Commission Services)	
Classified, as referred to in Commission Decision 2001/844/EC	

Table of contents

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

1. Summary

The main objective of this deliverable is the development and/or optimisation of live animal models for pathogenesis and/or host immunity studies, in four livestock species (pigs, sheep, cattle, alpaca/llama and chicken).

Animal models have been developed for Peste des Petits Ruminants virus (PPRV), MERS-CoV, Avian influenza virus (AIV) and tick-borne Orthonairoviruses, including Nairobi sheep disease virus (NSDV), Dugbe virus (DUGV) and Hazara virus (HAZ).

2. Rationale

Sheep PPRV infection models.

To optimise a PPRV infection model in sheep three different trials were carried out using different breeds. The viraemia profiles and the immunological parameters were measured and the procedures optimised. Furthermore, clinical signs and post-mortem lesions developed by PPRV infection were define and a clinical score quantification developed. The different samples collected (blood, PBLS, sera, tissues,etc) from these trials were stored and are available for potential future analysed by TNA or VetBioNet users.In addition to these analyses and to optimise this infection model for immunological purpouses, the IFN type I genes expression was studied. This provide relevan immunological information that can be used for design novel vaccines or evaluate the efficacy of vaccines against PPRV.

Alpaca/Llama MERS-CoV infection models.

To study immune responses in alpaca or llamas infected with MERS-CoV, transcription of 51 immune response genes was analyzed mediating a microfluidic qPCR assay (Fluidigm technology). In alpacas, the nasal mucosa and submucosa were analyzed individually by laser microdissection of methacarn fixed tissues while material from trachea and lungs was collected by scrapping thin slices of tissues fixed in glass slides. Alveolar macrophages and lymph-node cells of llamas were obtained from llamas previously infected by MERS-CoV and restimulated in vitro with infectious virus.

Sheep and cattle Orthonairovirus infection models.

Ruminants can play a major role in the natural transmission cycle of different orthonairovirus as Hazara (HAZV), Dugbe (DUGV), and Nairobi sheep disease (NSDV), all of them tick-borne arboviruses. Therefore, the development of experimentally infection models in sheep and

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

cattle could provide a better understanding of these important zoonosis. Furthermore, ruminants are considered as suitable indicator animals for serological monitoring studies to assess the risk for human infections. So here the infection models have been optimized together with the development of serological assays that allow the discrimination between different Orthonairoviruses.

Avian influenza germ-free chicken.

Emerging experimental and epidemiological evidence in mammalian species underscores a crucial crosstalk between the gut-microbiota (GM) and the lungs, dubbed gut–lung axis. The importance of the gut–lung axis has become more evident following the identification of GM-derived metabolites, such as short chain fatty acids - SCFA, as key mediators for setting the tone of the peripheral immune system. However, the gut-lung axis was not yet studied in poultry species, such as the chicken. We surmise that the chicken GM and its metabolites would be essential to ensure an adequate immune system development, notably at the innate arm of the immune system at peripheral sites. GM-derived metabolites, such as SCFA, may leave the chicken gut and reach peripheral organs (the gut-lung axis), where they would play a role in anti-pathogen immunity. Our research activities in WP7 Task 7.1 addressed the following three questions: (1) How does the GM regulate immune system functioning along the gut-lung axis in the chicken? (2) Do GM-derived metabolites reach peripheral organs and exert immunomodulating functions in chicken cells? (3) How do the studied mechanisms relate to resistance or susceptibility to avian influenza virus infection in the chicken?

3. Teams involved

INIA, IRTA, INRAE and FLI

4. Introduction

4.1 INIA (PPRV, SHEEP)

Peste des petits ruminants virus (PPRV) is a Morbillivirus that primarily affects domestic and wild ruminants. PPRV, the causal agent of peste des petits ruminants (PPR), a notifiable disease to the World Organization for Animal Health (OIE), produces considerable economic losses, predominantly in developing countries where livestock are one of the main economic resources. PPRV, like other morbilliviruses, induces immune suppression during the acute phase of the disease, which favors the establishment and aggravates the progression of secondary infections (1). Protective current PPRV vaccines are extensively used in countries

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

where PPRV is endemic (2). They are based on attenuation of live PPRV strains (Nigeria 75/1, Sungri'96, Arasur'87, and Coimbatore'97) by serial passage in Vero cells. The development of new vaccines together with DIVA test is quite relevant and requires the standardized infectious model that allow the efficacy assessment.

