# How does *Vaccinia* virus inhibit the detection of cytosolic DNA by the innate Immune system*?*

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I declare that this thesis is my own work and has not been submitted in part, or as a whole, for the award of a higher degree elsewhere

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## Acknowledgments

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## Abstract

Vaccinia virus (VacV) is a large dsDNA virus belonging to the *Poxviridae* family. Due to its size, the VacV replication cycle occurs entirely within the cytoplasm of infected cells. This exposes the virus to the many intracellular DNA sensors found in human cells. Since its discovery in 2013, cyclic GMP-AMP Synthase (cGAS) has been shown to be the predominant cytoplasmic DNA sensor in many cell types, including keratinocytes. Once activated, cGAS generates the second messenger cGAMP to trigger IFNβ production, through the adaptor protein STING. VacV can limit IFN production by inhibiting this pathway at multiple points, but little is known about whether VacV is able to inhibit DNA sensing directly. To investigate this, intracellular DNA sensing within keratinocytes was analysed during VacV infection. Results showed that within 4 hours of infection VacV was able to induce the loss of cGAS to undetectable levels, effectively blocking the cell IFNβ response. Additional DNA sensors that cooperate with cGAS such as IFI16 remained unaffected, showing that cGAS is essential for the IFN response to VacV. Inhibition of host degradation pathways did not prevent cGAS loss during VacV infection, suggesting cGAS is directly targeted for enzymatic cleavage. This demonstrates a potential new mechanism VacV employs to limit DNA sensing through cGAS, although the viral proteins responsible remain elusive. Identification of these proteins may provide new treatments for certain autoimmune disease where overactivation of cGAS contributes to disease phenotype.

# 1 Literature Review

## 1.1 *Vaccinia* Virus

### 1.1.1 History

*Variola* virus, the causative agent of smallpox, was once a devastating disease which resulted in epidemics in Europe up until the 19th century in addition to major outbreaks in Asia and Africa during the 20th century (WHO, 1979). At its highest incidence smallpox was prevalent worldwide and had an associated mortality rate of 30%, with survivors left permanently disfigured by the characteristic skin lesions (WHO, 1979). The origin of smallpox remains a mystery, but is believed to have emerged around 10,000 BC in northern Africa (Barquet and Domingo, 1997). Prior to the discovery of viruses and the immune system, it was common knowledge that smallpox survivors gained a lifelong immunity to the disease, which lead to the practice of disease survivors treating those affected (Gross and Sepkowitz, 1998). Consequently the first attempts at inducing smallpox immunity involved inoculating individuals with pustule debris from infected individuals, termed Variolation (Barquet and Domingo, 1997). Although often effective, the inherent risk of developing disseminated smallpox was still a concern, thus a new revolutionary treatment was needed; a treatment made possible by the work of Edward Jenner. For many years Jenner had heard stories of dairymaids who contracted minor infections with cowpox and subsequently gained a lifelong protection from smallpox (Barquet and Domingo, 1997). This lead to his conclusion that cowpox infections gave protection from smallpox, thus inspiring him to utilise this as a deliberate mechanism of protection (Gross and Sepkowitz, 1998). In 1796, Jenner took pustule material from a dairymaid with fresh cowpox lesions and used them to inoculate a young boy, who subsequently developed cowpox (Riedel, 2005). After recovery, Jenner then inoculated the boy with material from a fresh smallpox lesion but no disease developed. Jenner concluded that cowpox inoculation gave protection from smallpox. By using the Latin word for cow, *vacca,* and the term for cowpox, *vaccinia*; Jenner decided to call this process vaccination (Riedel, 2005). Due to the early work of Edward Jenner and a global immunisation effort lead by the World Health Organization smallpox was declared eradicated in 1980 (WHO, 2018). The key component was *Vaccinia* Virus (VacV).

