Detection and quantification of Equine type I Interferons

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Type I Interferons (IFNs), perhaps the most important of cytokines in fighting viral infections, have been target for detailed research only the past few decades and much is yet to be discovered. Hidden in the mysteries of Interferons might be powerful antiviral and antitumor therapies, alongside greatly increased understanding of vertebrate immunology. This degree project has been performed as part of an ongoing two-year research project concerning IFNs. The aim of the ongoing two-year project is to improve the understanding of the Interferon system in horses, possibly to aid in future developing of antiviral drugs and vaccines for an increasingly large population of horses in Sweden. The main question sought to be answered in this thesis is under which conditions IFNs are expressed. Whilst parasites, fungi and protozoa also may trigger Interferon production, this thesis aims at the virally induced Type I Interferons. The project involves in vitro stimulation of an equine cell line and studies of IFN expression in both healthy horses and horses diagnosed with Inflammatory Bowel Disease (IBD). In the field of equine diseases, IBD is of growing concern and scientific progressions within the project are, in many aspects, also applicable for human medicine.

LIST OF FREQUENTLY USED ABBREVIATIONS
IFN Type I Interferons, PKR Protein Kinase R, PRR Pathogen Recognizing Receptors, RNAseL Ribonuclease L, TLR Toll-Like receptors, RLR RIG-1-Like Receptors, NLR Nod-like Receptors, IBD Inflammatory Bowel Disease

1 INTRODUCTION
1.0 THE MICROBIAL BALANCE
Microorganisms constantly surround us, some essential for our survival while others pathogenic and yet other are potentially lethal. Most organisms, from bacteria to mammals, have some kind of immune system stretching from simple antiviral enzymes in simpler life forms to complex defensive systems in vertebrates. Whereas some pathogens infect sheep or perhaps pigs alone, other infect humans and yet other can be transferred across species. Throughout evolution, vertebrates in general and mammals in specific have developed a number of measures to maintain a healthy microbial balance, something far more complex than simply abolishing all interactions with microorganisms. Without gastrointestinal bacterial flora we could simply not function at all; advanced symbiotic systems are not only necessary but vital. This presents a great challenge in evolving defensive reactions towards pathogenic microorganisms whilst at the same time allowing for copious amounts of bacteria to inhabit discrete compartments of mammal bodies under certain conditions.

It is of common biological knowledge that we have a battery of defenses against pathogens, the first and perhaps most important factor being our physical barriers of mucosa's and skin, outermost layer being dead and thereby impossible to inhabit and use for synthesis of functional virions during viral infections. Amongst the groups of pathogens, viruses are unique in several features, one such feature is the strict dependency of living cells to reproduce. Reproducing in the host cells not only delivers efficient means of high-scale virion synthesis but also provides a shelter against components of the immune system. This makes viral infections even more difficult to clear from the host and to treat medically; most bacterial infections can be fought using antibiotics and fungal infections using anti-fungal drugs whereas anti-viral drugs are less efficient and more complex to develop since the aggressors reside inside the cells of the host and/or in parts of the body more difficult to reach for the components of the immune system.

Except for the physical barriers mentioned above, the more elaborate defensive mechanisms include the innate and the adaptive immune systems. The former is crude, wider in specificity and the latter is far more specific, slower in reaction but with greater potential. Breaching all barriers and/or evading immune systems, some microorganisms reach cells permissive for infection and invade them. Primarily for viral infections, a line of defense is of great importance if this happens; the Type I Interferons (IFNs). These are cytokines capable of inducing potent responses to invading virus through a number of signaling pathways and mediators. As will be described in this thesis, cells of the intrinsic immune system induce IFNs, but IFNs affect both the innate and adaptive immune systems, providing a crucial linkage between the two.
1.1 INTERFERONS

Interferons were first mentioned in the literature in the 1950s as something interfering with viral reproduction. The explorations of Interferons have however been hampered by insufficient technical solutions. Most of the details revealed about how Interferons are activated after viral recognition are results from the last decade of research, so this topic is still rather unexplored (Stetson and Medzhitov, 2006). The Interferon system is found uniquely in vertebrates and is most elaborate in mammals. Of the mammal Interferons there are three types: type I, II and III. Type II and III Interferons are less studied and understood, but are likely enhancers of the effects caused by Type I Interferons and regulators of immunologic systems.

The Type I Interferon system is an important field of immunological research, considering the fact that most cells produce these cytokines during almost all viral infections and many non-viral infections. In short, Interferons are vital anti-viral systems in vertebrates, activated by cellular receptors, and capable of inducing anti-viral states in both infected and neighboring non-infected cells. Innate immunity with its complement system, adaptive immunity with its lymphocytes and antibodies and the intrinsic immunity based on cytokines are all interlinked by Interferons.

1.2 PATHOGEN RECOGNIZING RECEPTORS (PRRs)

1.2.1 PRRs induce IFN production

The first step in viral invasion of a cell is attachment, during which the virus connects to receptors on the cell surface, receptors that normally have important regulatory effects in the cell but during infection might serve as back doors for invading pathogens. Each virus has its unique set of target receptors, sometimes only one receptor but most often a combination of receptors and co-receptors. Cellular receptors used to counter the infection are able to detect pathogens and induce IFN production and other defensive measures. These receptors are the Pathogen Recognizing Receptors (PRRs), which can be divided in two main categories; cytoplasmic and extra-cytoplasmic. The extra-cytoplasmic are membrane-bound and located either in the endosomes or in the cytoplasm membrane. The Toll-like receptors (TLRs) described in section 1.2.2 are located in endosomal membranes and cytoplasmic membranes. These are capable of inducing powerful immunologic responses, making them important regulators of the entire immune system. Amongst the cytoplasmic receptors are the Rig-1-like receptors (RLRs) described in section 1.2.3 and the Nod-like receptors (NLRs) described in section 1.2.4. All these receptors are PRRs, in total a large number of different receptors capable of detecting non-self structures and trigging the cellular alarm signals through production of IFNs and pro-inflammatory cytokines. (Cui et al,2010; Stetson and Medzhitov, 2006; Kawai and Akira, 2006; Garcia-Sastre and Biron, 2006; Kawai and Akira, 2011).