4.2 IRTA (MERs-CoV, ALPACA/llama)

Camelids are reservoirs for MERS-CoV but suffer a mild disease characterized by discrete nasal discharges and minimal focal microscopic lesions in the mucosa of the upper respiratory tract. Under experimental conditions, infections are resolved within 7 to 11 days leading to a solid antibody response. Despite high viral replication in nasal epithelial cells during the first two to three days of infection, the virus is completely cleared from the mucosa within few days. This fact suggests that potent innate and adaptive immune responses take place. To characterize these responses, 51 selected genes encompassing the main immune pathways were analyzed at the transcriptomic level: (1) in the upper (URT) and lower (LRT) respiratory tracts of alpacas undergoing an infection with clade A (EMC/2012) or B MERS-CoV (Qatar15/2015 and Jordan-1/2015) strains and (2) in alveolar macrophages and lymph-node cells of llamas collected 3 weeks post infection with the Qatar15/2015 or Egypt/2013 (clade C) MERS-CoV strains.

4.3 FLI (SHEEP, CATTLE)

Hazara orthonairovirus (HAZV) is a tick-borne arbovirus closely related to Crimean–Congo hemorrhagic fever orthonairovirus (CCHFV). Whereas CCHFV is a biosafety level (BSL) 4 agent, HAZV is classified as BSL 2, as it is not known to cause any disease in humans. Belonging to the same serogroup as CCHFV, HAZV might act as a model which can provide a better understanding of this important zoonosis. Furthermore, the serological relatedness may cause diagnostic problems if antibodies against HAZV interfere with current CCHFV serological assays. Therefore, sheep and cattle—important natural hosts for CCHFV—were experimentally infected with HAZV to prove their susceptibility and evaluate potential antibody cross-reactivities.

Dugbe orthonairovirus (DUGV) is a tick-borne arbovirus within the order Bunyavirales. DUGV was first isolated in Nigeria, but virus isolations in ten further African countries indicate that DUGV is widespread throughout Africa. Humans can suffer from a mild febrile illness, hence, DUGV is classified as a biosafety level (BSL) 3 agent. In contrast, no disease has been described in animals, albeit serological evidence exists that ruminants are common hosts and

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

may play an important role in the transmission cycle of this neglected arbovirus. In this study, young sheep and calves were experimentally inoculated with DUGV in order to determine their susceptibility and to study the course of infection.

Nairobi sheep disease orthonairovirus (NSDV) is a zoonotic tick-borne arbovirus, which causes severe gastroenteritis in small ruminants. To date, the virus is prevalent in East Africa and Asia. However, due to climate change, including the spread of transmitting tick vectors and increased animal movements, it is likely that the distribution range of NSDV is enlarging. In this project, sheep and cattle (hitherto classified as resistant to NSDV) were experimentally infected with NSDV for a comparative study of the species-specific pathogenesis.

4.4 INRAE (CHICKEN)

In poultry, the gut microbiota - GM is critical for the maintenance of intestinal homeostasis, whole body metabolism, immune system maturation, preservation of barrier integrity and pathogen resistance. Modern selection programs implemented by industrial chicken breeders favored high performance traits, such as rapid broiler growth, prolific egg production and efficient feed conversion. However, selection for a single trait may also affect other traits, with potentially negative effects on GM development and immune system maturation and functioning. Moreover, current poultry production systems have chicks hatched in very clean environments, where GM development is minimized by the absence of contact to the hens, together with intensive egg surface cleaning and disinfection procedures. Therefore, the establishment of a well-balanced GM in early life is believed to be hampered in industrial poultry production systems, with negative consequences to poultry's health in later life.

The development of the immune system goes hand in hand with the acquisition and maintenance of a complex GM. The GM promotes and calibrates multiple aspects of the immune system maturation and functioning via the release and production of various molecular motifs and metabolites, respectively. These molecules not only modulate local (gut) immunity, but also exercise remote functions on peripheral organs, including the lungs: a phenomenon referred to as the gut–lung axis. In mammals, changes in the constituents of the GM are linked to increased susceptibility to airway diseases and infections. Short chain fatty acids (SCFA) are the most abundant GM-derived metabolites in the gastrointestinal tract of mammalian and poultry species. They participate actively in modulating immune responses along the gut-lung axis. In mammals, there is a strong concentration gradient for each SCFA (acetate, butyrate and propionate) steadily decreasing from the gut lumen to peripheral organs, thus leading to differential cell and tissue exposures to SCFA. However, the significance of this biological

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

gradient for host physiology and immune competence is still poorly understood and completely unexplored in poultry.

