INFLUENZA A VIRUS INFECTION OF MURINE ALVEOLAR MACROPHAGES

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DECLARATION

I declare that all work included in this dissertation has been completed by myself, except where acknowledged in the main text.

Jairo Israel Tobar Cueva
ABSTRACT

Alveolar macrophages (AM) are one of the most abundant cell types in the airway space, but their role after IAV infection is not fully understood. Although these cells can exhibit a high plasticity, it is suggested that in steady state, they exhibit an ant-inflammatory phenotype. Since the activation state at the moment of infection would affect the macrophage response, we wanted to investigate IAV infection in AM as close as possible to its normal physiological state. Thus, we modeled an *ex vivo* infection by extracting AM from 129 Sv/Ev, IFNγR<sup>−/−</sup>, IFNα/βR<sup>−/−</sup> mice in the 129 Sv/Ev background, and immediately infected them with a mouse adapted laboratory strain of influenza A virus (A/WSN/33). After 2, 6, 12 and 24 hours post-infection (h.pi), cell lysates were prepared and samples were analysed by RT-qPCR, western blot and plaque assay, in order to estimate virus replication, release of infectious particles and cellular responses. We confirmed that IAV is able to infect alveolar macrophages, and the levels of PB1 RNA are increased after 2 h.pi in all of the AM groups, reaching a peak at 12 h.pi. Virus release was seen in all of the AM groups, however, it was low in 129 Sv/Ev and IFNγR<sup>−/−</sup> groups, and was further reduced after 12 h.pi. Conversely, IFNα/βR<sup>−/−</sup> AM showed an increase in virus titre at 12 h.pi, reaching a peak at 24 h.pi. Finally, where 129 Sv/Ev and IFNα/βR<sup>−/−</sup> groups showed an upregulation of Tnf-α after 6 h.pi, the IFNγR<sup>−/−</sup> group did not show a similar increase. These results showed that although 129 Sv/Ev and IFNγR<sup>−/−</sup> AM have a similar pattern in PB1 RNA levels and virus release, the apparent phenotype induced by the absence of IFN-γ downstream signaling diminishes the pro-inflammatory response. Furthermore, we confirmed that the absence of IFN-α/β stimulation in these cells, which is one the main cellular antiviral strategies, is associated with an increase of PB1 RNA levels, higher IAV productive replication and a higher pro-inflammatory response.

**Key words:**

Alveolar macrophages, Influenza A virus, A/WSN/33, 129 Sv/Ev, IFNγR<sup>−/−</sup>, IFNα/βR<sup>−/−</sup>.
INTRODUCTION

THE INFLUENZA VIRUS

Influenza type A, along with types B and C, are enveloped virus members of the family Orthomyxoviridae, with a negative sense single stranded segmented RNA genome and are widely recognized for causing respiratory disease (Flint & American Society for Microbiology, 2009). The Influenza A virus (IAV) is sub-classified according to the type of glycoproteins hemagglutinin (HA) and neuraminidase (NA) present on the virus surface. Currently 18 H and 11 N have been identified, which give a possibility for 198 subtype combinations (CDC, 2015). This high variability is a continuous cause for concern in public health, since different mutations and combinations of surface antigens have been associated with disastrous pandemics across human history. Amongst the most important were the Spanish flu (1918) caused by an H1N1 subtype, the Asian (H2N2) identified in 1957 and the Korean subtype H3N2 (1968) (Kilbourne, 2006). Behind these pandemics, cross-species transmission has played an important role in the virus circulation and spread. Wild aquatic birds, which are natural reservoirs of this virus, can spread it to domestic animals, such as birds or pigs. These animals then function as mixing vessels for the virus reassortment and mutation, enabling its spread and infection across the human population (Bengis et al., 2004). According to the World Health Organization, one of the main risk factors for the emergence of a human epidemic is the direct exposure to infected domestic birds (WHO, 2016). Just this year, the World Organization for Animal Health (OIE) has reported several outbreaks of avian influenza in numerous farm birds across Asia, Europe, Africa and North America (OIE, 2016).

INFLUENZA LIFE CYCLE

The influenza virus lacks essential cellular mechanisms to self replicate. Hence, it needs to get inside of cells in order to use their replication components to its benefit. Once the virus is in close proximity to host cells, it binds to the cell membrane by hydrogen bonds or Van Der Waals contacts between the viral hemagglutinin and sialic acids terminations, linked in α(2,3) or α(2,6), to galactose residues present on glycoproteins or glycolipids of cell membrane receptors (Skehel & Wiley, 2000; Weis et al., 1988). After absorption, the virus is internalized in endosomes, where changes
in the internal pH environment induce fusion of the viral and endosomal membrane, releasing the viral ribonucleoprotein complexes (vRNP) to the cytoplasm (Palese, P. & Shaw, 2007). These vRNP are the viral components that contain the different genome segments. The influenza A virus has a genome composed of 8 segments, encoding for at least 11 different proteins with specific functions in the virus replicative cycle (Jackson, Elderfield, & Barclay, 2011). Structurally, each vRNP consists of a specific viral RNA (vRNA) coated by the nucleoprotein (NP), attached to the RNA-dependent RNA-polymerase (RdRp) (Flint & American Society for Microbiology., 2009; Kapoor & Dhama, 2014). Once in the cytoplasm, these vRNP are translocated to the nucleus of the cell, where the vRNA is used as a template by RdRp to initiate the viral replication and transcription. The RdRp is a heterotrimer composed of three different viral proteins; polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA), each one having a specific role in the viral replication cycle (Fodor, 2013). The RdRp start the transcription of viral capped and polyadenylated mRNA, which are transported to the host ribosomes to start the translation of viral proteins (Tekamp & Penhoet, 1980). Another important function of RdRP is the replication of vRNA to a positive sense complementary RNA (cRNA), which will be further used to produced more negative sense vRNA. Besides being used as a template for synthesis of more viral mRNA, these vRNA will be assembled into new vRNP, to be transported to the cell membrane which, along with other viral structural components, will be assembled into new budding viruses (Flint & American Society for Microbiology., 2009; Kapoor & Dhama, 2014)

Influenza type A has a wide host range, able to infect several species including birds, humans, pigs, horses and marine mammals (Bengis et al., 2004). However, the outcome of an infection depends mostly on the antigenic variation of a specific strain and the host immunological state. One of the favorable factors for antigenic variation is the error-prone function of the viral RdRp, because it does not have the same proofreading mechanism as eukaryotic cells (Shirogane, Watanabe, & Yanagi, 2013). This apparently imperfect replication mechanism, along with the short generation time, lead to a high mutation rate, where changes as simple as a single nucleotide polymorphisms in a single gene could have an impact on the tropism of a determined
strain (Cauldwell, Long, Moncorgé, & Barclay, 2014; Palese, 2004). For instance, the tropism variability of different influenza subtypes is highly related to site-specific mutations in the receptor-binding region of hemagglutinin, which modifies the affinity to either α2,3 or α2,5 sialic acid linkages (Skehel & Wiley, 2000). Although the proportions of either sialic acid linkage type are related to the host species, both types of linkages are found in the membrane receptors of different cells across the body (Rosztoczy, Sweet, Toms, & Smith, 1975; Suzuki et al., 2000). However, low and high pathogenic strains have a strong tropism for cells of the upper and lower respiratory epithelium, respectively, causing cellular damage and necrosis (Kuiken, Riteau, Fouchier, & Rimmelzwaan, 2012).

**HOST INNATE IMMUNE RESPONSE TO INFLUENZA VIRUS**

In many instances, there are cellular mechanisms in place that function as sensors for the recognition of pathogen associated molecular patterns (PAMP), like the RNA genome of influenza virus. These sensors are cell surface or intracellular receptors, such as toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I) (García-Sastre, 2011). In the first case, double stranded and single stranded RNA are detected by TLR-3 or TLR7/8, respectively, present in endosomal compartments (Iwasaki & Pillai, 2014). On the other hand, RIG-I binds viral RNA free in the cytoplasm, by recognizing unique motifs like 5’- triphosphate of viral sequences (Iwasaki & Pillai, 2014; Kato et al., 2006), which in the case of the influenza virus, are found in both cRNA and vRNA (Fodor, 2013). These mechanisms activate several pathways, including an increment in recruitment of transcriptional factors, such as nuclear factor kappa beta (NF-κB) and Interferon regulatory factors 3R4F3/3R7. These factors will increase the expression of cytokines and chemokines, creating an antiviral environment, leading to a generalized activation of the immune system (Herold, Becker, Ridge, & Budinger, 2015; Hoffman, Schneider, & Rice, 2015). Among these factors, the interferon family is one of the most important cytokines in antiviral responses (Hoffmann et al., 2015). These cytokines are released to the extracellular space and, by autocrine and paracrine interactions with neighboring cells, induce a series of downstream pathways, leading to an increase in the transcription of antiviral genes (Randall, Goodbourn, & Richard Randall, 2008).
The interferon family and their receptors

Interferons are a family of cytokines with diverse biological functions, among them antiviral, anticarcinogens and immunomodulatory (Pestka, 1987). This family has been classified in three types. Type I interferon consists of nine classes, including IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-ω, IFN-κ, IFN-τ, IFN-ν and IFN-ζ. However, IFN-α/β are the ones mainly induced in response to viral antigens (Randall et al., 2008). On the other hand, Type II interferon is only comprised of IFN-γ and type III include IFN-λ1, IFN-λ2 and IFN-λ3 (De Weerd & Nguyen, 2012).