Figure 1.1 List of Pathogen-recognizing receptors (PRRs).

All the different PRRs are either membrane-bound or cytoplasmic. The membrane-bound PRRs are the TLRs, acting in homodimers e.g. TLR 4, 7 or in heterodimers e.g. TLR1+2 or TLR2+6. Note that TLR3 is present in both endosomal and cytoplasmic membranes. Of the cytoplasmic PRRs, the DAI are the only detecting dsDNA. However, TLR9 receptors have the same feature and together with DAI, and most likely a yet undiscovered receptor, they cover the need for dsDNA detection. TLRs are described in section 1.2.2, RLRs in section 1.2.3 and NLRs in section 1.2.4.

If successful in passing the cell membrane either by forcing the membrane or through endocytosis, the pathogen reaches either the cytoplasm or the inside of an endosome. Regardless of which, the interferon activating guardians are at post, ready to induce IFN production. The different families of PRRs detect different pathogens; together they cover endless possible combinations.

Directing PRR recognition towards just any structures in pathogens would be energetically and immunologically unfavorable due to the virtually unlimited number of variations. No immune system can be designed to recognize every individual microbe; a far better strategy is to target conserved regions in the pathogens. This reduces the number of combinations to be covered by the PRRs but also reduces the risk of losing affinity for a pathogen that often is undergoing rapid mutations. These conserved regions are pathogen associated molecular patterns (PAMPs), often crucial for metabolism, pathogenicity or other parts of the life cycle of the microbe.
Mutating other sequences of the genome might provide for evolutionary advantages whereas mutations in vital life cycle functions probably are evolutionary self-deleterious; these pathogens do not survive. Viral reproduction depends on many parts of the cellular machinery to replicate the viral genome and produce new virions. During this process, virally associated structures will be present in the cytosol and/or in the nucleus depending on the type of virus. These structures may be dsRNA, proteins or e.g. parts of membranes such as LPS of bacteria (Kumar and Kawai 2011). Although most cells have PRRs of varying subtypes and amounts, dendrite cells (DCs) are sentinels equipped with large numbers of these receptors, further discussed in section 1.11.

1.2.2 The TLR part of the puzzle

Both in the cellular membranes and in the walls of endosomes are TLRs of different subtypes (10 for humans), all capable of inducing production of IFN. TLRs are membrane-bound receptors with an external ligand-binding part containing leucin rich repeats (LRRs) and an internal signal-mediating Toll/Interleukin-1 receptor domain (TIR). For TLRs bound in the cytoplasm membrane (TLR 1, 2, 4, 5 and 6), the ligand-binding part is extracellular, whereas TLRs located in endosomes (3, 7, 8, 9) have their ligand-binding part in the interior of the endosome (Kawai Akira Nature 2011). For the TLRs to be able to bind PAMPs of pathogens, the pathogens are likely degraded in the endosomes (Garcia-Sastre 2006).

TLRs are usually dimers in their active formation, most are homodimers but some are heterodimers (e.g. TLR2 that dimers with TLR1 or TLR6) and some are linked to a co-receptor e.g. TLR4 that has the coreceptor MD2 (Kawai and Akira, 2011). The TLR appears to be oriented in U- or M-shapes, producing grooves in which substrates can bind. Once bound, TLRs initiate a signaling pathway by recruiting adaptor molecules such as MyD88, TIRAP or TRIF. In common for these are their binding action to the intracellular TIR domain of the activated TLR, which eventually activates transcription factors like NF-κB and MAP-kinases (fig 1.2). The choice of adaptor molecule and signaling pathway depends on which TLR subtype that is activated. Of the different cytosolic membrane TLRs, TLR 2 and 4 recruit the adaptors TIRAP and MyD88, whereas of the endosome-localized TLRs, TLR3 does not recruit MyD88 at all (McWhirter et al, 2005). Recruitment of MyD88 induces a following signaling pathway (in all TLRs except TLR3) by using IRAK signaling proteins. The details of MyD88-dependent signaling are complex but in short IRAK4 of the IRAK-family signaling proteins is recruited to MyD88 upon MyD88-activation of the TLR. This activates IRAK1 and IRAK2, these two bind MyD88 and recruit another signaling protein (TRAF3) and further down the pathway also TRAF6. A number of enzymes and other proteins are involved and the outcome is activation of NF-κB, several MAP-kinases and a regulation of the TLR-inducible genes. The effects of activated NF-κB and MAP-kinases are production of Type I Interferons and pro-inflammatory cytokines.

Instead of utilizing the MyD88 pathway, TLR3 activates the TRIF-dependent pathway. Due to the high number of TLRs, most cells have both MyD88-dependent and TRIF-dependent pathways working in parallel. There are feedback mechanisms and signal mediators that produce stimulating effects in one pathway but inhibiting effects in the other. One such example is TRAF3 which suppresses TRIF-dependent signaling in one pathway but increases MyD88-dependent signaling in another. The TLR subtypes can detect dsRNA (e.g. TLR3), ssRNA (e.g. TLR 7,8), dsDNA (e.g. TLR9), viral proteins or PAMPs on other structures such as lipopolysaccarides, fungi and protozoan (McWhirter et al, 2005; Kumar and Kawai, 2011).

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**Figure 1.2** MyD88-dependent signaling pathway of TLRs

After binding their ligand (the pathogen), TLRs activate an adaptor molecule (i) or a combination of adaptors. All TLRs except for TLR3 activate MyD88 which activates and recruits IRAK-family proteins (ii) which starts a signaling pathway that eventually activates NF-κB and MAP-kinases (iii). These up-regulate the expression of IFNs and pro-inflammatory cytokines, together producing a response to the infection (iv) that triggered the TLR activation.
1.2.3 Rig-1-Like receptors (RLRs)

Of the different PRRs, the DExD/H box RNA Helicases are ubiquitous detectors of double-stranded non-self RNA but also other nucleic acids of pathogens (table 1.1). This system has very specific targets and fills in the gaps of TLR pathogen-recognition. Included in the DExD/H box RNA Helicases are the Rig-1-like receptors (RLRs) which include three groups of receptors: melanoma differentiation-associated protein 5 (MDA-5), retinoic acid inducible gene 1 (RIG-1) and Laboratory of genetics and Physiology 2 (LGP-2). All RLRs are RNA Helicases with important regulatory functions in both infected and non-infected cells. The RLRs have a helicase domain responsible for binding the PAMP and two caspase activation and recruitment domains (CARDs), structures used for downstream signaling through MAVS (section 1.7) (McWhirter et al, 2005; Kumar et al, 2011; Kawai and Akira, 2006).