VacV has a long and shrouded history mainly due to its unknown origins. Analysis of VacV DNA has further complicated this, as it has been shown to be both closely related, and yet distinctly different from other members of the *Orthopoxvirus* genus including *Variola* and cowpox (Esposito and Knight, 1985). Further investigation into VacV based vaccines has shown that they are comprised of a mixture of multiple, closely related viral strains, termed a quasispecies, as well as bacteria and bacterial debris contaminants (Fenner et al., 1988). This knowledge, paired with the fact that the VacV genome shows striking instability and

high rates of recombination between different virus strains (Coulson and Upton, 2011), makes identification of VacV origin an almost impossible task. Despite its unknown history, VacV has been effectively used in smallpox eradication since the introduction of the live-virus vaccine Dryvax (Poland et al., 2005). Although effective, VacV based vaccines were phased out due to serious side effects, especially when a history of eczema was present (Fulginiti et al., 2003). This vaccine was eventually replaced by the safer, Modified *Vaccinia Ankara* (MVA) based vaccines which, due to the restrictive replication of MVA, could be given to immunocompromised patients (Stittelaar et al., 2001). The combination of the efficacy of the VacV based vaccines and the apparent lack of an animal reservoir for *Variola* resulted in the eradication of smallpox in the 1980s (Fenner et al., 1988).

Although smallpox has been eradicated for almost 40 years, research into VacV and MVA is still of great importance, with the requirement for safe VacV and smallpox vaccines being driven by concerns of re-emergence. Serological based evidence show that VacV outbreaks involving bovines, horses and rodents have been reported in South America, with evidence of zoonosis to humans (Borges et al., 2018). Although discovery of a previously unknown natural reservoir is doubtful, re-emergence of smallpox via bioterrorism is an unlikely but valid concern (CDC, 2016). The most promising research avenue for VacV is its use in recombinant vaccines. By utilising recombinant DNA technology and exploiting the genomic features of VacV, recombinant vaccines could provide promising results against diseases such as HIV and Influenza (Sánchez-Sampedro et al., 2015).

### 1.1.2 Structure and Genome

*Poxviridae* is a large family of viruses comprised of two subfamilies *Entomopoxvirinae* (insect hosts) and *Chordopoxvirinae* (vertebrate hosts), with 41 species that have vertebrate hosts. However the genus of greatest interest is *Orthopoxvirus*, which contains both VacV and *Variola* virus (ICTV, 2018). All members of the *Poxviridae* family share the same characteristics; a large linear double stranded DNA genome, a replication cycle that occurs entirely in the cytoplasm, and a complex virion structure. The most notorious member of the family is the causative agent of smallpox; *Variola,* and the most studied and widely used in laboratories is VacV.

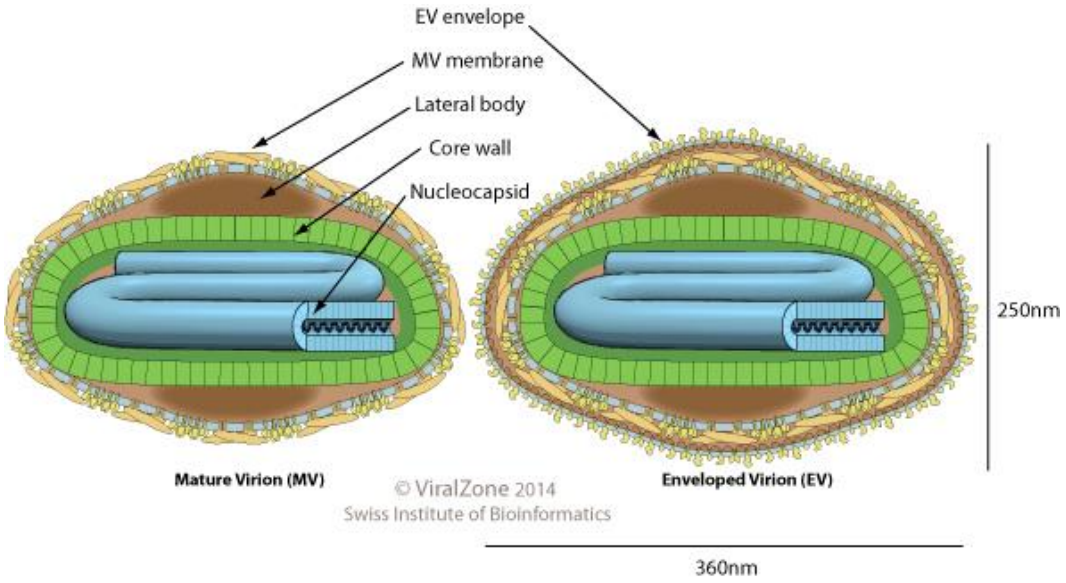


**Figure 1 : Morphology of the VacV mature and enveloped virion**

*The structure of the VacV virion. Shown is the single lipoprotein bilayer only present on the enveloped virion (EV). The VacV virion, like all poxviruses, has the unique biconcave oval shape with flanking lateral bodies. The large nucleocaspid is surrounded by the core wall which is characterised by a lipid core membrane and a protein palisade layer. This protects the nucleic material in the virion (SIB, 2018).*