Interferons produce their biological effect by interaction with specific transmembrane receptors; all type I interferons produce their action by binding to a common receptor, which is different than type II interferon receptors. The type II receptor (IFNγR) is comprised of 2 ligand-binding and signal transducer subunits chains. In a similar way, interferon Type-I receptor (IFNα/βR) is a heterodimer formed by the union of subunits IFNα/βR-1 and IFNα/βR-2 (De Weerd & Nguyen, 2012; Pestka, Krause, & Walter, 2004). Although both interferon types have unique transmembrane receptors, in both cases interactions within their receptor subunits lead to the internalization of the ligand and transduction of a biological response through a common pathway, the janus activated kinase (JAK)/signal transducers and activators of transcription signaling (STAT) Pestka et al., 2004). Typically, this interaction promotes the tyrosine phosphorylation of several STAT proteins with a subsequent formation of complexes. These complexes are translocated to the nucleus to bind specific DNA motifs associated with promoter regions of interferon stimulated genes (ISG) (Darnell, Kerr, & Stark, 1994). Nevertheless, each interferon type also induces different molecular interactions as part of complementary cascades involving CRK proteins, phosphatidylinositol 3-kinase (PI3K), mitogen activated protein kinase p38 and mammalian target of rapamycin (MTOR) signaling, which together produce different interferon type biological responses, through an increase in the transcription of hundreds of ISG (Platanias, 2005).

Interferon stimulated genes

The main outcome of the IFN response is the activation of diverse pathways leading to the stimulation of a wide range of genes. While some genes are stimulated by both
type I and II interferon, others are only stimulated by specific type interferons (Platanias, 2005). Consequently, the interaction among these pathways in the antiviral response is still a major subject of research. Furthermore, not all of the viruses are controlled by the same mechanism (Pestka et al., 2004).

Type I and II IFN induce ISGs that restrict the virus replication cycle at different levels. Firstly, at the virus entry, there are ISGs such as IFTM3. This protein is required at basal levels to inhibit viral host receptor binding (Brass et al., 2009). Secondly, the synthesis and trafficking of virus components can be blocked by proteins like Mx, which can reduce the concentrations of virus polymerase transcripts (Pavlovic, Haller, & Staeheli, 1992) or human MxA, preventing the nuclear import of vRNP from the cytoplasm (Xiao, Killip, Staeheli, Randall, & Jackson, 2013). Another example is the interferon-inducible RNA-dependent protein kinase (PKR), which produces its antiviral functions by phosphorylation of key host proteins, like eIF-2α, IRF1 or p53, altering viral translation and induction of apoptosis in infected cells (García et al., 2006). Lastly, there is a group of ISGs that inhibits the budding of new viral particles, such as viperin and tetherin (Wang, Hinson, & Cresswell, 2007; Yondola et al., 2011). However, the aforementioned ISGs only represent one of the most studied groups and are not an exhaustive list. A microarray analysis of susceptible mice infected with PR8 H1N1 showed that 41 different ISG were highly up-regulated after infection (Alberts et al., 2010).

**INFLUENZA VIRUS DYSREGULATION OF THE IMMUNE SYSTEM**

On one side, the activation of the cellular defensive mechanism is beneficial to the host to establish a strong antiviral response. However, some high pathogenic strains, in addition to the primary cytopathogenic effect, will stimulate a disproportionate activation of the innate immune system, characterized by an excessive release of pro-inflammatory cytokines and chemokines, such as TNF-α, Interferon type I/II and several interleukins, causing severe and even lethal effects on the host (Herold et al., 2015; Kuiken et al., 2012). This inflammatory response can be further exacerbated by the presence of bacterial components, like lipopolysaccharide within the alveolar environment, which will increase the expression of potent pro-inflammatory mediators, like TNF-α (Nain et al., 1990; Peschke, Bender, Nain, & Gemsa, 1993).
These can trigger additional inflammatory mediators, like cyclooxygenase-2, leading to a self-sustaining cytokine cascade (Lee et al., 2008), producing an over-reactive and uncontrolled inflammatory response, which may cause a phenomenon known as “cytokine storm”, characterized by high fever, leucopenia, accumulation of oxidative species, cell damage and shock (Beutler & Ceramit, 1989; Herold et al., 2015; Offner et al., 1990). Furthermore, an acute lung injury is induced by an excessive accumulation of nitric oxide (NO) and oxygen radicals (O2-), leading to a secondary pneumonia, which can be aggravated with superinfections caused by opportunistic bacteria (Akaike et al., 1996; Li, Molledo, & Moran, 2012). In vivo studies in macaques infected with an adaptation of the 1918 (H1N1) influenza strain showed acute lung damage with severe edema and accumulation of hemorrhagic exudates in the alveolar space. These lesions were accompanied by an increase of pro-inflammatory cytokines and chemokines, like IL-6, IL-8, CCL2 and CCL5, along with many interferon stimulated genes such as Ifitm, Irf, Mx, Pkr, among the most important (Kobasa et al., 2007). On the other hand, influenza resistant pigs showed a reduced pro-inflammatory signaling mediated by a higher expression of suppressor of cytokine signaling 3 (SOCS3)(Nelli et al., 2012). In the lung environment, respiratory epithelial cells and alveolar macrophages are the main producers of chemokines and cytokines in the early phase of infection (Aldridge et al., 2009). These cells play an important role in the pathogenesis of IAV.

MACROPHAGES: THE CRITICAL PLAYERS DURING INFECTION

Macrophages are one of the body’s most important cellular defense mechanisms against pathogen invasion. These cells can be divided into a number of subpopulations, which can be found in almost every tissue of the body, exhibiting a high plasticity and a wide diversity of functions. These cells show different activation states; among the most important are the M1 or classical pro-inflammatory, which are triggered mainly by exposure to IFN-γ, IL-12 and IL-18. This state is characterized by an increased microbial killing capacity, with high phagocytosis and high production of toxic oxygen species as well as pro-inflammatory markers, like TNF-α, iNOS, IL-6 and IL-1. On the other hand, M2, or alternative activated macrophages, are a broad range population of cells showing anti-inflammatory and tissue repair properties. This phenotype is
primarily induced by IL-4 and IL-13. These cells are recognized as having a high expression of Mannose receptor, MHC II and Arginase-I (Gordon, 2003).

The lung macrophage population in the normal physiological state is comprised of alveolar macrophages (AM), interstitial macrophages, intravascular macrophages and dendritic cells. Within these groups, AM are one of the most abundant types in the lung alveoli (M-L. Lohmann-Matthes, C. Steinmüller, 1994). These cells are under constant regulation by airway epithelial cells and it is believed that at steady state they are induced to express the alternative activated phenotype. However, upon pathogen invasion they are able to adapt in order to contribute to pathogen clearance. (Hussell & Bell, 2014)

Their importance in the innate protection against IAV was demonstrated by studies in knock out mice for GM-CSF (Csf2−/−), which lack alveolar macrophages. These mice showed severe clinical symptoms and fatal respiratory failure after infection (Schneider et al., 2014). Similarly, AM-ablated mice infected with PR8 IAV strain showed a higher spread of the virus within the lungs, along with dramatic weight loss and reduced survival rates (Purnama et al., 2014). However, specific information about the alveolar macrophage response to IAV infection is limited and the available information is under debate, since most of the research has been carried out using different types of macrophages. Seo et al (2004) reported that pig bronchi-alveolar lavage (BAL) derived macrophages infected ex vivo with several human influenza A viruses strains, among them A/Sydney/5/97(H3N2) and A/New Caldedonia/1/99 (H1N1), supported virus replication, with high expression of Tnf-α (Seo, Webby, & Webster, 2004). On the other hand, a comparison of the infection of HPAIV H5N1 between human CD68+ BAL macrophages and macrophages cultured from blood monocytes showed that BAL macrophages had decreased levels of infectivity, with a reduced expression of Tnf-α and an abortive virus replication (van Riel et al., 2011). Although the ability of IAV to infect and productively replicate in alveolar macrophages is not clear, some evidence suggests that highly pathogenic flu strains are more able to productively replicate in these cells, and the level of immune response not only relies on the pathogenicity of the strain but also on the macrophage phenotypic state of activation (Cline, Karlsson, Seufzer, & Schultz-Cherry, 2013;
Nicol & Dutia, 2014; Yu et al., 2011). In a recent study, alternatively activated bone marrow derived macrophages (BMDM) infected with A/WSN/33 IAV showed a higher susceptibility to infection than classically activated BMDM. However, the levels of expression of pro-inflammatory markers were lower than in the classically activated ones, suggesting that the original state of activation could have an important effect on the pathogenesis of the disease (Campbell et al., 2015).