Many details concerning binding of RIG-1 and MDA-5 to foreign genome are still to be revealed and the “map” of the system is frequently renewed. It was previously thought that both cytosolic and endosomal viral recognition targets single-stranded 5’ phosphate bearing RNA as a PAMP (Pichlmair 2006). This because a 5’ phosphate-end is a hallmark of viral RNA, unlike cellular RNA which is equipped with a 5’ cap. However, recent studies have cast new light over this area, proving that 5’ P-equipped ssRNA is not recognized as a PAMP whereas dsRNA is (Kumar et al, 2011). It has been shown that RIG-1 detects dsRNA regardless of the 5’ ends having a triphosphate or monophosphate group. Also, it seems as if both RIG-1 and MDA-5 are capable of detecting dsRNA but different lengths of it, which may contribute to the understanding of how MDA5 and RIG-1 detect different viruses. The functions of LGP2 are less clear, but this family member of the RLRs lacks the CARD-domain that both RIG-1 and MDA-5 have. LGP2 (named after laboratory of genetics and physiology 2) seem to be important regulators of RIG-1 and MDA-5 signaling, both positive and negative in its regulations. LGP2 positively regulates RLRs in different ways depending on the virus detected. LGP2 also has the ability to negatively regulate RIG-1-mediated signaling through competitive inhibition of RIG-1, blocking PAMPs from binding, thereby possibly preventing over-stimulation of RIG-1 (Kumar et al, 2011; Broquet et al, 2011). As IFNs regulate the expression of RLRs, they are important in enhancing the anti-viral responses through feedback-looping which accelerates the production of RLRs and IFNs (Onoguchi et al, 2011). Being cytosolic, RLRs detect mainly virus and viral products; bacteria are mainly extracellular problems, but there are a few exceptions of bacteria that replicate intracellularly and do stimulate MDA-5.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genome</th>
<th>Type species</th>
<th>RLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picornaviridae</td>
<td>(+)ssRNA</td>
<td>Théllier’s virus</td>
<td>MDA-5</td>
</tr>
<tr>
<td>Caliciviridae</td>
<td></td>
<td>Mengo virus</td>
<td>MDA-5</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td></td>
<td>Murine norovirus-1</td>
<td>MDA-5/RIG-1</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>(+)ssRNA</td>
<td>Murine hepatitis virus</td>
<td>MDA-5/RIG-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>West Nile Virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dengue Virus</td>
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<tr>
<td></td>
<td></td>
<td>Japanese encephalitis virus</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Hepatitis C virus</td>
<td></td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>(-)ssRNA</td>
<td>Influenza A virus</td>
<td>RIG-1</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td></td>
<td>Newcastle disease virus</td>
<td></td>
</tr>
<tr>
<td>Rhabdoviridae</td>
<td></td>
<td>Sendai virus</td>
<td></td>
</tr>
<tr>
<td>Reoviridae</td>
<td>dsRNA</td>
<td>Reovirus</td>
<td>MDA-5/RIG-1</td>
</tr>
<tr>
<td>Poxviridae</td>
<td>dsDNA</td>
<td>Vaccinia virus</td>
<td>MDA-5</td>
</tr>
<tr>
<td>Herpesviridae</td>
<td>dsDNA</td>
<td>Myxoma virus</td>
<td>RIG-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epstein-Barr virus</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 List of ligands for MDA-5 and RIG-1, reconstructed from table in (Onoguchi et al, 2011). By targeting different PAMPs, RIG-1 and MDA-5 cover a large number of viral families and strains.
1.2.4 Nod-like receptors regulate IFN production and signaling

The NOD-like receptors (NLRs) are described having a nucleotide binding and oligomerization domain (NOD) together with Leucine Rich Repeats (LRR). A more detailed description of NODs comprises three domains instead of two; a C-domain, an N-domain and an intermediate domain. The C-domain is thought to be the PAMP-recognizing part with the LRRs (just like the TLRs); the N-domain has a death effector domain (DED), a pyrin domain (PYD), a CARD and baculovirus inhibitor repeats (BIR); the intermediate domain contains several NACHT-domains important for formation of the receptors. The many different actions of NLRs remain unclear, but they seem to be important regulators of IFNs and NF-kB. Regulation can be either positive or negative, which underlines the importance of NLRs in balancing the production and action of IFNs. Negative regulation includes blocking phosphorylation of IKKα and IKKβ which inhibits production of NF-κB (see section 1.3). Positive immunologic regulations by NLRs have several possible outcome, either stimulated production of pro-inflammatory cytokines through the action of NF-κB and/or MAP kinases or activation of the inflammasome (Kumar Kawai 2011). The inflammasome is a protein complex composed of one or several NLRs and other proteins. The complex converts pro-interleukins to active IL through caspase activation or degradation and is also capable of initiating apoptosis. NLRP3 inflamasomes are the so far most studied inflamasomes, and they are able to recognize a large number of pathogens. In summary, NLRs are important immunologic regulators, both positive and negative in action. (Kumar et al, 2011; Cui et al, 2010). The many effects and connections of NLRs remain to be discovered, and in the understanding of the system lies possibly great advances in understanding not only infections, but also other immune related conditions. Most cancers, MS, diabetes, asthma and neurodegenerative conditions such as Alzheimer’s are but few examples of the many diseases concerned by malfunctioning immune systems, all of them more or less under the control of NLRs.

Some examples of NLRs are NOD1 (CARD4) and NOD2 (CARD15), recognizing different peptide structures in pathogenic microbes. NLRX1 is another NLR, localized in the mitochondria and regulating the adaptor protein IPS1 (MAVS), important in interferon activation. NLRC5 is a strong negative regulator of NF-κB and IFN, but its own expression is down-regulated by the activation of NF-κB clearly pointing to the intricate signaling strategies of NLRs (Cui et al, 2010).