Influenza infection has been shown to cause dysbiosis in mammalian species (via type I interferon-mediated effects on the GM composition). Viral infections such as those caused by low pathogenic avian Influenza viruses (LPAIV) are relatively prevalent in European poultry flocks and some strains are known to infect humans (e.g. H7 and H9). Although LPAIV may favor secondary infections (bacterial, fungal), LPAIV itself generally cause subclinical disease and have limited impact on poultry performance. However, dysbiosis due to an unbalanced GM composition and altered immune system development and functioning could favor respiratory and/or intestinal LPAIV infection with increased severity and negative consequences to the poultry production sector. This two-way dialogue was never addressed in poultry. Exploring its complexity may provide important insights into the role of the GM in determining chicken resistance to economically important viral pathogens.

5. Results

5.1 PPRV infection models in sheep (INIA)

Experimental infection of different sheep breed showed that Churra breed are more susceptible to Peste des Petits Ruminants virus (PPRV) infection. The animals were inoculated by intravenous or intravenous plus nasal route. Sheep did not show significant pyrexia neither clinical signs. However, viremia and seroconversion were detected. The post-mortem examination showed congestion along the caecum, colon and rectum, and more remarkable in the ileo-cecal valve. Pulmonary congestion was observed just in some animals, with fibrin depositions in the pleura surface. All animal developed pyrexia (40.5-41.5ºC) and clinical signs (mild depression, mucopurulent nasal discharge, red conjunctives, loss of appetite and dull look of wood). We found that IFN-g decreased by 20-fold starting at day 5pi and kept low until day 13pi. RIG-I showed an expression pattern similar to IFN-g. Other genes related to type I IFN responses such as MXA, ISG15 and IFIT3 showed an increased expression by day 5 pi but a sharp decline starting at day 7pi until the last day tested. Genes related to type II IFN responses, IFN-g and CXCL10 showed a decreased expression by day 7 pi, according to the immunosuppression observed in PPRV infected animals by day 7 pi, and did not recovered the expression by day 13 pi.

In regard of 3Rs rule, this in vivo infection model was connected to the development of an ex vivo model for the study of PPRV infection. PPRV-infected MoDCs decreased costimulatory molecules as well as MHC-II on their surface. This lack of stimulatory molecules correlated with the lack of presenting antigen observed in MLR assay. The transcriptomic analyses showed 453 up-regulated and 179-down-regulated genes on PPRV-infected MoDCs

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

compared to mock-infected counterparts. KEGG analysis revealed 31 different up-regulated pathways, including some involving autophagy or mitophagy mechanisms and signalling pathways related to viral infection responses like TNF, mTOR or IL-17 pathways, among others. Interestingly, PPRV-infected MoDCs showed a prominent induction of cell death receptors. Taken all together, the observed prominent expression of apoptosis-induced death receptors, together with the strong expression of T-cell attracting chemokines may contribute to the pronounce lymphopenia observed during PPRV infection in vivo.

5.2 MERS-CoV infection models in alpaca/llama (IRTA)

Primer pairs to amplify genes involved in immunity were validated in camelid peripheral blood mononuclear cells activated with non-specific stimulators. A prepublication has been posted in BioRxiv (doi.org/10.1101/2023.01.15.524100) and will be submitted to a specialized journal. MERS-CoV experimentally infected alpacas, independently of the strain used, mount an efficient innate immune response characterized by an early production of interferons (IFN) only detected in the mucosa of the URT. As opposed, antiviral IFN stimulated genes (ISG) are readily produced in both the URT (including the submucosa of the URT) and LRT. Pro-inflammatory cytokines are kept to nearly basic levels or even downregulated, probably due to the action of the anti-inflammatory cytokine IL-10 and type III IFN. In lungs, a moderate recruitment of macrophages is observed and this correlates with the induction of chemokines (CCl2 and CCl3). Innate immune responses provoked by MERS-CoV clade B strains are of higher intensity than those induced by the clade A strain EMC/2012. These results have been published in PLOS pathogens (DOI: 10.1371/journal.ppat.1009229) and Emerging Microbes and Infection (DOI: 10.1080/22221751.2021.2019559) they also give a rational explanation on why MERS-CoV clade B strains have replaced early epidemic clade A strains in the Arabian Peninsula by acquiring mutations influencing viral replication in the nasal mucosa. The role of alveolar macrophages in clearing MERS-CoV particles in lungs has been established in llamas. These studies performed in vitro indicated that viral particles are engulfed by macrophages obtained by broncho alveolar lavages and subsequently destroyed within vesicles/vacuoles as assessed by electron microscopy examination. Lymph-node cells restimulated with infectious virus (clade B and C strains respectively) mounted within 24 to 48 hours innate and adaptive Th1 immunes responses without viral replication. Type I, II (IFN gamma) and III (IFN lambda 3) were strongly induced as ISG. However, none of the pro-inflammatory cytokines or inflammasome components were significantly upregulated at the transcriptomic level. The clade B strain (Qatar-15/2015) provoked more intense immune responses than the clade C (Egypt/2013) strain. These results will be shortly submitted to