VacV, like all poxviruses, has an ovoid shaped membrane with multiple internal structures including a biconcave walled core that is flanked by lateral bodies (**Figure 1**). The enveloped virion (EV) is around 360 x 250 nm and is encapsulated by a single lipoprotein bilayer (Cyrklaff et al., 2005). The genome of VacV is around 200kb which encodes more than 200 proteins that function in viral entry, replication, virion assembly and host immune evasion (Johnson et al., 1993). The large size of the VacV genome allows multiple foreign genes to be inserted to create a recombinant virus. Although the maximum foreign gene size that can be inserted into the VacV genome is yet to be determined, poxvirus vectors have been shown to have the capacity for at least 25kb DNA inserts, without affecting their replication (Smith and Moss, 1983). This, paired with the high immunogenicity of poxviruses and their high levels of gene expression, makes them an ideal candidate for the production of recombinant vaccines. Due to poxvirus replication being solely in the cytoplasm there is also a low risk of interaction with the host's genome. Moreover genetically engineered VacV is also yielding promising results for cancer therapy (Thorne et al., 2005). Recombinant VacV virons preferentially propagate in cancer cells resulting in lysis of tumour tissue and can be engineered to express anti-cancer genes (Haddad, 2017).

Due to possible of side effects when wild type VacV is used, most recombinant vaccines use the attenuated strain Modified *Vaccinia Ankara* (MVA). MVA is distinctly unique from all known strains of *Vaccinia* and other members of the *Orthopoxvirus* genus. It does not occur naturally and displays reduced virulence in humans and other mammals (Mayr et al., 1975). The immunogenicity of MVA is the same as VacV, hence its interest in vaccine development. MVA was derived from 570 serial passages of Chorioallantois *Vaccinia* virus *Ankara* (CVA) in chicken embryo fibroblasts (Mayr et al., 1975). The MVA genome is much smaller than VacV or CVA, around 178kb compared to 200kb (Antoine et al., 1998). This is because during cell passage, six major deletions occurred within the CVA genome which resulted in the loss of around 24,000 nucleotides and caused 51 fragmented open reading frames (Meisinger-Henschel et al., 2007, Meisinger-Henschel et al., 2010). These deletions removed many human innate immune evasion proteins, hence its low virulence phenotype.

### 1.1.3 Replication

VacV has been shown to infect and replicate within a wide range of cell types such as; keratinocytes, fibroblasts, and leukocytes (Sánchez-Puig et al., 2004, Liu et al., 2005). However, VacV shows preferential replication within dermal cells, specifically fibroblasts and keratinocytes, causing the characteristic skin lesions in the infected host (Liu et al., 2005). VacV has also been shown to infect T cells and dermal dendritic cells, although this results in an abortive infection (Liu et al., 2005). HaCaT cells were the chosen cell line used in this project for VacV and MVA infections. HaCaT cells are a spontaneously transformed immortal keratinocyte cell line, that is similar to normal human keratinocytes when grown *in vitro* (Boukamp et al., 1988).

VacV has two main infectious forms, mature virion (MV) and extracellular/enveloped virion (EV), therefore the virus has different cell entry mechanisms dependent on the infectious form. The main process in which VacV facilitates host cell entry is via macropinocytosis as it is the most appropriate for endocytosis of large particles (Mercer et al., 2010). The process of endocytosis begins when virus-receptor interactions occur, resulting in intracellular transportation prior to membrane fusion (Liu et al., 2014). This process is also similar between VacV strains, however each strain uses a distinct form of macropinocytosis relying on different host receptors and entry mechanisms (Mercer et al., 2010).