1.3 MAVS LINKING VIRAL RECOGNITION TO IFN PRODUCTION

Regardless of which of the PRRs that are activated, there are converging pathways activating a number of transcription factors, often including IRF3, IRF7, ATF2-c-Jun and NF-κB. As described in section 1.4 TLRs most often use MyD88 for its signaling (fig 1.2). RLRs on the other hand have a different system including the CARD and MAVS proteins (figure GHG). The CARD-like domains of the RLRs also exist in a signaling protein called mitochondrial anti-viral signaling protein (MAVS), also known as IPS-1, VISA or Cardif. MAVS, associated with mitochondria, uses its own CARD to bind to the CARDs of RLRs, acting as adaptor protein and binding the transcription factor TRAF6. TRAF6 regulates the IKK gene regulator, a complex controlling the expression of NF-κB, which in turn is translocated to the nucleus to regulate the IFN expression (fig 1.3). In summary, transcription factors are activated as RIG-1 binds pathogen structures; CARD domains of RIG-1 and MAVS bind and the CARD domains bind TRAF6. TRAF6 in turn controls the IKK complex leading to increased production of NF-κB (Broquet et al, 2011). As a result of combined actions of NF-κB and other transcription factors, expression of IFN is thereafter up-regulated. A second important feature of MAVS, except for inducing IFN production, seems to be inhibition of apoptosis during early stages of viral infection. This is a strategy to increase the cytokine production in the early warning system before the cell becomes apoptotic and cytokine production, together with all cellular functions, seizes (McWhirter 2005, Broquet 2011). This strategy is not unique to the case of viral infections alone, IRF3 and IRF7 are triggered by viral infections whereas ATF2-c-Jun and NF-κB can be triggered by numerous stimuli. As mentioned NF-κB is activated through the action of MAVS, so it seems to be active in both viral and non-viral infections (Kawai and Akira, 2006). According to McWhirter et al (2005), it is still unclear whether MAVS is linked not only to intracellular pathogen recognition through the action of RIG-1/MDA-5 but also to recognition of extracellular pathogen structures through TLRs.
The mitochondrial protein MAVS is an important adaptor in downstream signaling of RLRs. Both MAVS and the RLRs are equipped with a CARD domain, during signaling these CARD domains interact and join in connections to e.g. TRAF6, which eventually leads to the activation of NF-κB. NF-κB is translocated to the nucleus where it up-regulates translation of IFN-β.

**1.4 PKR INDUCES APOPTOSIS AND CONTRIBUTES TO IFN PRODUCTION**

Another receptor able to detect foreign genome is protein kinase R (PKR), an enzyme normally present in most cells but inactive until activated by binding dsRNA. Virally produced dsRNA binds PKR which then is autophosphorylated, becoming activated and able to phosphorylate its substrate: eukaryotic translation initiation factor 2 (eIF2). Under normal conditions, eIF2 mediates the contact between tRNA and the ribosomal subunits during translation, but if phosphorylated eIF2 becomes inactive and unable to promote translation. So viral infection causes PKR to phosphorylate eIF2, which no longer can facilitate translation, inevitably leading to apoptosis of the infected cell (Nakayama et al, 2010). Both viral and cellular translation is inhibited, eventually causing the cell to become apoptotic, a sacrifice worth the cost to stop the viral infection.

As with all parts of the immune system, simply upregulating PKR would not be beneficial. The many cellular functions controlled by PKR most often act through the activation of NF-κB and are delicately balanced. Examples of such cellular processes are the suppression of T-cell proliferation, regulation of cellular differentiation and balanced apoptotic pathways. Over-expression of PKR disturbs many of these processes and induces oncogenic effects (Sen G., 2001; Nakayama et al, 2010). Interestingly, PKR genes are amongst the ISGs (section 1.10) under control of IFNs. Further, IFNs can also act as substrates for PKR and as mentioned previously in this section PKRs contribute to the production of IFNs; PKR and IFNs stimulate each other.

**1.5 THE OUTCOME OF IFN PRODUCTION THROUGH THE JAK-STAT PATHWAY**

IFN-α and IFN-β are the first of all cytokines to be produced at higher concentrations during viral infections. IFN-γ and interleukins follow. These IFN-α and IFN-β bind receptors on the very cell from which they were secreted (auto secretion), but also on surrounding cells in amounts decreasing with distance from the original cell. The receptors bound are, for human type I IFNS, a heterodimer of IFNα receptor 1 and 2 (IFNAR1/IFNAR2). The dimers are trans-membrane proteins with an intracellular domain connected to Janus kinase 1 (Jak) and tyrosine kinase 2 (TYK2). As IFN-β binds the IFN receptors, Jak/TYK2 are phosphorylated, in turn phosphorylating STAT. The STAT1 and STAT2 trimerize with the interferon regulating factor 9 (IRF9), producing interferon-stimulated gene factor 3 (ISGF3), STAT1 also homodimerizes to produce IFN-γ-activated factor (GAF). ISG3 and GAF are re-localized to the nucleus, where they act as transcription factors on sequences containing IFN-stimulated response elements (ISREs) and IFN-γ-activated sites (GAS), both interactions lead to increased expression of the interferon-stimulated genes (ISGs) (Ganes Sen 2001, Garcia Sastre 2006).
The ISGs are more than 300 in total, encoding a vast number of different proteins. Most importantly, the expression of PRRs, PKR and Ribonuclease L (RNase L) are genes being up-regulated but also ISG15 and the protein Mx1. The increased number of PRRs aid in detecting foreign cytoplasmic genome and material, the increased levels of PKR inactivate more of the eIF2, resulting in reduced translation and eventually apoptosis. The RNase L is an enzyme that degrades foreign (and cellular) RNA after being activated by the action of a second enzyme; 2’5’-oligo-A-synthetase (OAS) which itself is activated by dsRNA; a uniquely viral compound. So in one sense, the OAS functions as a type of PRR by binding dsRNA and activating RNaseL which in turn degrades cytosolic RNA which inevitably kills both the intruder and the host cell.