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

publication in specialized journals. Two PhD thesis were successfully defended at the Universidad Autonoma de Barcelona based on the work described above.

5.3 Orthonairovirus infection models (FLI)

Hazara orthonairovirus (HAZV) is a virus closely related to Crimean-Congo hemorrhagic fever (CCHFV) since HAZV is a BSL2 agent it can be a good model for study of these zoonotic viruses. Sheep and cattle were experimentally infected with HAZC to prove their susceptibility and evaluate potential antibody cross-reactivities. The results showed that neither sheep nor cattle are susceptible to experimental infections with HAZV, so this infection can be clearly differentiated from CCHFV infections.

Young sheep and calves were experimentally inoculated with Dugbe orthonairovirus (DUGV) to determine their susceptibility and study the course of the infection. Following subcutaneous inoculation none of the animals developed clinical signs or viremia, but all the ruminants seroconverted.

Nairobi sheep disease orthonairovirus (NSDV) infection of sheep developed a course of infection with severe clinical signs, including hemorrhagic diarrhea. In contrast, infection of cattle with virus was only subclinical. However, all the infected animals, sheep and cattle showed seroconversion suggesting that both species are susceptible for NSDV.

5.4 Avian Influenza infection model in chicken (INRAE)

Using the germ-free (GF) chicken model, we were already able to validate, for the first time, the functional existence of a gut-lung axis in an inbred White leghorn chicken line **(INRAE Figure 1)**. Conventional or GF birds were sacrificed at 3 weeks of age (when immunocompetence can be reached with the decrease of maternal antibodies) and lungs were recovered for the quantification of SCFA and for assessing the expression of selected innate immune genes between the two conditions. Germ-free chickens presented a growth rate similar to conventional animals raised under the same housing conditions and same type of diet **(INRAE Figure 1 A)**. As expected, conventional animals presented average concentrations of SCFA in their caecal contents, as measured by proton nuclear magnetic resonance (^{1}H-NMR). However, most SCFA are undetectable in the caecal contents of GF animals **(INRAE Figure 1 B)**. The only exception is acetate, which is reduced to half of the concentration found in conventional animals. The presence of acetate is believed to be of dietary origin since sterility tests revealed the complete absence of bacteria in germ-free animals. Interestingly, we identified for the first time the presence of SCFA in the lung tissue of conventional chickens **(INRAE Figure 1 C)**, although in concentrations significantly lower as compared to those found in caecal contents. These metabolites are absent (or below

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

detection threshold) in the lungs of germ-free chickens, therefore validating our hypothesis that metabolites from bacterial fermentation, possibly from the GM (since healthy lungs will harbour no fermentation processes), may reach peripheral organs.