After the virus enters the cell and disassembles, infection-specific concentrated cytoplasmic domains form; referred to as “viral factories” (Tolonen et al., 2001). These factories form immediately following host cell entry, before viral replication, and consist of cellular derived components, specifically an endoplasmic reticulum derived membrane cisternae which

facilitates viral replication (Tolonen et al., 2001). DNA replication, protein translation and virion assembly all occur within these viral factories (Katsafanas and Moss, 2007). After viral replication and assembly, scaffold proteins are removed and membrane restructuring occurs resulting in formation of the mature virion. Many viral and host proteins have been found to play a role in this restructuring, but the exact process remains controversial (Liu et al., 2014). Mature virions are then transported out of the viral factories to be further processed into an enveloped virion. This is assisted by host microtubules trafficking these MV to the cell surface. During this translocation the virion becomes encapsulated in a double membrane derived from the trans-Golgi network, this results in the formation of the EV (Liu et al., 2014, Roberts and Smith, 2008). When at the cell surface, fusion with the plasma membrane occurs, where the formation of an actin tail below the membrane associated EV promotes its release from the cell (Horsington et al., 2013).

## 1.2 Innate Immunity: Overview

The Innate immune system is a non-specific host defence mechanism that responds rapidly to microbial exposure. The aim of this response is to limit the spread of any potential pathogens within the body as well as activating the more specific adaptive immune response (Iwasaki and Medzhitov, 2015). Microorganisms are initially recognised by the host germline-encoded pattern recognition receptors (PRRs), by interacting with their corresponding pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). These PAMPs are usually essential components of the microorganism and are therefore rarely altered, a factor exploited by PRRs. There are several PRRs each tailored towards activating a specific branch of the immune system; of which the family of Toll-like receptors (TLRs) are one of the largest and most studied (Medzhitov and Janeway, 2000). This, paired with the constitutive expression of PRRs, is the conserved initiation point of innate immune processes.

The innate immune system consists of several parts and mechanisms that interplay to limit microbe entry, dissemination and ability to cause disease. The key elements of the innate immune system include physical barriers, humoral anti-microbial products and cell-mediated defence mechanisms (Romo et al., 2016). A summary of key components in the innate immune system is represented in **Figure 2**.