The aim of this research was to investigate the influenza virus infection in AM, determine the ability of AM to support productive virus replication and study the physiological response of different AM phenotypes after infection. We hypothesised that murine alveolar macrophages can be infected by a mouse adapted strain of influenza A virus and, once infected, they will block the virus by limiting its replication and reducing the expression of pro-inflammatory cytokines. AM are one of the most important groups of cells in the protection against not only influenza virus infection, but also many other respiratory pathogens. However, specific studies about influenza A infection in alveolar macrophages are rare and those that exist are inconsistent. Thus, the results of this work increase the understanding of the role of alveolar macrophage in the pathophysiology and control of the IAV infection.
METHODS

MDCK Cell culture
Madin Darby canine kidney cells (MDCK) were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% foetal calf serum, 100U/ml Penicillin/Streptomycin and 2mM L-glutamine (complete media) and cultured until reaching confluence at 37°C in 5% CO₂ before being transferred to fresh flasks. Cell monolayers were passaged by washing twice with warm PBS, followed by treatment with 10 ml of 0.25% Trypsin-EDTA solution at 37°C in 5% CO₂ until detachment of adherent cells from the surface. Then, 10 ml of DMEM-complete media was added to the flask to neutralize the trypsin. Cell suspension was centrifuged at 458 g for 5 minutes. Supernatants were discarded and the cell pellet was resuspended in 10 ml of DMEM-complete media. Cell concentration and viability were determined using a haemocytometer and trypan blue; 5 µl of the cell suspension were added to 45 µl of trypan blue and mixed by vortexing. 10 µl of this solution were placed between a haemocytometer and its coverslip. A cell count was performed and the cell concentration was calculated by multiplying the cell concentration by the dilution factor and the correction factor, derived from the size of the chamber (1 x 10⁴). Once the amount of cells was calculated, new 175T flasks containing 35 ml of DMEM-complete medium were seeded with 2.8 x 10⁴ cells/cm² and incubated at 37°C in 5% CO₂.

A/WSN/33 influenza virus working stock.
MDCK cells were seeded in a T25 flask at a density of 2.8 x 10⁴ cells/cm² and cultured at 37°C in 5% CO₂ until 90 % confluence was reached. The flask was washed twice with warm PBS and the cell monolayer was infected by adding A/WSN/33 influenza A virus (2.75 x 10⁸ PFU/ml) at a multiplicity of infection (MOI) of 0.01 diluted in serum-free DMEM. After incubation of the flask for 1 hour at 37°C in 5% CO₂, the unabsorbed virus was removed by washing the cell monolayer twice with warm PBS. Pre-warmed serum-free DMEM (5 ml) containing N-Acetyl Trypsin (2.5 µg/ml) was added to the flask. Incubation was continued for 48 hours or until cytopathic effect was seen in most of the cell monolayer. Then, culture supernatants were transferred to
20 ml universal tubes and clarified by centrifugation at 1270 g for 5 minutes. Virus samples were aliquoted in 25 µl to 1 ml volumes and stored at -70°C.

**Plaque assays**

MDCK cells were seeded in three six-well tissue culture treated dishes at a density of 1x10^5 cells/cm², maintained in 4 ml of DMEM-complete media, supplemented with 10% fetal calf serum. The cells were incubated at 37°C in 5% CO₂ until reaching over confluence. Then, media was removed from the wells and the cell monolayer was washed twice with pre-warmed PBS. 10-fold serial dilutions were prepared with virus inoculum or cell supernatants in serum free-DMEM up to 1 ml each dilution, and 450 µl of each serial dilution was added in duplicate into each well of six-well dishes, starting from the most diluted to the most concentrated dilution. Virus inoculum was allowed to absorb by incubation of the dishes for 1 hour at 37°C in 5% CO₂ with manual rocking every 10 minutes. The media with virus was aspirated and each well was covered with 2 ml of overlay medium (0.4% BSA fraction V (w/v), 0.3% NaHCO₂ (w/v), 20 mM Hepes, 20% 10x Minimmal esstential medium (vol/vol), 70% H₂O (vol/vol) 2% L-glutamine (vol/vol) 2% Pen/strep (vol/vol), 1% agarose (w/v))

The plates were left at room temperature for 15 min and inverted upside down before they were incubated at 37°C in 5% CO₂. Plates were incubated for 76 hours, then fixed with 10% formalin for 6 hours and stained with a solution of 0.1% toluidine blue for 40 min. The plaques formed on the cell monolayer were counted and the number of plaques forming units (PFU) by milliliter were calculated using the formula:

\[
\text{PFU/ml} = \frac{\text{Number of plaques}}{\text{Volume of inoculum (ml)} \times \text{dilution of virus}}
\]

**Isolation and culture of murine alveolar macrophages**

Murine alveolar macrophages (AM) were isolated by bronchoalveolar lavage (BAL) of 129 Sv/Ev, IFN-γ R⁺ and IFN-α/β R⁺ mice on the 129 Sv/Ev background (6-8 mice per group). Mice were euthanized in a CO₂ chamber by Dr. Marlynne Q Nicol according to the Schedule 1 of UK Home Office Animal Act. After confirmation of death, dissection was performed to expose the trachea along with the thoracic cavity.
A small incision (5 mm) was performed along the middle line on the ventral side of the trachea at ⅔ from the larynx. A small cannula attached to a 23G (0.6 x 16 mm) needle along with a 1 ml syringe was introduced to the trachea. The lungs were flushed with 1 ml of 1x Hanks buffered saline and 3mM EDTA solution and the bronchoalveolar lavage (BAL) transferred to 5 ml polypropylene tubes, and placed on ice. The same procedure was repeated 4 times until a total volume of 5 ml/mice was obtained. Mice BAL was centrifuged at 453 g at 4 °C for 15 min. The supernatants were discarded and the cell pellets corresponding to each mouse were pooled and resuspended in 12 ml of cold RPMI, supplemented with 100 U/ml Penicillin/Streptomycin, 10% FCS and 2mM L-glutamine. A cell count was performed in haemocytometer and the cells were split in 12 wells (1ml/well) of a 24-well tissue culture dishes at an approximate density of 5 x 10⁴ cells per well. AM were incubated for 1 hour at 37°C in 5% CO₂, washed 8 times with pre-warmed serum-free RPMI to remove non-adherent cells. Then AM were immediately infected with virus.

**In-vitro Influenza virus infection of cells.**

MDCK cells or AM were infected with A/WSN/33 influenza virus strain, in serum-free medium at different multiplicity of infections (MOI), for 1 hour at 37°C in 5% CO₂. For MDCKs, we infected confluent cells plated in 24-well tissue culture wells at a MOI of 0.01 in DMEM-serum free media. AM were infected with a MOI of 5 in RPMI serum-free media. The unabsorbed virus inoculum was removed and the cells were washed 3 times with either warm serum-free RPMI (AM) or DMEM medium (MDCK cells). DMEM or RPMI supplemented with 14% bovine serum albumin (BSA) fraction V and N-Acetyl Trypsin (1 µg/ml) was added, to allow cleavage of virus hemagglutinin allowing multiple cycles of infection. Cells were left to continue infection by incubated them at 37°C in 5% CO₂ for 2, 6, 12 or 24 hours according the experimental design.

**RNA extraction.**

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Cat # 74104) with on column DNAse treatment (RNase-Free DNase set (Qiagen, Cat # 79254)), following manufacturers guidelines. Briefly, AM or MDCK cells were disrupted by direct addition of 350 µl or 500 µl of RLT buffer onto the cells, respectively. Lysates
were homogenized by vortexing for 1 min and either stored at -70°C or immediately processed for extraction. RLT lysates stored at -70°C were thawed for 15 minutes at 37°C before continuing with the extraction protocol. Eluted RNA samples (50 µl) were transferred to microcentrifuge Eppendorf tubes. RNA samples were quantified using the nanodrop spectrophotometer to estimate the total RNA concentration and quality. RNA samples were stored at -70°C.