All these pathways have the same final aim: to stop the viral infection. This is achieved through a number of measures like inhibiting cell growth, degrading viral genome, down-regulating MHC II, up-regulating MHC I, inducing apoptosis and preparing the surrounding cells by inducing them in an antiviral state. The secreted IFNs bind all surrounding cells regardless of whether they are infected or not. As mentioned above one of the effects produced by IFNs is increased levels of PKR, a hallmark of the anti-viral state, which itself does not trigger any effects unless the PRK is activated through the binding of dsRNA. So a cell non-infected but induced in an antiviral state contains no viral dsRNA, leaving the PKR inactive but still at high concentrations, being ready to act swiftly upon infection.

In addition to the proteins mentioned, IFNs can stimulate production and alteration of many other genetic products, one example is the Mx proteins mentioned above, these are directed towards Influenza virus. By inhibiting the cap-snatching feature of Influenza virus, the Mx proteins inhibit Influenza from replicating thus fighting its spread in the body. Except for controlling the ISGs, IFNs also activate and/or regulate a number of immune cells such as dendritic cells (DCs), cytotoxic killer cells (CTLs) and natural killer cells (NKs) (Garcia-Sastre et al, 2006). Once the transcription of Interferons has been up-regulated, the process is accelerated through feedback looping and a number of transcription factors, encoded by ISGs, further increase the transcription (Sadler and Williams, 2008). See section 1.8 concerning IFN-PKR feedback looping (fig 1.4)
1.6 DENDRISTIC CELLS (DCs) ARE IMPORTANT CARRIERS OF PRRs AND TLRs
DCs are present in large amounts in peripheral tissues e.g. mucosal membranes and skin, but also to some extent in muscles, scanning the surroundings for pathogens. Pathogens, parts of them or debris from dead or dying infected cells are engulfed by DCs which are capable of producing cytokines such as IFN upon pathogen recognition. Massive IFN production with the aim of inducing antiviral states in infected and surrounding cells is one strategy of the DCs in fighting viral infections. A second strategy, in parallel, is linking this detection to stimulation of an adaptive immune response. This occurs when the DCs have engulfed proteins or debris from pathogens or apoptotic infected cells and they initiate a process of self-maturation. After maturing, DCs are no longer capable of endocytosis and change their repertoire of receptors, also inducing a homing movement of the DCs towards lymph nodes. Arriving at a lymph node the DCs now carrying pathogen-associated structures activate T cells resident in the node. Through their dendrites, the DCs connect to T-cells and aid in this activation process. The dendrites connect to T-cells and help inducing an adaptive immune response towards the pathogen. Further contributing to the immune response is the secretion of pro-inflammatory cytokines from the mature DCs, a process different from the secretion of IFNs by the immature DCs.

1.7 MICRO RNA CONTROLS IT ALL
The interferon system is, of course, by far more complex than described so far in this thesis. The myriads of pathways, receptors and signaling molecules are overwhelming and the mere fact that the ISGs are more than 300 by numbers is frightening for anyone who tries to overview the entire system. One factor, still rather unexplored in its actions and connections, is micro RNA (miRNA). These are tiny stretches of cellular non-coding RNA (19-25 nucleotides), possibly induced by interferons and used to enhance antiviral effects, but also to regulate ordinary cellular gene expressions.

For miRNA there are two different strategies of regulating genetic expressions, either by degrading mRNA or by inhibiting its translation. The latter was previously considered as the more important strategy, it is however now believed that degrading mRNA is of greater importance (Guo et al, 2010). By binding the 3´UTR of target mRNAs (viral or cellular), translation of the mRNA is inhibited. Amongst miRNAs studied so far, nr 122 alone regulates expression of more than 400 genes. Many of these genes are important for cholesterol synthesis; down-regulating them could be harmful. However blocking this miRNA renders hepatocytes far less permissive for Hepatitis C viral infection (O´Neill et al, 2011). This interesting observation, the negative effect on one vital process together with one positive effect on anti-viral defense, depicts the complexity of miRNA and its actions.

Of the various targets for miRNA identified, a few are single proteins (e.g. MyD88, IL-10, and IRAK1) and several are TLRs. TLR2, 3 and 4 are targets of miR-105 and miR-223 respectively and most likely many other TLRs are targeted by other miRNAs. The aim of the miRNA targeting TLRs is, most likely, to regulate the TLR-mediated effects in order to avoid unnecessary pro-inflammatory effects. Many pathogens stimulate more than one TLR subtype, with the risk of inducing too high levels of inflammation as response to infection. Except for targeting the TLRs (more specifically the mRNA expressing the TLRs), the TLR pathways and/or transcription factors may be targeted by miRNA. Not only IFNs stimulate the production of miRNAs, but many different inducers are possible e.g. transcription factors. miRNAs can function in negative or positive feedback e.g. reducing the expression of the transcription factor that induced the miRNA itself (O´Neill et al, 2011).

1.8 INFLAMMATORY BOWEL DISEASE (IBD)
IBD is a category of gastro-intestinal inflammatory conditions with no solely clarified cause in common for patients, whether human or equine. As for the frequently diagnosed irritable bowel syndrome (IBS), the symptoms are of varying sort and degree all affecting the GI-tract. For both IBS and IBD, constipation or diarrhea, loss of appetite, discomfort and pain are common but for IBD there are by definition also inflammatory course of events. These inflammations often cause severe symptoms in addition to the bowel function abnormalities described for IBS; bleedings, vomiting, severe pains, cramps and diversified secondary effects due to inflammatory mediators and tissue damage. Amongst the types of IBD are two main diagnoses: Crohn’s disease (CD) and Ulcerative Colitis (UC). The two conditions have many similarities but CD is often more severe, more widespread along the GI-tract and histologically deeper in its inflammations whereas UC normally is limited to affecting the end parts of the intestine (colon and rectum) and causing inflammation in the mucosa alone. From an immunological point of view, IBD in general and CD in specific, causes mayhem in the normally delicately balanced GI-tract. Regardless of what initiated the disease, IBD weakens the intestinal epithelium, eventually to the point where the mucosa is leaking and luminal contents pass the barrier (fig 1.5).
The extensive amounts of microbes present in the lumen pose a serious threat when reaching beneath the mucosa; inflammatory responses are instant and may lead to chronic disease. Or does the chronic disease lead to weakening of the epithelium? As with autoimmune diseases the order of actions and whether the disease causes the abnormalities or vice versa is unclear, but once the disease is onset it nurtures itself. Observed in human IBD patients are altered expressions of TLRs, which is interesting and may contribute to understanding IBD. As noted by (Baumgart and Carding, 2007), TLR5 is normally not reachable by luminal pathogens due to the basolateral position of TLR5 in the epithelial cells, but in IBD, TLR5 is exposed and thereby possible to stimulate. TLR3 is down-regulated during Crohn’s Disease but not during Ulcerative Cholitis and TLR4 is up-regulated during both. TLR4 is known to induce IFN production (Kawai and Akira, 2010), so an increased expression of IFNs are likely during IBD. As depicted in this thesis, the vertebrate immune systems are however very complex, so the detailed effects of the different TLRs being altered may be difficult to determine but they may provide clues.