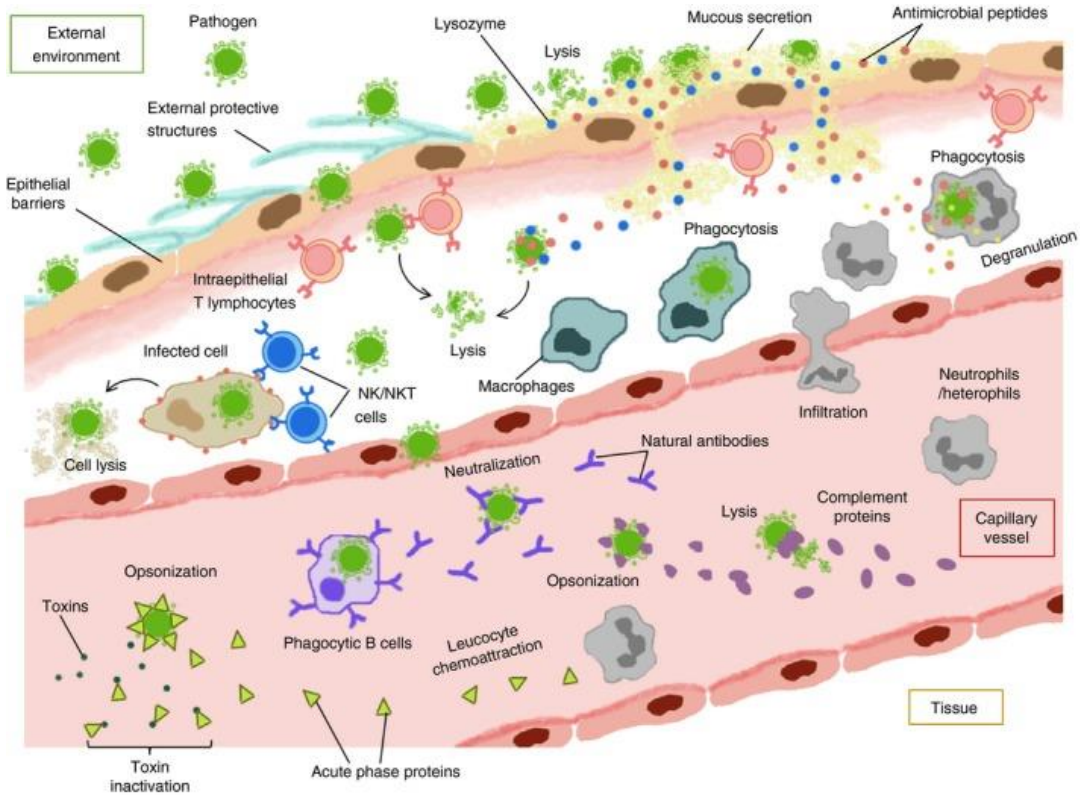


**Figure 2: An overview of the innate immune system.**

*An overview of innate immune components within humans. The function of physical and chemical barriers, humoral immunity, and cell mediated immunity are shown. The pathogen shown is non-specific and may represent either bacterial, viral or fungal, depending on the mechanism shown (Romo et al., 2016).*

### 1.2.1 Physical barriers

Physical and chemical barriers have a vital role of isolating the internal environment from external elements, such as potentially pathogenic microorganisms as well as harmful substances (Elias, 2007). The main physical barrier in humans is the epidermis, specifically the stratum corneum (Elias, 2005). The epidermis functions as a barrier against a broad set of stimuli; for example, pathogenic microorganisms, foreign chemicals, oxidative stress, and is able to act as a cutaneous inflammation interface (Elias, 2007).

### 1.2.2 Humoral innate immunity

The humoral innate immune response encompasses all soluble macromolecules found in extracellular fluids that contribute to antimicrobial activity. This is one of the largest branches of the innate immune system as it consists of multiple components, including pentraxins, natural antibodies, and the complement system (Shishido et al., 2012).

**Pentraxins**

Pentraxin are a family of evolutionary conserved, multimeric proteins characterised as a class of PRR. They are acute phase proteins that are synthesised rapidly during infection, therefore are often used as biomarkers for infection and inflammation (Du Clos and Mold, 2004). There are two main types of pentraxins; long and short. This is denoted by the presence or absence of further domains, in addition to the common C terminal domain. Short pentraxins include C-reactive protein and serum amyloid P, whereas the primary long pentraxin is pentraxin 3 (Pepys and Baltz, 1983). The main role of pentraxins is to recognise and bind to pathogens and apoptotic cells. This interaction is then recognised by macrophage Fc receptors facilitating clearance of cell debris and potential pathogens (Mold et al., 2001).

**Natural Antibodies**

Produced mainly by the B1 subset of B lymphocytes, Natural antibodies (NAb) are non-specific immunoglobulins synthesised by cells in the absence of pathogens (Baumgarth et al., 2005). Their function is similar to that of pathogen specific antibodies, however NAb can recognise a wide range of epitopes but exhibit low binding affinity. Despite this they are essential for clearance of cellular debris, recruitment of complement components and modulating the adaptive immune response (Ochsenbein et al., 1999).

**Complement**

The complement system is a complex cascade composed of serum proteins that interact with pathogens resulting in inflammation, opsonisation and lysis of microbes (Stoermer and Morrison, 2011). Activation can occur via three converging pathways; classical, alternate, and mannose binding lectin pathway (Dunkelberger and Song, 2010). Although each pathway differs mechanistically, they all result in the cleavage of C3 and C5 by their respective convertase enzymes causing C5b deposition, initiating the common terminal pathway (Dunkelberger and Song, 2010). The action of the complement cascade results in the formation of a membrane attack complex, which results in lysis of either microbes or infected cells.

### 1.2.3 Cell mediated innate immunity

Cell mediated innate immunity is a major part of the innate defence against intracellular and extracellular pathogens, with the main cellular components functioning as either phagocytic cells or cytotoxic cells (Alberts et al., 2008). The vast majority of these innate immune cells derive from myeloid precursor cells, with the exception of natural killer cells and B1 lymphocytes; both of which arise from a common lymphoid progenitor (Alberts et al., 2008).

The main cellular components of the innate immune system are professional phagocytes; macrophages and granulocytes, and natural killer cells (NK cells).