**Two-step Reverse transcription qPCR**

**Reverse transcription**
cDNA was synthesised from total RNA samples according to manufacturer protocols using SuperScript III First strand system for real time PCR (Invitrogen Cat. No: 18080-051). The RNA samples were thawed on ice, and a maximum volume of 9 µl were added to a nuclease-free microcentrifuge tube along with 1 µl of Oligo(dT)15 Primer (0.5µg/ µl), 2 µl of random hexamers (50 µM) and 1 µl of dNTPs mix (10 mM). The tubes were heated at 65°C for 5 min and placed on ice for 1 minute, followed by a brief centrifugation to remove any condensation. 7 µl of a master mix (4 µl 5X first-strand buffer, 1 µl of 0.1 M DTT; 1 µl RNase OUT (40 U/µl); 1 µl of SuperScript III RT (200 U/µl)) were added to each tube. Additionally, negative reverse transcriptase controls were included by adding a master mix containing nuclease free H2O instead of SuperScript III. The contents in each tube were mixed by gentle pipetting up and down. The tubes were incubated at 25°C for 5 minutes and followed by incubation at 50°C for 50 minutes. The reaction was inactivated by heating the tubes at 70°C for 15 minutes. The tubes were placed on ice and 80 µl of nuclease free H2O added to each cDNA sample. The samples were stored at -20°C.

**Relative quantitation for PB1**
A qPCR reaction mix was carried out using FastStart Universal SYBR Green Master (ROX) (USA, Roche)(Cat: 04913914001) by mixing 10 µl of the reaction mix along with 2.5 µl of each forward and reverse primer obtained from Dr. E Gaunt, Digard group (see table 1). 15 µl Of the master mix were loaded in each one of the qPCR reaction tubes. 5 µl of sample cDNA was added to the respective tubes in duplicate along with no template (H2O), and negative reverse transcriptase controls. The tubes
were capped and transferred to a 72 rotor-disc Corbett Research 3000 machine. The relative levels of PB1 were calculated using the $2^{-\Delta\text{CT}}$ method using succinate dehydrogenase (SDHA) as a normalizer (Livak & Schmittgen, 2001).

**Absolute quantification for cytokines**

Purified DNA samples for standard curve were made by preparing seven serial dilutions of the gene of interest in TRIS (10mM). A master mix was prepared with Fast-start Universal SYBR Green Master (ROX)(USA, Roche) following previous protocol with respective primers(see table 1). 15 µl Master mix in addition to 5 µl of sample cDNA, serial dilution, or negative controls were loaded in duplicate using a fresh tip each time. The tubes were capped and transferred to a 72 rotor-disc Corbett Research 3000 (Qiagen). The levels of each gene of interest were calculated by the relative standard curve method using the mock infected samples as calibrators and SDHA as endogenous controls (Giulietti et al., 2001).

**Cycling conditions**

The cycling conditions for all of the analysis were: one cycle of 95°C for 100 seconds, followed by 40 cycles (see Table 1), then 94 °C for 20 seconds and a ramp from 60°C to 99°C.

**Western blot**

Total cell lysates were prepared from mock or virus infected AM or MDCK cells at different time points (indicated on the text) by adding 1:1 Laemmlı loading buffer. Lysates were stored at -20°C or immediately processed by boiling them at 90°C for 5 minutes before separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a stacking gel (4% Bis-acrylamide (v/v), 1.2 mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), H2O, 0.1% Ammonium persulfate (w/v), 0.1% N,N,N’N’-tetramethylethylenediamine (v/v)) and a resolving gel (12% (v/v) Bis-acrylamide, 23.7 mM Tris-HCl, 0.1% (w/v) SDS, 43.5% (vol/vol) H2O, 0.1% (w/v) Ammonium persulfate, 0.1% (v/v) N,N,N’N’-tetra-methylethylenediamine). Here, between 5-15 µl of samples, positive controls (purified virions A/Puerto Rico/8 H1N1 and A/WSN/33 H1N1 were prepared by Mariya Goncheva) and protein ladder (PageRuler Prestained Protein Ladder, Thermo Scientific) were loaded in respective wells. The electrophoresis was run at 150 V for 90 minutes.
After SDS-PAGE, proteins were transferred by semidry blotting to a nitrocellulose membrane in Tris-glycine transfer buffer (20 mM Tris containing 153 mM glycine and 20% [vol/vol] methanol) using a Bio-Rad transblot device at 25 V, 1.0 A for 30 minutes. Once completed, the membrane was blocked for 1 hour with PBS containing 5% (wt/vol) BSA and 0.1 % (vol/vol) Tween 20. The membrane was then incubated overnight at 4°C with primary antibody (see below) diluted in 4 ml of washing buffer (PBS containing 0.1% [vol/vol] Tween 20) on a rocking platform. The membrane was washed three times with washing buffer, all washes carried out for 5 minutes on rocking platform at room temperature, to remove residual primary antibody, then incubated for 45 minutes with 10 ml of a 1/10,000 dilution of secondary antibody (see below) diluted in washing buffer solution. Once the membrane was exposed to the secondary antibody, it was kept in the dark with an aluminum foil covering. After the incubation time was completed, the membrane was washed 5 times. Then, the membranes were imaged using LiCor Odyssey Software.

A/WSN/33 proteins were detected using a goat polyclonal anti-H1N1 AbD serotec (1/1,000). Nucleoprotein (NP) was detected using a mouse monoclonal anti-NP [9G8] (ab43821) (1/500). Non-structural protein 1 (NS1) was detected using a rabbit polyclonal anti-NS1 (PR8) “V29” (1/500) (Carrasco et al., 2004). Bound primary antibodies were detected by Li-Cor IRDye® secondary antibodies: donkey anti-goat 800 CW (green), donkey anti-rabbit 800 CW (green), Donkey anti-mouse CW 800(green) and Donkey anti-mouse 680 CW(red). Mouse monoclonal anti-NP, rabbit polyclonal anti-NS1 and secondary antibodies were obtained from Prof. P Digard.

**Statistics**

Statistical analysis for differences between groups in virus titer, PB1 and cytokine expression were determined using two-way analysis of variance, adjusting for interactions between mice type and time. Pairwise comparison was done using Tukey multiple comparative analysis or paired t test. All of the analysis and figures were designed on GraphPad Prism 7.0.
Table 1. Primers sequences and cycling conditions

<table>
<thead>
<tr>
<th>Primers</th>
<th>Conc. nM</th>
<th>Sequences (5’ to 3’)</th>
<th>Cycling conditions (40x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sdha</td>
<td>Forward 400</td>
<td>GCT CCT ACT GAT GAA ACC TG</td>
<td>Step 1: 95 °C - 10 secs</td>
</tr>
<tr>
<td></td>
<td>Reverse 400</td>
<td>AAC TCA ATC CCT TAC AGC AA</td>
<td>Step 2: 62 °C - 50 secs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Step 3: 75 °C - 5 secs</td>
</tr>
<tr>
<td>Pb1</td>
<td>Forward 300</td>
<td>GGA ACA GGA TAC ACC ATG GA</td>
<td>Step 1: 95 °C - 10 secs</td>
</tr>
<tr>
<td></td>
<td>Reverse 200</td>
<td>AGT GGY CCA TCA ATC GGG TT</td>
<td>Step 2: 60 °C - 50 secs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Step 3: 77 °C - 5 sec</td>
</tr>
<tr>
<td>Tnfα</td>
<td>Forward 500</td>
<td>CAC CAC CAT CAA CGA CTC AA</td>
<td>Step 1: 95 °C - 10 secs</td>
</tr>
<tr>
<td></td>
<td>Reverse 400</td>
<td>GAC AGA GGC AAC CTG ACC AC</td>
<td>Step 2: 65 °C - 50 secs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg-1</td>
<td>Forward 400</td>
<td>GGC CTT TGT TGA TGT CCC TA</td>
<td>Step 1: 95 °C - 10 secs</td>
</tr>
<tr>
<td></td>
<td>Reverse 400</td>
<td>ATG CTT CCA ACT GCC AGA CT</td>
<td>Step 2: 62 °C - 50 secs</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward 400</td>
<td>TGC TAC TGA GAC AGG GAA</td>
<td>Step 1: 95 °C - 10 secs</td>
</tr>
<tr>
<td></td>
<td>Reverse 500</td>
<td>GAC AGT CTC CAT TCC CAA</td>
<td>Step 2: 60 °C - 50 secs</td>
</tr>
</tbody>
</table>
RESULTS

The main research question of this work was: Is the influenza A virus able to infect and productively replicate in alveolar macrophages? Alveolar macrophages are a particular subpopulation of macrophages, which can rapidly change their phenotype and functionality in response to changes in their environment (Campbell et al., 2015). Therefore, we wanted to model an *ex vivo* infection in AM as close as possible to their steady state *in vivo* by infecting these cells after only one hour of plastic adherence, in order to reduce the changes in their phenotype before infection (Tomlinson et al., 2012). Thus, we isolated alveolar macrophages (AM) from 129 Sv/Ev (Wild Type), IFNγR−/− (Huang et al., 1993) and IFNα/βR−/− (Muller et al., 1994) mice in the 129 Sv/Ev background, and *ex vivo* infected them with a laboratory strain of influenza A virus (A/WSN/33). In order to asses the viral replication and the different stages of virus cycle, cell lysates were prepared after different time points post-infection and analyzed by RT-qPCR to asses viral replication, western blot (WB) in order to detect viral protein translation and plaque assays to detect viral assembly and release. Furthermore, cellular response was assessed by measuring gene expression by RT-qPCR.