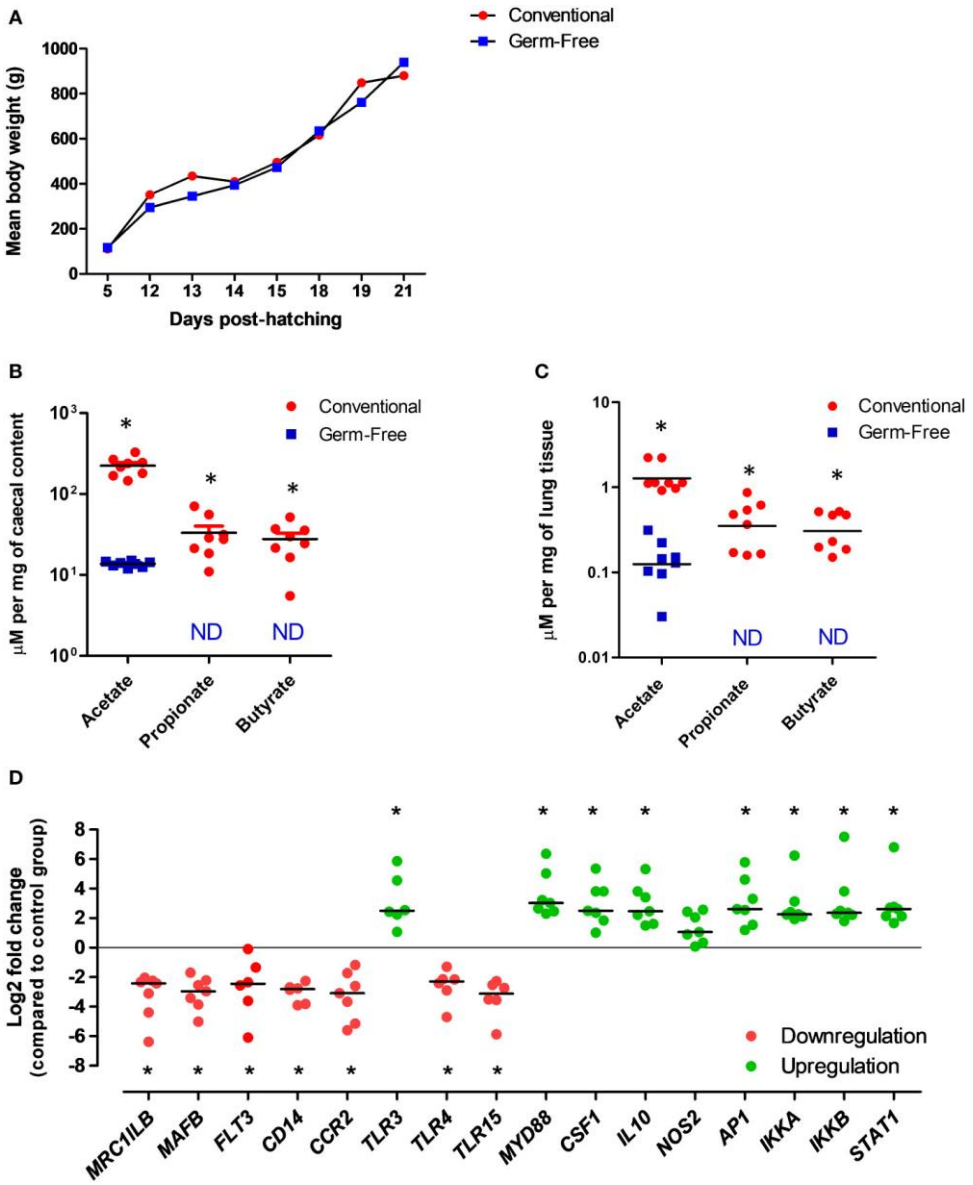
Finally, we carried out a gene expression analysis in lung samples from conventional and germ-free animals to test the hypothesis that the GM and its metabolites may regulate immune mechanisms at the respiratory mucosa. Targeting selected genes related to immune cell development and function, as well as pathogen detection, we observed that different patterns of gene expression can be observed in the lungs in the absence of a GM (INRAE Figure 1 D). Certain genes linked to mononuclear phagocytic cell functions (*MRCL1B, MAFB, CD14, CCR2*), bacterial (*TLR4*) and yeast (*TLR15*) molecular pattern recognition are significantly downregulated in the lungs of germ-free chickens. On the other hand, genes linked to viral molecular pattern recognition (*TLR3*), key immune transcription factors (AP1, IKKA and IKKB, STAT1), regulatory cytokines (*CSF1, IL10*), and the gene coding for the universal TLR adapter protein MyD88 (*MYD88*) are significantly upregulated **(INRAE Figure 1 D)**. These data indicate that at least cells of the mononuclear phagocyte system and TLRs in the respiratory mucosa of chickens are likely to be regulated by molecular signals coming from the GM.