**Phagocytes**

The main cellular components of the innate immune system are myeloid phagocytic cells, more commonly referred to as professional phagocytes. These type of leucocytes include neutrophils, monocytes, macrophages, basophils and eosinophils (Alberts et al., 2008). The main role of these cells is to engulf (phagocytose) and destroy pathogens, as well as secrete cytokines and soluble mediators, such as histamine, lysozymes, and interleukin 12 (IL-12) (Tripp et al., 1993). As well as destroying pathogens, the soluble mediators released, especially IL-12, drive NK and T lymphocytes to produce interferons (IFN), specifically IFNγ. Which in turn leads to further activation of macrophages, increasing their antimicrobial activity (Tripp et al., 1993).

**Natural Killer cells**

Natural killer cells are a large granular lymphocytes within the innate immune system and are remarkably similar to T and B lymphocytes of the adaptive immune system. Unlike T and B lymphocytes, NK cells do not undergo somatic hypermutation, therefore do not alter receptors from their germline structure (Caligiuri, 2008). Instead NK cells express a repertoire of receptors, both inhibitory and activating, that are tailored to ensure self-tolerance while rapidly responding to microbial challenge. Once activated NK will initiate apoptosis in virally infected cells as a means to limit viral replication and spread (Vivier et al., 2011). As well as this, NK cells have also been shown as the major producer of cytokines, both inflammatory (TNFα) and immunosuppressive (IL-10) (Moretta and Moretta, 2004).

## 1.3 Innate immunity to viruses

Due to their mechanism of action, phagocytes are more tailored to destroying extracellular pathogens, mainly bacteria and parasites, whereas NK cells are more tailored to intracellular pathogens such as viruses. Primary infected cells, such as keratinocytes during VacV infection, have their own intracellular innate processes to detect and limit viral infections. Host cells are able to recognise viral infection and mount a strong antiviral response to destroy and limit the spread of the pathogen. This occurs by the innate immune system recognising viral components through pattern recognition receptors (PRRs) (Medzhitov, 2007). There are three main classes of PRRs responsible for recognition of viral components, Toll-like receptors (TLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs),and retinoic acid-inducible gene-I-like receptors (RLRs) (Hansen et al., 2011) . These PRRs can recognise a wide variety of viral components including DNA, single

stranded RNA, 5'-triphosphate capped double stranded RNA, and soluble viral proteins (Hansen et al., 2011). Of the aforementioned receptors, TLRs and RLRs are important for the production of type I interferons and other various cytokines, whereas NLRs have a more regulatory role.

### 1.3.1 Toll-Like Receptors

Toll-like receptors are a family of membrane spanning, non-catalytic receptors commonly expressed on sentinel immune cells such as macrophages. There are currently ten known TLRs in humans each activated by a specific ligand, covering a wide range of different molecular patters found in microorganisms (Tartey and Takeuchi, 2017). They are found in two primary locations within cells, either at the cell surface or compartmentalised within the cell inside an endosome (Botos et al., 2011). All TLR family members are structurally similar with the presence of a leucine-rich repeat domain within their extracellular region, a transmembrane domain, and a conserved Toll IL-1 receptor (TIR) domain; named due to their homology with IL-1 receptor signalling domains (Botos et al., 2011). Irrespective of their ligands, the TIR domains of TLRs converge and activate several common signalling pathways, resulting in either the activation of mitogen activated protein (MAP) kinase and the transcription factor NF-κB to stimulate cytokine production or activating interferon regulatory factors (IRFs) to promote type I interferon production (Beutler, 2004). For viral infections the most important TLRs are TLR3, TLR7 and TLR9, as these detect double stranded RNA, single stranded RNA, and unmethylated CpG oligodeoxynucleotides, respectively (Tartey and Takeuchi, 2017). However, TLRs are limited in their capacity to detect viral components, as they can only detect viruses within the extracellular and endosomal spaces. However other PRRs, NLRs and RLRs, are able to cover this potential gap.