**Analysis of A/WSN/33 proteins in infected AM**

The aim of this experiment was to detect influenza proteins in alveolar macrophages as an indirect assessment of viral replication. In this study, we used western blot (WB) to detect IAV proteins in *ex vivo* infected AM or MDCK cells. First, we infected confluent MDCK cells with A/WSN/33 influenza virus strains at a M.O.I of 0.01 as a positive control to validate the protocol. MDCK cells were cultured with or without FCS in order to evaluate possible serological inhibitory effects on viral replication, since FCS is commonly used as a growth factor in cell cultures. Cell lysates were prepared at 4 and 24 hours post infection (h.pi) and processed for detection of IAV proteins using a polyclonal anti H1N1 (Fig 1). All of the infected samples at 24 h.pi including positive controls showed 2 bands (50 - 70 kDa), which are likely to be similar in size to NP (NP=58 kDa) and cleaved hemagglutinin 1 (HA 1=56 kDa). The lower band is not seen in PR8 virus uncleaved control, confirming that this is likely to be HA1. At the bottom of the gel, there were 2 strong bands (25 - 30 kDa) present in positive controls. This signal is likely to be cleaved HA2 (26kDa) and M1 (matrix
protein) (27 kDa). Since the uncleaved sample showed only a single band at the same position, the identity of this band is likely to be M1 protein, which is also seen in 24 h.pi samples. This protein, along with HA and NP, comprises approximately 90% of the virion particle (Oxford & Corcoran, 1981).

**Fig 1.** Western blot for detection of IAV proteins in MDCK samples.

As expected, these results showed that MDCK cells effectively allowed A/WSN/33 viral protein translation and these proteins could be detected using the polyclonal H1N1 antibody. Furthermore, differences in the viral protein signal, from low band intensities at 4 h.pi to a higher intensity at 24 h.pi, indicated increases in virus quantities, and hence virus replication. The same results were observed in a replicate experiment using only 24 h.pi and mock infected samples.

Because the signal detected for HA, NP and M1 could account for protein already present in the inoculated virus, but not from newly translated viral mRNA, we looked for proteins that can be more specifically associated with replicative stages, such as non-structural protein 1 (NS1). This protein is present only in small amounts in the
virion and has to be synthesized \textit{de novo} after the vRNP has entered the cell nucleus and initiated viral transcription (Hutchinson et al., 2014). We also looked specifically for NP, since a previous report showed that synthesis of this protein is inhibited in IAV infected AM (Cline, Karlsson, Seufzer, & Schultz-Cherry, 2013). Although the previous experiment could indicate the presence of this protein, because the antibody was raised against whole virus, these results need to be confirmed with a monospecific antibody.

Thus, we used a monoclonal antibody anti-NP and polyclonal antibody anti-NS1 to detect these proteins in MDCK 24 h.pi and mock infected samples (Fig 2). The target proteins NP (56 kDa) (Shaw, Stone, Colangelo, Gulcicek, & Palese, 2008) and NS1 (25 kDa) (Palese, P. & Shaw, 2007) were detected in all of the infected samples. The membrane incubated with NS1 antibody also showed a band of 40 kDa in both mock and infected samples, which we suggest is an unspecific binding of the primary antibody.

\textbf{Fig 2.} Western blot for detection of IAV NP and NS1 in MDCK samples.

![Western blot](image)

Fig 2. MDCK cell lysates prepared at 24 h.pi were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with 1) mouse monoclonal anti-NP or 2) rabbit polyclonal anti-NS1.

We then looked at the presence of viral proteins in AM using this validated WB protocol, by analyzing cell lysates prepared at 24 h.pi. Since the amount of AM/well was low, we prepared the cell lysates for protein extraction with a low volume of lamelli buffer (see methods) to increase the concentration of cellular proteins and
maximize the signal in the WB. Although different bands corresponding to several IAV proteins (Fig 3.1) along with NP (red) and NS1 (green) (Fig 3.2) can be detected in MDCK infected samples, no virus specific bands were detected in AM samples. Although these results showed a lack of viral protein production in infected alveolar macrophages, suggesting that the virus is not replicating, the number of cultured AM was low in comparison with the MDCK cells, which could limit the amount of total protein available for detection by this technique. Repeating this using more samples and as much protein lysates as we could load in the gel, we still could not detect any significant signal for viral proteins in AM samples. Thus, in the present experimental conditions, this test was not sensitive enough to detect proteins present in infected alveolar macrophages.

**Fig 3.** Western blot for detection of IAV proteins in MDCK and AM samples.

1) Membrane was incubated with polyclonal anti-H1N1. Different IAV structural proteins were detected in infected MDCK and positive control (A/WSN/33) 2) Nucleoprotein 56 kDa (RED) is only detected in positive control (A/WSN/33) and infected MDCK sample. In the lower area of the membrane, NS1 (25 kDa) (GREEN) is detected only in the positive control and infected MDCK sample.

**A/WSN/33 PB1 RNA levels are increased in infected alveolar macrophages.** Since the previous experiments showed inconclusive results, we followed a different approach aimed at the detection of viral nucleic acids. Thus, we looked for the presence of PB1 RNA using reverse transcription quantitative polymerase chain reaction (RT-qPCR), which is a very sensitive method, widely used in diagnostic virology or pathogen detection.
Segment 2 of the influenza A virus, also known as PB1, is the structural and functional core of the RdRp heterotrimer, which has the main function of working as a viral polymerase (Honda & Ishihama, 1997). Models of viral replication suggest that once the vRNP are translocated to the nucleus, the transcription of viral proteins precedes the replication of the viral genome (Chu et al., 2012). Although the replication of all viral segments begins simultaneously, only specific genes are transcribed during this first phase of transcription, including NP, PB1, PB2, PA, and NS1 (Hatada, Hasegawa, Mukaigawa, Shimizu, & Fukuda, 1989). Moreover, the overall transcription of mRNA peaks at the first hours of infection, between 2-6 hours, while the genome replication continues until the late phase of infection (Fodor, 2013). Thus, the levels of expression of PB1 can be a good estimation of the viral replication cycle, starting at the early phase of infection.

In order to measure this segment, we tried two different approaches. First, we used a one-step RT-qPCR, where the reverse transcription and segment amplification were performed in the same tube. We adapted a published method, where IAV PB1 was detected in purified influenza virions (Gaunt et al., 2016). However, after adapting this technique to detect PB1 in infected MDCK samples, we observed that the control without reverse transcriptase (RT -) (Fig 4.1 Red lines) showed relatively high levels of amplified product (Fig 4.1 Black lines). Moreover, the melting curve for RT - was very similar to those of the RT+ samples, suggesting that they were the same product. In order to discard the possibility of cross contamination during the manipulation of the samples, we repeated the experiment using more MDCK samples, including one RT- control for each of them. However, we still saw this atypical amplification, even after several repetitions of the test and after changing the reagents of the kit. As the melting curves suggested amplicons of the same size for RT – and RT+ samples, we tested this by running them on agarose gels, where we found no apparent difference between the size of the RT - and RT + amplicons (Fig 4.2). Although this technique allowed us to see changes in the viral replication, as there was a difference in PB1 levels between 2h and 24h samples, the lack of specificity did not permit a correct interpretation of the results.
**Fig 4.** One-step RT-qPCR amplification curves for PB1 in MDCK samples and gel electrophoresis of the amplicons.