![Figure 1.5](from Baumgart and Carding 2007)

The left figure shows normal, well-balanced immune activities in proximity of the gastrointestinal lumen. The right figure shows cause of events during IBD; degraded epithelium provides a port of entry for microbes, dendritic cells are matured, interferons produced, chemo attractants recruit leukocytes from the blood, interleukins and histamine together with bradykinin produce tissue damage, pain and acute inflammation.
2 MATERIALS & METHODS

2.1 THE ORIGINAL PLAN

Before starting this project, the plan was to culture cells, infect them with different immunologic inducers, harvest some of the cells and extract RNA for gene expression analysis after six hours and the rest of the cells after 24 hours for protein analysis. Thereafter IFN expressions on protein and gene level were to be compared for the different infection conditions. Of the ten genes analyzed, three were housekeeping genes: GAPDH, B2M and 18S. The remaining seven were IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-ω and IFN-μ. As stated below, plans were slightly changed along the way but the project was still within Type I Interferons, using the same protocols for gene expression analysis as for the original plan.

2.2 CELL LINES AND INDUCERS

In investigating the connections between infection and expression of IFN and the genes encoding IFN, a number of different cell lines were used. The T888, equine embryonic leukocytes (EEL) and (prior to this experiment) peripheral blood monocytes (pBMCs) were cultured and split every 48 hours until stable conditions were established. Cells were infected with a number of commonly used immunological inducers; live equine herpes virus (EHV), Sendai virus, PolyI:C, PU, ODN. After infection, cells were incubated for six hours before a part of each suspension was extracted and stored in -20°C for gene expression analysis. The rest of the cell suspensions were incubated for another 18 hours (24 hours post infection), the incubation was stopped also in these samples and they were kept in -20°C for protein expression analysis. Six hours should be enough time for the cells to express mRNA encoding IFNs, whereas 24 hours is needed for the cells to express detectable amounts of the actual proteins (the IFNs).

2.3 RNA PURITY ANALYSIS

The samples incubated for six hours were centrifuged, cells were lysed and RNA extracted using Trizol before the purity of the RNA samples were to be measured using Nano Drop. Nano Drop is based on spectrophotometric analysis comparing the absorbance of the sample at different wavelengths. The quote of absorbance at 260 nm divided by the absorbance at 230nm indicates the purity of the RNA extract since RNA has its absorbance peak at 260nm and other compounds e.g. proteins have higher absorbance at 230nm. A second quote of abs at 260nm/abs at 280nm is calculated to determine possible contamination of EtOH, which is possible after washing the samples using EtOH. High or very low quotes are indicative of contamination and the RNA extraction is evaluated, possibly repeated to ensure the quality of the experiment. A quote of 2.1 indicates perfectly pure RNA.

2.4 ELISA ANTIBODIES

For the ELISAs and the ELISPOTs, the coating antibodies and the primary antibodies to be used were previously constructed during cooperation between SVA and Cornell University, US. The coating antibody 29B, a monoclonal anti-equine IFN-α antibody derived from rabbit, was previously indicated as the most efficient in combination with the biotinylated primary antibody 240-2 BioT (Wagner et al 2008).
2.5 ELISA OPTIMIZATION USING DOTBLOT
Having problems with degrading standard curve samples, I started evaluating and optimizing the ELISA using DotBlot, a method based on Western Blot though faster and easier as gel separation is not used. The sample is dotted on a nitrocellulose membrane, the membrane is blocked and incubated with primary antibodies having affinity for the protein in the sample, secondary antibodies are added after washing and finally a substrate is added. The primary antibodies are conjugated with a protein (in this case Biotin) to which the secondary antibodies bind with very high affinity. These secondary antibodies are conjugated with an enzyme, for this experiment Horse Radish Protease (HRP), this enzyme catalyses a reaction with the substrate added in the last step producing detectable changes on the membrane. Two different substrates were used; DAB producing visible colorations and the more sensitive ECL producing light emissive reactions detectable with photographic film.

Initially the DotBlot was set up using concentrations of antibodies and protein standard dilutions identical to those used in the ELISA. A second run was made using only the highest concentration of IFN-α standard (200U/mL), the 240-2 BioT were used at the same concentration but from a new batch and the secondary ab were switched from Streptavidin-HRP to polyclonal anti-mouse Ig-HRP derived from rabbit. A new run was made using stock concentration IFN-α of 1000U/mL + diluted 200U/mL and separate strips were made using either 29B (the coating ab) or 240-2 (the primary ab) as primary antibodies to the IFN-α and either of the two HRP-conjugated secondary antibodies (see fig). The aim was to cover more possibilities of revealing the problem in the ELISA setup. Due to negative results, the developing method was changed from the DAB to the more sensitive ECL that is developed using photosensitive film.