Next, using the same GF chicken model, we assessed whether the microbiota would influence the orchestration of antiviral immune responses using a model of LPAIV infection with a highly virulent H7N1 strain. GF or conventional chickens at 21 days of age (immunocompetent birds) were infected by the intra-tracheal with the H7N1 LPAIV strain. Between days 1 and 3 post-infection, lung samples were collected for viral load assessment and immune responses analysis. There were no clear differences in clinical signs related to the disease between the 2 groups, both groups consistently developed mild disease symptoms (short of breath, prostration) from day 1 p.i. onwards. As for the viral load in the lung tissue, no differences were observed between the two groups, with both possessing sustained viral load from day 1 to 3 p.i. **(INRAE Figure 2A)**. To ask whether tissue response to LPAIV infection were pathologically similar as well, we observed that cumulated score for macroscopic lesions found in the lungs during necropsy are similar in both groups, with lesions being more apparent from day 2 p.i. onwards **(INRAE Figure 2B)**. Overall, the absence of a GM from birth does not interfere with viral replication and the clinical course of this H7N1 LPAIV model of infection. We next asked whether the expression of genes of antiviral immunity are differently regulated in both groups during infection. For most genes related to the interferon response, such as *IFNA* and *IFNB*, or ISGs such as *MX1*, no differences were observed. However, for a regulatory ISG with ubiquitin functions, *OASL*, its expression is significantly altered in the lung of GF chickens at day 3 p.i. **(INRAE Figure 2D).** The same was true for the gene coding for the inflammasome-related cytokine IL1-beta. **(INRAE Figure 2D).** Other analyses are still being carried out but,

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

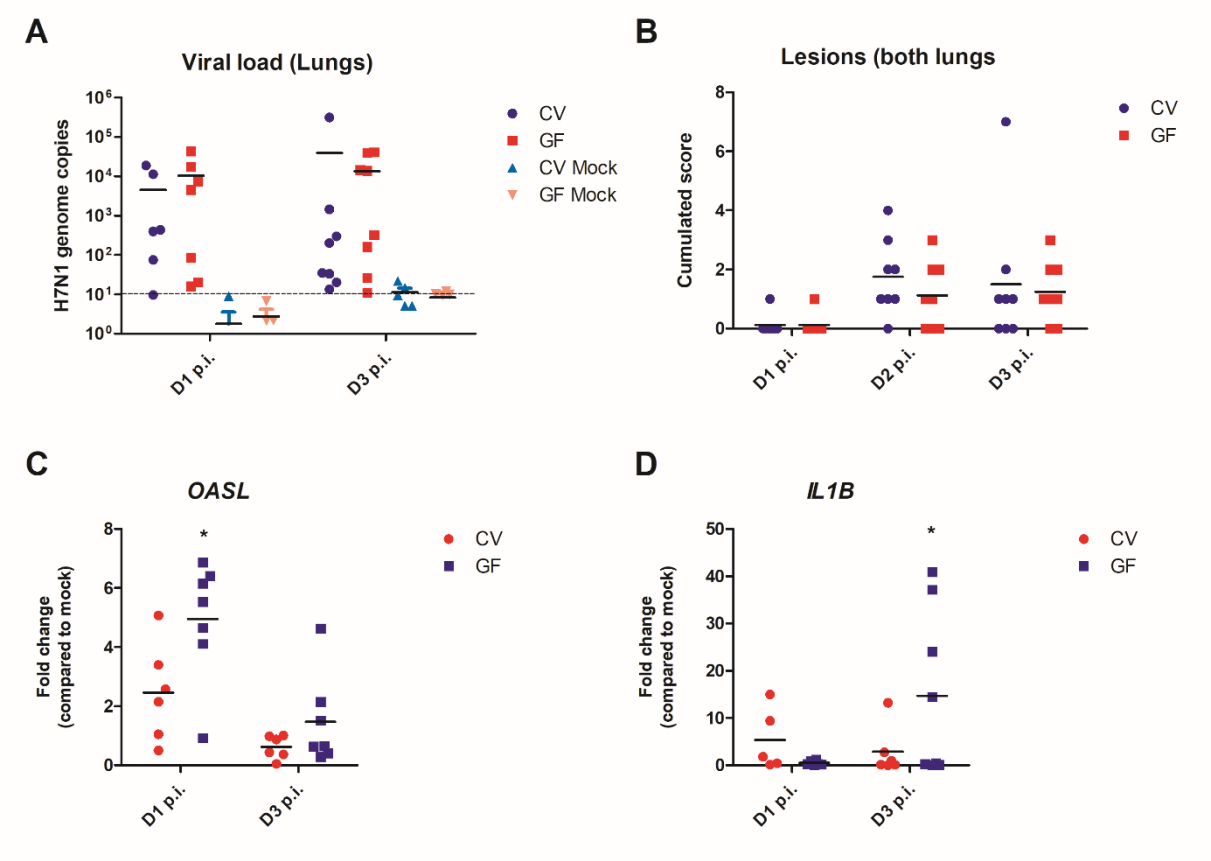
overall, the absence of a GM has no pivotal role in antiviral responses during this specific model of LPAIV infection. However, since GF show different regulation for selected innate immune genes during infection, this may alter the course of disease for pathogens other for this H7N1 LPAIV, which would merit considerable attention.