![Amplification curves diagram]

**Sample** | **Ct**
--- | ---
24 h.pi (Blue) | 9
2 h.pi (Black) | 27
24 h.pi (RT -) (Red) | 21

1) MDCK cells were infected with A/WSN/33 MOI: 0.01, cell lysates prepared at 2h and 24h for RNA extraction and One-step RT-qPCR analysis (each sample was tested in duplicate). Bellow this picture, melting curve analysis is shown. 2) Amplicons were loaded in separate wells of a 1.5% agarose gel and separated by electrophoresis (PB1 amplicon size=116 bp).

Next, we replaced the previous method with a conventional two-step RT-qPCR, where the reverse transcription (RT) and qPCR amplification were performed in separate tubes (described in Methods section). Although the multiple manipulation of the samples could increase the possibility of cross contamination, one of the main advantages of this protocol is that we had more control in the developing of the
reaction, thus we could optimize different steps that were not possible to be changed in the one-step RT-qPCR.

Since the original number of alveolar macrophages was substantially low compared to MDCK samples, we optimized this method using MDCK cells and murine lung samples (provided by Dr. MQNicol). First, we optimized a reverse transcription protocol intended to achieve the highest possible cDNA yield in order to increase the sensitivity of the qPCR amplification. In order to do this, we first looked at the effect of using different primers in the RT reaction. First strand cDNA can be synthesized by priming poly(A) RNA or total RNA using Oligo(dT), random hexamers or gene specific primers. Since the starting material from AM was limited, we wanted to use the same cDNA to detect virus genome and multiple cellular genes, thus we were unable to use gene specific primers. On the other hand, as previously reviewed during the influenza virus reproductive cycle, the vRNA is used to generate mRNA with poly(A) tail and cRNA, which can be detected using either Oligo dT or random hexamers, respectively, or a mix of both in the same reaction. Thus, we tested these alternatives by reverse transcribing RNA from lung RNA and MDCK samples, using the different primers in separate reactions, as well as a combination of both in the same reaction. After qPCR amplification of SDHA in lung samples and PB1 in MDCK samples, we found that the combination of Oligo(dT)15 primer and random hexamers gave the best results for gene expression and virus detection (Fig 5). Overall, these conditions showed good efficiency in qPCR amplification for SDHA (>0.98) and for PB1 (99%) with a strong linear correlation (>0.99). Furthermore, we did not detect qPCR amplification in the RT - controls.
Fig 5. Effects of Oligo(dT) and Random hexamers in reverse transcription and qPCR amplification.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1µl Oligo(dT) + 2µl Rh</td>
<td>16.5</td>
</tr>
<tr>
<td>1µl Oligo(dT)</td>
<td>17.3</td>
</tr>
<tr>
<td>1µl Rh</td>
<td>23.1</td>
</tr>
<tr>
<td>2µl Rh</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Fig 5. 1) qPCR for SDHA amplification in lung samples. RNA from a single sample was reverse transcribed in four different reactions. **Blue**: 1µl Oligo(dT) + 2µl Random hexamers(Rh), **Pink**: 1µl Oligo(dT), **Orange**: 1µl Rh, **Green**: 2µl Rh, **Yellow**: No template control. 2) qPCR for PB1 detection in MDCK 24 h.pi with A/WSN/33. RNA from a single sample was reverse transcribed in three different reactions. **Blue**: 1µl Oligo(dT) + 2µl Rh, **Orange**: 1µl Rh, **Green**: 2µl Rh, **Yellow**: No template control.

Then, we evaluated the use of ribonuclease H (RNase H) at the end of the RT reaction. This enzyme is an endonuclease part of retrovirus reverse transcriptase protein and enhances the generation of viral double stranded DNA by degrading the RNA, which is hybridized to newly synthesised viral cDNA during the reverse transcription process (Schultz & Champoux, 2008). Therefore, when using it at the end of first strand cDNA synthesis, it removes residual RNA that could inhibit the PCR. In order to assess the effect of this enzyme, we measured the levels of expression of a house, keeping gene
(SDHA) in infected murine lung samples and AM (24 h.pi), which were reverse transcribed with and without RNase H. After qPCR amplification, we found that the use of RNase H (Fig 6. Lung: Blue, AM: Pink) had no difference or even reduced levels of detection for SDHA, compared to RNase H negative samples (Fig 6. Lung: Red, AM: Green). Since our priority was to obtain the highest possible sensitivity, the use of this enzyme did not fit our experimental conditions.

**Fig 6.** RNase H effects in reverse transcription and qPCR amplification of SDHA.

During the qPCR amplification for PB1 detection, we used degenerate primers. These primers are designed to tolerate mismatches among related sequences, reducing false negative results (Kwok, Chang, Sninsky, & Wang, 1994), and in this case they were designed to detect PB1 from different influenza viruses (Gaunt et al., 2016). However, after being tested in multiple qPCR reactions, the mock infected samples showed a tendency to form high levels of primer dimers with melting curves, showing multiple peaks and overlapping with the melting curves from infected samples (Fig 7.1). These effects generated false positive values, reducing the specificity of the test. Therefore, we tried to reduce this non-specific primer annealing by testing different primer and cycling conditions. After testing 25 different primer concentrations, we found a few combinations that improved the reaction by reducing the area under the melting curve in the mock infected samples. After modifying the qPCR cycling conditions in order
to acquire fluorescence after 77 °C, we were able to avoid the quantification of most of these non-specific signals. (Fig 7.2). Although we did not completely eliminate the primer dimers, this optimized method reduced false positive results and improved the specificity of the test, particularly at the early timepoints.

**Fig 7.** Melting curve analysis for different conditions in qPCR detection of PB1.

Fig 7. 129 Sv/Ev AM mock and A/WSN/33 infected samples were reverse transcribed according to previously optimized protocols and tested for PB1 amplification. 1) qPCR initial conditions 2) primers and cycling conditions optimized (see methods). **Red:** AM mock infected samples, **Blue:** AM infected samples, **Green:** no template controls.

Next, we used this optimized qRT-PCR protocol to compare the levels of expression of the PB1 segment in virus infected alveolar macrophages. AMs obtained by bronchoalveolar lavage (BAL) from 129 Sv/Ev, IFNγR−/− and IFNα/βR−/− mice were infected with A/WSN/33 virus at MOI=5. This high MOI was used to allow a single cycle of infection for all of the cells present on the well, and rule out the possibility that this IAV strain is not able to continue multiple rounds of infection in AM(T. D.
Mock and infected cells were collected at 2, 6, 12 and 24 hours post infection (h.pi) for analysis. Overall, PB1 cDNA was detected in all of AM groups at every time point (Fig 8.1), showing a trend to increase the levels over the subsequent time points ($r=0.69; p<0.001$). This increase reached a peak at 12 h.pi., after which production decreased or plateaued, as was seen at 24 h.pi. Although we showed the levels of PB1 at the 2 h.pi, we did not compare the differences between groups at this time point, because much of the amplicon detected was probably related to residual virus bound to cell surfaces or recently absorbed. Therefore, this observation could be taken as a baseline to assess any further increase. After comparing the PB1 levels between the different groups, 129 Sv/Ev (wild type) AM showed a peak in production at 12 h.pi (Fig 8.2), which was barely changed after 24 h.pi. IFNα/βR−/− AM showed higher levels of PB1 than IFNγR−/− at 12 h.pi ($p<0.05$)(Fig 8.2). When comparing IFNγR−/− to the wild type, it showed significantly higher levels at 6 h.pi ($p<0.05$) and at 12 h.pi ($p<0.01$). Lastly, IFNγR−/− AM PB1 production was partially restricted at early time points (2h p.i). However, the levels were normalized with the other cell types at 6 h.p.i, showing a similar behavior to the wild type in the subsequent time points (Fig 8.2). These results showed that the virus was able to infect the AM, increasing the amount of PB1, which was a seen at a higher level in IFNγR−/− AM.

**Fig 8.** A/WSN/33 PB1 levels in infected murine alveolar macrophages measured by RT-qPCR.

![Fig 8.](image_url) 1) Growth curves in PB1 levels 2) multiple comparison between groups. Alveolar macrophages were extracted from 6-8 mice/group and assayed in independent experiments. Data represents mean values of $2^{-ΔΔCt}$ (log10) and standard error (bars) from duplicate samples. *$=p<0.05$, **$=p<0.01$
Virus productive replication is partially restricted in alveolar macrophages

Virus productive replication is an important characteristic of the influenza virus. Although continuous and primary cell cultures, such as MDCK, Vero, airway epithelial or endothelial cells, allow a high release of virus (M. C. W. Chan et al., 2009; Genzel & Reichl, 2009; Zeng et al., 2012), the permissiveness of alveolar macrophages to allow viral productive replication is under debate. Where some groups have reported abortive viral replication (Rodgers & Mims, 1982; van Riel et al., 2011; J. Wang et al., 2009, 2012), others have found different levels of virus productive replication and related these observations to different factors, such as the virulence of a specific strain, or even the type of macrophage used in the experiment (R. W. Y. Chan, Leung, Nicholls, Peiris, & Chan, 2012; Cline, Karlsson, Seufzer, & Schultz-Cherry. 2013; Riser, Maassab, Riser41, Maassab, & Arbor, 1990; Yu et al., 2011).