### 1.3.2 RIG-I-like Receptors

Upon host cell entry, many viruses are able to avoid exposing their genomic material to endosomal TLRs, specifically viruses that contain a lipid envelope. Despite this, activation of the type I IFN response to viral infection is achieved by cytoplasmic detection of non-self RNA by the RLR family of receptors. There are three known RLRs; retinoic acid-inducible gene (RIG-I) (Yoneyama et al., 2004), melanoma differentiation-associated antigen 5 (MDA5) (Kang et al., 2002), and laboratory of genetics and physiology 2 (LGP2) (Miyoshi et al., 2001). Both RIG-I and MDA5 belong to a DExD/H-box helicase family with an N-terminal caspase activation and recruitment domain (CARD) that senses viral RNA with its helicase domain. After sensing, a downstream signal is transduced by CARD, therefore these two proteins share homologous function (Yoneyama et al., 2005). In contrast, LGP2 lacks a CARD domain and was originally identified as a negative regulator of RLR signalling by

interfering with viral RNA recognition by RIG-I and MDA5 (Yoneyama et al., 2005). However, recent studies have shown LGP2 as a potential cofactor of MDA5 which can assist in MDA5-mediated IFN signalling (Satoh et al., 2010, Hei and Zhong, 2017).

Both RIG-I and MDA5 are able to directly bind viral RNA via their helicase domain, however they display different binding specificity. MDA5 binds to long (greater than 2kb) double stranded RNA species (Kato et al., 2008), whereas RIG-I binds shorter double stranded RNA and 5'-triposphate capped RNA (Hornung et al., 2006). Activated RLRs interact and activate mitochondrial antiviral-signalling protein (MAVS), also known as IPS-1, which then forms prion-like aggregates that convert other inactive MAVS into its functional multimeric state (Hou et al., 2011). This results in the activation of two distinct signalling pathways. One pathway involves the activation of TBK1 and IRFs, leading to IFN production, whilst the other pathway signals via the kinase complex IKK, resulting in the activation of NF-κB and upregulation of proinflammatory genes (Belgnaoui et al., 2011).

### 1.3.3 NOD-like Receptors

The final class of PRRs important for recognition of viral infections are the nucleotide-binding oligomerisation domain-like (NLR) family of receptors. The NLR family of receptors consist of multi-domain proteins, each with a variable N-terminal caspase recruitment domain (CARD) and pyrin domain (PYD), a central nucleotide-binding oligomerisation domain (NOD) essential for receptor activation, and a C-terminal leucine-rich repeat region responsible for PAMPs sensing (Harton et al., 2002). When a PAMP is sensed by the C-terminal leucine-rich repeat region, a conformational change occurs that results in the oligomerisation of the NOD domain (Inohara et al., 1999, Kanneganti, 2010). This conformational change results in exposure of the CARD and PYD domains, which in turn recruits and activates CARD and PYD containing effector molecules (Inohara et al., 1999). As a result of this, NLRs activate many signalling pathways such as nuclear factor-κB (NF-κB), mitogen activated protein kinase (Kanneganti et al., 2007), and IRF dependent IFN production via mitochondria associated antiviral signalling protein (MAVS) (Sabbah et al., 2009). As well as activating downstream signalling proteins, NLR family members specifically NLRP1, NLRP3 and NLRC4 can form large protein complexes called inflammasomes (van de Veerdonk et al., 2011). These NLRs form inflammasomes by activating caspases, specifically caspase 1, which in turn generates the active proinflammatory cytokines interleukin-1β and IL-18 (Fantuzzi and Dinarello, 1999). High levels of IL-1β and IL-18 have been shown to lead to an inflammatory form of programmed cell death, pyroptosis (Martinon et al., 2002).

All three of these aforementioned PRRs work in tandem to detect intracellular and extracellular viruses and activate further innate immune signalling. A summary of each is shown in **Figure 3.**