INRAE Figure 1



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

INRAE Figure 2



6. Conclusions

6.1 PPRV infection models in sheep (INIA)

Different sheep breeds show different susceptibility to PPRV infection. The model in Churra breed has been optimized and has allowed the evaluation of a novel vaccine against PPRV developed by a TNA user from Parma University (Dr.G.Donofrio). The procedures for analyses of immunological parameters including humoral and cellular response have been developed, as well as the optimization of virus detection after animal infection in different samples.

https://doi.org/10.3389/fimmu.2021.705539

6.2 MERS-CoV in alpaca/llama (IRTA)

All camelid species are susceptible to MERS-CoV. So far, clade B strains (lineage 5) are the most infectious leading to pronounced innate and Th1 type immune responses. Interferon responses are timely induced and limited to the mucosa of the URT and lymph-node cells, they are accompanied by a transcriptional upregulation of ISG. Strikingly, and despite high viral loads observed in the nasal mucosa of infected animals, proinflammatory cytokines are at the

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

best weakly induced or kept at basal levels. Alveolar macrophages (known to be the drivers of a cytokine storm in humans suffering MERS, SARS or COVID-19) participate in clearing the virus without engagement of inflammatory or canonical innate anti-viral pathways. These results highlight the existence of host factors in animal reservoirs able to limit inflammation without compromising effective innate and adaptive immune responses.

https://doi.org/10.1371/journal.ppat.1009229

6.3 Tick-borne Orthonairoviruses infection models (FLI)

Successful development of specific animal models and diagnostic tools for tick-borne Orthonairoviruses (CCHFV, NSDV, DUGV, HAZ; BSL2,3&4). Pathogenesis and immunization studies in these models.

https://www.mdpi.com/1999-4915/13/7/1398

https://www.mdpi.com/2076-2607/9/7/1493/htm

https://www.mdpi.com/1999-4915/13/7/1250

https://www.mdpi.com/1999-4915/13/3/372

https://www.mdpi.com/2076-2607/8/12/1927)

6.4 Avian influenza infection in germ-free chicken (INRAE)

The highly regulatory tone of the neonate immune system and the action of early-life gut commensals in the development and training of this system lead to the establishment of a durable and homeostatic host/commensal relationship beyond the gastrointestinal tract, which led to the discovery of very complex physiological dialogues such as the gut-lung axis. SCFA, key metabolites involved in the regulatory functions of the GM within the gut and the peripheral system, has been show as pivotal signaling molecules in bridging gut health and resilience to pathogens in both the respiratory and gastrointestinal tracts. Nevertheless, the importance of early gut colonization for the chicken was largely neglected despite the particular situation in modern poultry farming where chicks are prevented from acquiring a well-balanced and essentially healthy GM from the hen. Moreover, only recently new technologies and tools have been developed and used for a better understanding of the particularities of the chicken mucosal immune system. Thus, it is becoming increasingly clear that in-depth analyses of the impact of the GM on immune system development and resilience to environmental stressors and pathogenic challenge will generate new knowledge and pave the way for the development of novel GM modulation strategies (e.g. GM transfer and tailored feed formulations) to be applied in poultry farming, thereby mutually benefitting animal health and welfare and

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

consumer safety. Our data presented here point to the existence of a functional gut-lung axis in chickens, which must now be further explored in greater details. In a nutshell, the gut-lung axis starts to become a fully exploitable concept in the field of poultry sciences.

Uncovering the core principles of the gut-lung axis to enhance innate immunity in the chicken (Front. Immunol., 04 October 2022):
https://www.frontiersin.org/articles/10.3389/fimmu.2022.956670/full

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014