Here, we determined the ability of alveolar macrophages to support productive viral replication by looking the release of competent virus, using plaque assay on supernatants derived from infection assays. Since we quantified the virus titers at 2 h.pi, these values were considered as a baseline for virus quantifications, as a newly formed virus starts being released from infected cells only after a period of 5-6 hours (Werner Henle, 1949). These results showed that the virus titer in wild type AM increases after 6 h.pi (Fig 9). On the other hand, IFNγ R− and IFNα/β R− showed an increase in virus titer later on, after 12 h.pi. Wild type and IFNγ R− showed a reduction in the virus titer after 12h.pi, while IFNα/β R− on the contrary increased the virus titer, reaching a peak at 24 h.pi, with concentrations significantly higher than the other mice type (p<0.001). Wild type and IFNγ R− groups showed similar viral titers, with the exception of 6 h.pi, where there was a higher virus concentration in the wild type group (Fig 9). When comparing the virus titers in supernatants of the first and last time point, between each AM group and MDCK cells, we found that the levels of virus in MDCK supernatants were much higher than those from alveolar macrophages (Fig 10), corroborating the popularity and wide use of this cell line in influenza virus propagation. The apparent low titer in the MDCK sample at 2 h.pi came from the initial low MOI (0.01) which was used to allow multiple cycles of infection. The fact that MDCK are a highly susceptible cell line, if using the same MOI than in AM(MOI=5),
the virus would destroy most of the cell monolayer in the first cycles. Since the virus titer is reduced in wild type and IFNγ R−/− after 12 h.pi, no differences can be detected within these groups between 2h and 24h (Fig 10). On the other hand, the increase in virus titer is evident in the IFNα/β R−/− (Fig 10). Overall, we saw an increase in the virus titer in relation to the 2 h.pi titer in all of the AM groups and the IFNα/β R−/− showed a higher virus titer in the last time point.

Fig 9. A/WSN/33 Virus titers in cell supernatants of infected alveolar macrophages.

Fig 9. Left: supernatants were recollected at 2, 6, 12 and 24 hours, and assayed for virus titres by plaque assays on supernatants of infected AM. Right: Representative plaque assay pictures shown as a matrix of AM group vs Time point. Data represents PFU/ml mean values and standard error (bars) of four observations from duplicate samples at each time point. *=p <0.05, **=p <0.01, ***=p <0.001.
**Fig 10. Comparison of A/WSN/33 virus titers at 2 and 24 hours post-infection in alveolar macrophages and MDCK cells.**

Fig 10. Virus titers on supernatants of infected AM (MOI=5) and MDCK (MOI=0.01) cells at 2 and 24 h.pi. Boxplots represent PFU/ml (log_{10}) mean values and standard error of four observations from duplicate samples. **p<0.01, ***p<0.001.

**Inflammatory responses in influenza infected alveolar macrophages**

Our previous experiments showed that the virus was infecting AM. In this experiment, we wanted to assess the level of inflammatory responses induced in these cells by IAV infection, by evaluating the expression of cytokines. Also, cytokine production is directly related to the state of macrophage activation, where alternative activated macrophages will produce different cytokines than classically activated macrophages (Gordon, 2003). Thus, we investigated the mRNA expression for *Tnf-α* (pro-inflammatory marker), *Arg-1* (alternative activation phenotype marker) and *iNOS* (classical phenotype marker) in each AM group, by a RT-qPCR at various time points post virus infection. The levels of *Tnf-α* in IFNγ R−/− were lower than those in the other AM groups at all of the time points, the only detectable difference being at 12 h.pi (p<0.05) (Fig 11). On the other hand, wild type mice and IFNα/β R−/− showed a peak in the expression of this cytokine at 12 hours. However, At 24 h.pi there was a decrease in the expression. Surprisingly, *iNOS* was not detected in any of the groups at any time point. Conversely, *Arg-1* was only detected at 2 and 6 hours (Fig 11). The only detectable difference was at 12 h.pi, where the levels in IFNα/β R−/− were higher than the other groups (p<0.05).
Fig 11. Expression of pro- and anti-inflammatory markers in A/WSN/33 alveolar macrophages.

Fig 11. AM samples infected with A/WSN/33 were recollected at the previous indicated time points and tested by RT-qPCR for gene expression in separate experiments. TNF-α and Arg-1 fold changes were calculated by the corresponding mock infection at each time point (1) and normalized to the SDHA expression.
DISCUSSION

In the present work we have modeled an ex vivo IAV infection in murine alveolar macrophages, showing that a mouse adapted IAV (A/WSN/33) strain was able to infect AM, increasing PB1 RNA levels at different degrees, depending on the AM group. Although the levels of PB1 were similar in wild type and IFNγ R−/− AMs, the expression of Tnf-α was lower in the last group, indicating a reduced pro-inflammatory response. An increase in the release of infectious virus was detected in all of the mice groups, but productive replication was higher in IFNα/β R−/− AMs, which also showed higher expression of Tnf-α, demonstrating the important role of IFNα/β as a cellular antiviral mechanism.

One of our main limitations during this work was the amount of AM available for the assays, since it is not possible to obtain a high number of cells from the BAL of a single mouse’s lungs. However, we improved these conditions by pulling the cells extracted from different mice and by increasing the sensitivity of our detection assays, such as our qRT-PCR protocol. Although our western blots analysis failed to detect viral protein in the AM samples, this does not necessarily mean an absence of viral transcripts or protein translation or a complete absence of cell. Although in the present work a loading control was not used, the signals generated by the unspecific bounding of the secondary antibodies allowed us to detect a band in the lanes, corresponding to the AM samples. These unspecific bands were possible to be detected only after overexposing the membranes, and although the identity of these bands is unknown, they suggest the presence of cellular components in very small quantities. We speculated that if we increased the over exposition, we could have detected some viral signals, but after increasing the over exposition, the membranes got oversaturated with light, making it impossible to see any further signal. Other reports have found several IAV proteins in infected macrophages by using this technique (Cline, Karlsson, Seufzer, & Schultz-Cherry, 2013; Londrigan et al., 2015). However, in these studies they have used a continuous cell line (RAW.267), which grows at confluence in cell culture and is more likely to produce more viral protein. A report of influenza A virus infection (PR/8) in rat alveolar macrophages showed several structural proteins by western blot (Nain et al., 1990). Nevertheless, the amount of cell used (2x10^6 cells)
was considerably higher than in our assays (see methods). Although virus protein translation could not be confirmed using this method, we could verify that the virus was able to enter the AM, and once there, the vRNP containing the PB1 segment was translocated to the cellular nucleus and starts replication, which was evidenced by the increasing levels of PB1 across the different time points, detected by qRT-PCR.

The levels of PB1 RNA are a good estimation of virus RNA replication and transcription, since it is the functional viral polymerase. The fact that PB1 is essential for the generation of viral transcripts and genome replication implies that any block or reduction in the levels of this segment would affect the synthesis of other viral components. However, our results showed that the production of PB1 is increased in all of the groups in the first hours of infection. Although there is a reduction in the detection of this segment in the last time point, it is most likely related to a reduction in cell viability after 12 h.pi (Campbell et al., 2015).

Then, when comparing all of the groups, we saw higher levels of PB1 and higher virus titers in the supernatants of IFNα/β R−/− AM. This response could be directly correlated with a reduction of the IFNα/β downstream signaling. This is one of the main cellular antiviral strategies and its depletion will have a deep impact on the cellular control of virus replication (Hoffmann et al., 2015). On the other hand, 129 Sv/Ev and IFNγ R−/- AM showed a similar pattern characterized by a limited productive virus replication and comparable levels of PB1 RNA across the different time points. Since IFNγ R−/− AM cannot follow a classical activation driven by IFNγ R+ stimulation (Schroder, Hertzog, Ravasi, & Hume, 2004), it is implied that the induction of this phenotype is not necessary for limiting virus productive replication by alveolar macrophages. Similarly, past reports showed that the absence of IFNγ signaling in IAV infected mice does not change the kinetics of viral replication in comparison with wild type mice, and it is not necessary for the development of humoral or cellular responses (Graham et al., 1993; Price, Gaszewska-Mastarlarz, & Moskophidis, 2000).