In parallel with DotBlots, an ELISA was set up with new reagents to exclude the possibility of degraded or contaminated chemicals. The coating antibody (29B), the primary antibody (240-2 BioT) and the secondary antibodies (Streptavidin-HRP or anti-mouse-HRP) were all evaluated for their affinities and functions. The results were not totally uniform so the decision was made to retry the ELISA using a total of 81 different conditions to determine optimal setup. All antibodies were used in three different concentrations (pure IFN-α at 1000U/mL or samples previously measured using ELISA, having 1000U/mL or 1889U/mL) and samples were added in a strategic manner to determine the sensitivity of the ELISA at different protein concentrations.

2.6 SLIGHT CHANGE OF PLANS
When finally being ready to run all samples to investigate their contents of IFN, new problems arose. Due to delivery problems of our antibodies, we finally decided to go for a second plan. A collaboration was initiated with a research group at the University of Agricultural Sciences (SLU), also performing immunological research in horses. My plan was slightly changed, from investigating type I Interferons in equine cell lines to investigating them in equine intestinal biopsies. The aim of the “Intestinal project” was to study samples of horses diagnosed with Inflammatory Bowel Disease (IBD). So my project was diverted to analyzing gene expression of the Type I Interferons we had been working with, using the same reagents, equipment and protocols as previously.

2.7 BIOPSY SAMPLES
Samples were collected by a veterinary, for the dead horses this was performed at a slaughterhouse and within 40 minutes after time of death, samples stabilized in RNAlater. Initially, rectal biopsies from six healthy horses were analyzed to determine gene expression of IFNs under normal physiological conditions. Then biopsies of five horses diagnosed with IBD (table 2.1) were analyzed, finally two different sets of biopsies from the first six healthy horses were also analyzed. In total, biopsies from three different positions (jejunum, colon, rectum) in six healthy horses and rectal biopsies from five sick horses were included in the project.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Health status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>Healthy</td>
</tr>
<tr>
<td>7</td>
<td>IBD; chronic, unspecific eosinophilic proctitis</td>
</tr>
<tr>
<td>8</td>
<td>IBD; mild, unspecific chronic superficial proctitis</td>
</tr>
<tr>
<td>9</td>
<td>IBD; severe transmural ulcerative, eosinophilic colitis with vasculitis</td>
</tr>
<tr>
<td>10</td>
<td>Acute cholic; mild, superficial chronic active unspecific proctitis</td>
</tr>
<tr>
<td>11</td>
<td>IBD; mild superficial active unspecific proctitis</td>
</tr>
</tbody>
</table>

Table 2.1 Three biopsies (one jejunal, one rectal, one colonal) were gathered from each of the six healthy horses. One rectal biopsy was taken from each of the five diseased horses.
2.8 REAL-TIME QUANTITATIVE PCR (RT-qPCR)

The RT-PCR was performed in three major steps: purification, reverse transcription and amplification with real-time detection (qPCR). The purification involves using the enzyme DNase to denature any DNA present in the samples. The pure RNA is then combined with the enzyme reverse transcriptase (RT) and again run in a thermal cycler, now producing cDNA, ready to be amplified and analyzed in the qPCR. Three housekeeping genes (HKG) were used; the glycolysis enzyme GAPDH; B-2-Microglobulin, part of the MHC complex; the ribosomal subunit 18S. The purpose of the HKGs is to provide a standard level of genetic expression, the genes of interest are then related to the expression of the HKGs. This is important because under varying circumstances, the entire translational machinery may be down-regulated or the cells may for any other reason have reduced translation which would produce false negative results unless gene expression always is related to HKGs. All primers were previously set up by my supervisor for equine IFN research. The DNAse treatment was made with Qiagen DNAse I kit containing DNAse, DNAse buffer and stop buffer. 1 µL of DNAse was used for every unit of RNA treated according to the manufacturer’s instructions. The RT reaction was performed using a complete Reverse- Transcriptase Mastermix from Applied Biosciences. The qPCR was run using a SYBR Green system (see section 2.9).

2.9 CT-VALUES

SYBR Green is a fluorescent that intercalates double-stranded DNA and emits detectable light during the qPCR run. As the genes of interest (GOI) are amplified through both forward and reverse primers, complementary strands of the GOI are formed, SYBR Green intercalates and the amounts of the GOI can be detected. The qPCR runs in cycles, each new cycle increases the CT value by one. When the amounts of DNA for a GOI are sufficient to produce a detectable signal in the qPCR, the sample is dedicated its CT-value. Low amounts of DNA take many cycles to amplify to detectable levels, thus resulting in high CT-values. For samples with high concentrations of DNA, low CT-values are registered. By relating these CT-values to the CT-values of samples of housekeeping genes, a relative gene expression can be determined.

All tables in the results section are lists of CT-values obtained by qPCR analysis on cDNA produced and amplified from mRNA coding equine IFNs. Of the ten genes analyzed, three were housekeeping genes as mentioned previously: GAPDH, β2M and 18S. The remaining seven were IFN-α, IFN-β, IFN-δ, IFN ε, IFN K, IFN-ω, IFN-μ. Plus signs indicate RT positive samples, in which reverse transcriptase was used to convert RNA to cDNA. Negative signs indicate RT negative samples in which non-RT was used to produce negative controls of the cDNA-reaction. CT-values should ideally be 40 or above for RT negative samples.

In every cycle, the amounts of DNA are duplicated so the quantities increase exponentially. Each complete cycle represents a CT-value in the qPCR, starting at one. In my project the qPCR was set to stop at 45 cycles at which the amounts of DNA have been amplified $2^{45}$ times which is about 35,000 billion more DNA than in the starting material.

Difference in CT-values of the HKG and the gene of interest (GOI) is denoted delta ($\Delta$). The expression of the GOI compared to the HKG is calculated as $2^{\Delta CT}$, because each CT-value represents a duplication of amounts of DNA (fig 2).
3 RESULTS AND DISCUSSION

3.1 ONLY IFN-ε, IFN-Κ AND IFN-μ WERE DETECTABLY EXPRESSED IN HEALTHY HORSES
Comparing the expressions of the different IFNs related to that of housekeeping genes in rectal biopsies from the first six healthy horses investigated, a distinctly higher level of IFN-Κ was observed for several samples (fig 3.1). IFN-ε was also noted as interesting although in modest levels compared to IFN-Κ. Further experiments were aimed at investigation of the expression of these two genes. For all other IFNs the levels of expression are close to undetectable. This is not surprising since IFNs are primarily anti-viral cytokines and therefore should not be quantitatively expressed in healthy animals. These first samples were used to investigate the overall expression of IFNs and to narrow down the following experiments.