Previous studies have shown that IAV infected AM increased the expression of Tnf-α, which was correlated with the amount of virus infectious particles released (Yu et al., 2011). Our observations indicated that 129 Sv/Ev and IFNα/β R−/- upregulated the
expression of $Tnf-\alpha$ upon IAV infection, and the levels of expression were correlated not only with the amount of virus released, but also with the amount of PB1 RNA found in these cells. However, an important observation was that although wild type and IFN$\gamma$ R$^{-/-}$ showed similar kinetics in virus productive release, the degree of the pro-inflammatory response was lower in the IFN$\gamma$ R$^{-/-}$ group than in the other ones. This response could be associated with the activation state of these cells at the start of infection. Campbell and colleagues (2015) suggested that the initial macrophage activation state before a virus challenge is an important factor associated with the type of cellular response generated, since they observed that alternative activated macrophages showed a lower expression of pro-inflammatory cytokines, such as $Tnf-\alpha$ and $iNOS$, and were more susceptible to IAV infection than classical activated macrophages (Campbell et al., 2015). Our results agree with those findings by showing that the lack of IFN$\gamma$-induced classical activation reduces the levels of an important pro-inflammatory cytokine such as $Tnf-\alpha$ after IAV infection of AM.

Although, it is suggested that in vivo AM exhibit an alternative activation phenotype, induced by a close regulation of airway epithelial cell, mainly by CD200/CD200R interactions (Snelgrove et al., 2008). This normal interaction state could be disrupted after IAV infection of the airway epithelial cells, creating an inflammatory environment, that will evidently affect macrophage responses (van Riel et al., 2006; Wang et al., 2009). A study in alveolar macrophage heterogeneity, after challenged with influenza A virus (H3N1), showed that a subpopulation of AM exhibited mixed surface markers from M1 and M2 phenotypes, along with upregulation of $Arg-1$, $iNOS$, $Il-6$ and $Il-10$ (Duan et al., 2012). These results imply a mixed inflammatory response between an alternative and classical activated phenotype. Although, our results showed a population of alveolar macrophages where only $Arg-1$, but not $iNOS$, was detected. An alternative activation state cannot be defined, since the signal for $Arg-1$ was lost after 6 h.pi and all other AM groups upregulate the expression of $Tnf-\alpha$.

According to Gordon et al (2003), IFN$\alpha/\beta$ and IFN$\gamma$ stimulation induce distinct but partially overlapping activation states. Thus, if we differentiate all of the macrophages that are not activated by IFN$\gamma$ in a single group, this will hide important differences, given the wide diversity of responses in the alternative activation group (Gordon,
Therefore, although an exact phenotypic state would be hard to define, it is evident that the priming through one or another state would have a big impact on the initial host response to the disease.

Past reports agree that although the virus can infect macrophages, its normal replication is blocked later during the virus cycle (Campbell et al., 2015; Cline Karlsson, Seufzer, & Schultz-Cherry., 2013; Londrigan et al., 2015). Similarly, we observed an increase in the levels of viral RNA, but a limited release of virus particles in the 129 Sv/Ev and IFNγ R−/− groups, suggesting that a restriction of the synthesis of viral products at an early stage is not responsible for the lack of virus release, and this restriction can be related to later stages in the cycle as illustrated by other groups. For instance, one of the last steps in the virus replicative cycle is the nuclear assembly and exportation of vRNP to the cell membrane. Although, the vRNP uses host chaperon proteins, such as NPI-1/NPI-3 or Hsp90, to be translocated between different cellular compartments (Naito, Momose, Kawaguchi, & Nagata, 2007; P. Wang, Palese, & O ’neill, 1997). It is suggested that NP interaction with host cell proteins is essential for an efficient vRNP trafficking in and outside of the cellular nucleus (Portela & Digard, 2002). Previous research showed that abortive replication of H1N1 infecting alveolar macrophages was accompanied by a reduction of NP concentration in the cell nucleus within 4 hours post infection. On the other hand, infection with a highly pathogenic H5N1, which did not show this NP nuclear decrease, replicated productively in these cells (Cline, Karlsson, Seufzer, & Schultz-Cherry., 2013).

Although we did not have information about NP variations in alveolar macrophages, we did observe productive replication of a low pathogenic influenza strain in the absence of IFNα/β downstream signaling, suggesting that the capacity of a highly pathogenicity strain to complete a productive cycle is correlated with its ability to neutralize interferon downstream antiviral effects. Such effects are clearly different from the other AM groups, where the sole presence of a normal IFNα/β receptor in wild type and IFNγ R+ AM restricted the viral productive release. These different responses observed with each system could be explained by the fact that type I and II stimulates not only similar ISGs, but also many other type-specific ISGs (Der, Zhou, Williams, & Silverman, 1998). Since IFN-α/β is one the principal inducers of a
cellular antiviral state (Randall et al., 2008) and AM are one the primary producers of these class of cytokines after exposition to RNA viruses (Kumagai et al., 2007), the lack of IFN-α/β signaling will limit the ability of these cells to control the virus replication. Likewise, Kopf and collaborators (2005) suggested that once AM are infected, they are able to reduce the virus spread and pro-inflammatory signaling by upregulation of IFN-α/β (Kopf, Schneider, & Nobs, 2015). Human bone marrow derived macrophages, treated with IFN-α and exposed to influenza virus, showed low levels of virus replication. This high levels of IFN-α were associated with increased expression of MxA (Ronni, Sareneva, Pirhonen, & Julkunen, 1995). Although MxA is an orthologous gene to the Mx1 from mice, both have an antiviral effect. However, their sole activity is not enough for a full virus restriction (Pavlovic et al., 1992; Xiao et al., 2013). Since macrophages upregulate dozens of ISGs after influenza infection (Alberts et al., 2010; Wang et al., 2012), the interaction and the sum of these genes effects could be affecting several viral steps. We have shown that the virus is able to replicate an important segment of its genome in AM. Therefore, we could speculate that this restriction is affecting a stage after viral genome replication. Although, we cannot conclude a direct link between the effect of interferon signaling on the inhibition of a particular virus cycle blockage. Our results showed a productive viral replication of a mouse adapted H1N1 influenza strain in alveolar macrophages lacking IFN-α/β effector signaling. Thus, it is possible to make a direct association between an absence of this signal with a reduction in the transcription of dozens of interferon stimulated genes.

Overall, we showed that whether IFN-α/β stimulation is involved in limiting virus replication, a classical activation phenotype induced by IFNγ R-/- does not play an equal role. Furthermore, the absence of this signaling can be associated with a reduction of important pro-inflammatory markers, such as Tnf-α, involved in harmful secondary effects, like hypercytokinemia or secondary pneumonia, supporting the theory that the macrophage activation state is will affect the severity of the IAV infection.
Future perspectives

In this study we have demonstrated that the AM can be infected by IAV, with a productive viral replication at different levels. However, we do not know the fate of these macrophages after infection; whether they are readily killed by the virus or if they are signaling to enter in apoptotic pathways, and the time this takes. Campbell and colleagues showed that alternative activated macrophages are killed faster than classical activated ones after IAV infection. Thus, it would be interesting to assess the cell viability of the different groups of the AM used in this experiment after IAV infection. The virus used in these experiment is a mouse-adapted IAV strain and it is not considered a HPAI virus. Previous reports have shown that the infection of macrophages with HPAI strains like H5N1 would induce productive replication in alveolar macrophages with a higher induction of pro-inflammatory cytokines (de Jong et al., 2006; Yu et al., 2011). Since the IFNγ R−/− group was nonresponsive to pro-inflammatory stimulus induced by an apparent alternative activation, it would be also helpful to determine if this attenuated pro-inflammatory response and low virus productive replication are maintained after a challenge with a HPIA.

Another important effect of HPAI infections in vivo is the induction of an excessive leukocyte infiltration of the alveolar space, which is mediated by high production of chemokines, such as CCL2, CCL3, CCL5 or CXCL10 (J. Wang et al., 2012; Zhou et al., 2006). Since the IFNγ R−/− AM showed low levels of expression of Tnf-α, we could hypothesize that the levels of these chemokines will also be low, however further investigation would be needed to address this question.

Although the techniques used in this work helped us to address our research question, they can be improved or other alternatives can be used. For instance, during our assay we could not verify virus protein translation by western blot. However, a viable alternative could be the use of immunocytochemistry and fluorescence microscopy in order to detect virus proteins in infected cells. Our RT-qPCR method adapted from a previous work was a good tool that allowed us to measure the viral PB1 kinetics. However, the presence of high levels of primers was a nuisance during the whole
experiment. The only solution is to try to redesign the primers or different segment primers.

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