![IFN expression in healthy horses 1-6](image1)

Figure 3.1
Expression of IFNs measured in rectal biopsies from six healthy horses (named H1G-H6G) related to the average expression of housekeeping genes. All IFNs are at undetectable levels; Kappa and Epsilon being the only exceptions.

3.2 DISEASED HORSES ALSO SHOWED LOW LEVELS OF OVERALL IFN EXPRESSION
Proceeding to diseased horses, all having GI-infections, very low levels of IFN expressions were surprisingly observed (fig 3.2). Some degree of elevated IFN expressions due to inflammation was expected, at least for some of the IFNs. Once again, the expression of IFN-Κ was higher than for other IFNs as previously noted for the healthy horses. A plausible explanation to the low levels of IFN expression is the down-regulation of IFNs achieved by pathogens during prolonged infections. It is also possible that the amounts of pathogens in the horses were already reduced enough for the immune system to inhibit further infection. If so, the present inflammation could be caused by a combination of pro-inflammatory cytokines, tissue damages and the effects of mast cells and other immuno-mediators. Having very low levels of pathogens reduces the need for IFNs, which could explain why they are not highly expressed.

![IFN expression in diseased horses](image2)

Figure 3.2
All IFN genes investigated in diseased horses H7-H11 displayed using the same scale as in figure 3.1. As for the healthy horses, the diseased display close to undetectable levels of IFNs except for IFN-Κ and possibly IFN-ε. The expressions of IFN-Κ and IFN-ε are further analyzed in section 3.3-3.5.
As mentioned in section 1.2.4, NLRs are important positive/negative regulators of both pro-inflammatory cytokines and IFNs, so a higher NLR-activity could be associated with increased inflammation; possibly in combination with a reduced expression of IFNs, if IFNs are not needed they will be down-regulated to maintain balance. Further mentioned in 1.2.4 is the inflamasome; if this complex is initiated during infection to produce an inflammatory response, perhaps the inflammation itself is prolonged until the inflamasome and its produced pro-inflammatory cytokines are inhibited and degraded. And since IFNs are more likely to be expressed during earlier stages of the infection to produce its response, the low levels of IFNs might be understandable.

3.3 IN THE HEALTHY HORSES, IFN-κ SHOWS HIGHER EXPRESSION IN RECTUM
Focusing on IFN-κ and the three different regions of biopsies, samples from rectum display the highest IFN-κ expression (fig 3.4). This higher expression in rectal biopsies may have several explanations. The rectum is by far more exposed to external threats than is jejunum or colon, thus making the need for pathogenic protection higher in this area; a greater activity of the Interferon-system. A simpler explanation could be differences in biopsy procedures; rectal tissue samples are due to the anatomy of the horse (or any mammal) differently excavated compared to those of the inner intestines which always are taken from deceased animals. Having the intestine placed on a hard surface, easier available for sample collection, also makes a difference in which cell layers are included in the biopsy. Tissue layers having higher concentrations of IFN-producing cells may thus be included in different amounts depending on how the biopsy is performed.
3.4 COMPARING EXPRESSIONS OF IFN-ε AND IFN-κ IN THE DISEASED HORSES
For the rectal biopsies of the diseased horses investigated, levels of IFN-K are clearly higher than those of IFN-ε but compared to the expressions in healthy horses (fig 3.3), levels are low.

![Figure 3.5](image)

Expressions of IFN-K (red) and IFN-ε (blue) in diseased horses, all biopsies from rectum. Both IFNs are expressed at low levels in these diseased horses, compared to that of

3.5 EXPRESSIONS OF IFN-κ VARIED IN ALL GROUPS
Returning to the expression of IFN-K, now averaging levels of expression of both the six healthy horses and the five diseased horses examined, results are varying (fig 3.6). Despite using 95% CI, the results lack statistical significance. Reasons for this have already been considered in sections 3.1-3.2 and include polymorphism, variations in biopsy procedures and individual differences of the horses and their conditions.

![Figure 3.6](image)

Comparison of the average expression levels of IFN-K in all biopsies, 95% CI. In total, 18 biopsies were taken from healthy horses; one from each intestinal position on six horses (blue). Five rectal biopsies were taken from diseased (red) horses. Clearly, there is no statistically significant correlation between IBD and levels of IFN-K in this experiment but a variation larger between individuals rather than between groups.
4. SUMMARY AND CONCLUSIONS

No statistically significant correlations were found between mRNA levels of Type I Interferons and IBD in the horse biopsies analyzed. As mentioned in section 3, this may depend on variations in biopsy procedures and/or down-regulation of IFNs either caused by normal regulation as IFNs are not highly expressed in late stages of inflammation or due to IFN-downregulating mechanisms of pathogens involved. Large individual variations of IFN expression were also observed, to some extent explained by the complex regulatory systems of IFNs. The higher degree of IFN expression in rectal samples as compared to biopsies from jejunum or colon might be explained by the elevated need for immunologic protection in parts of the intestine more exposed to external threats.

The variations in results depict the reality of studying cytokine expressions in live animals rather than in cell cultures, having large biological variations rather than homogenous conditions. Clearly, however, IFN-κ is expressed in both healthy and diseased horses. Future experiments could be based on more biopsies from diseased horses, preferably taken from different parts of the intestine as for the healthy horses, in order to increase the possibility of finding statistically significant correlations. If there still are no significant correlations, one could set out to trace the factors effecting the large individual variations in order to understand the complex IFN systems.

*IFNs are of great importance in both veterinary and human biomedical sciences; all efforts in revealing its secrets are worth the costs.*

5. ACKNOWLEDGEMENTS

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Equine biopsies were kindly provided by our collaborating research group at the Department of Biomedicine and Veterinary Health Science, University of Agricultural Sciences (SLU), Uppsala; Lic.Vet., PhD Student Karin Olsson; Professor Ronny Lindgren; Professor Caroline Fossum.
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