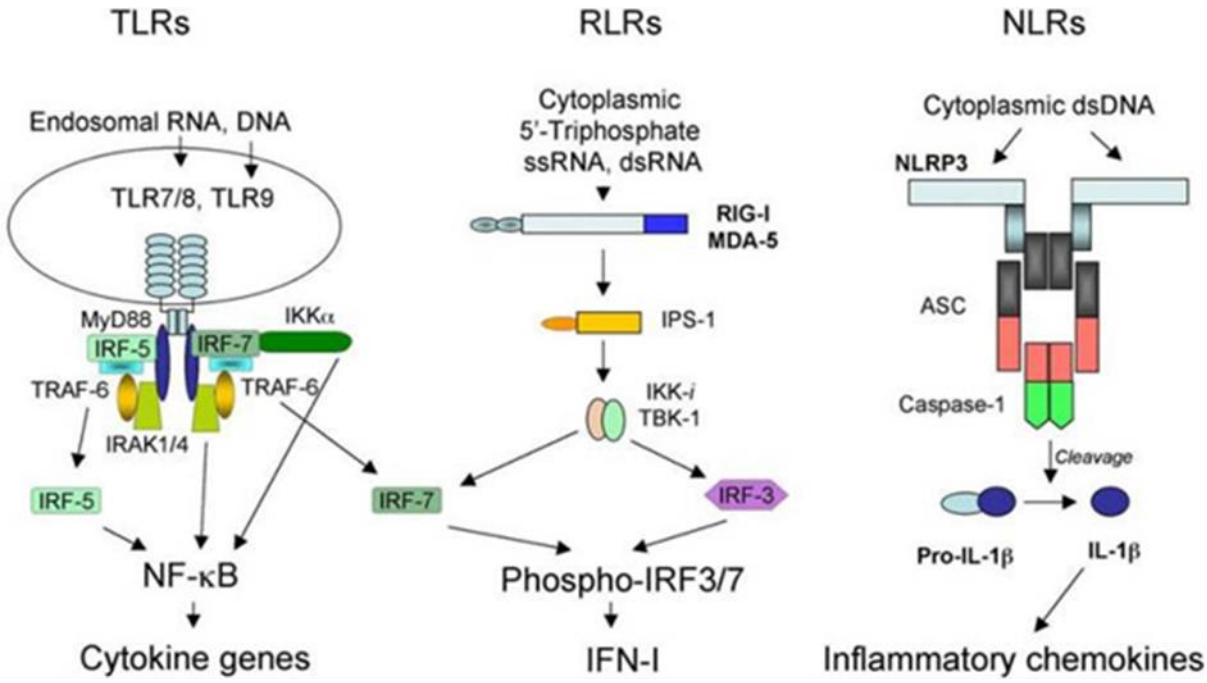


**Figure 3: The roles of TLRs, RLRs and NLRs in pathogen sensing**

*Three classes of pattern recognition receptors involved in intracellular and extracellular viral sensing. Shown are the main downstream effectors when each receptor is activated. See text for a more detailed description of their structure and role in sensing viral infection (Shayakhmetov, 2010)*

## 1.4 Interferons

Interferons are secreted glycoproteins which possess potent antiviral properties. Within humans, there are three classes designated Type I, II and III. Type I IFNs are the largest class of the three and include primarily 13 IFN-α subtypes and one IFN-β gene, as well as other more specialised IFNs such as IFNs δ, ε, κ, ω, and τ, and all bind to the same ubiquitously expressed receptor IFNAR (McNab et al., 2015). Type II IFN (IFNγ) is secreted by activated immune cells, primarily NK and T-cells, resulting in the activation of the cell mediated adaptive immune response. Finally, Type III IFNs (IFNλ) share similar function to type I IFNs as they activate the same intracellular signalling pathway and perform many of the same anti-viral activities (Kotenko et al., 2003, Sheppard et al., 2003). However, expression patterns of type III IFN differ from type I, as it is mainly restricted to cells of epithelial origin (Donnelly and Kotenko, 2010). All three classes of IFNs play an important role in the antiviral effects against VacV infection.

The IFN response is initiated by the recognition of PAMP by cellular PRRs, which include TLRs, RLRs and cytosolic DNA sensors. Each PRR has its own signalling process, but all result in the activation of transcription factors and their translocation to the nucleus (McNab et al., 2015). The transcription factors activated during the IFN response include IRFs 1 3 and 7, activator protein 1 (AP-1) and NF-κB (Akira et al., 2006). Each IRF is able to activate transcription of different IFNs; IRF3 induces transcription of IFNβ genes (Sato et al., 2000), IRF7 induces transcription of both IFNα and IFNβ genes (Sato et al., 2000), and IRF1 promotes IFNλ transcription (Siegel et al., 2011). While both IRF7 and IRF3 can activate transcription from IFNβ genes, IRF7 is the master regulator of type I IFN response, as it is essential for the full function of all elements of the IFN response (Honda et al., 2005). Furthermore, the transcription factors NF-kB and AP-1 work alongside IRFs in cytokine production to regulate the transcribed genes (Iwanaszko and Kimmel, 2015).

When produced, IFNs are secreted from the cell and promote either autocrine or paracrine signalling through their respective interferon receptors. When bound to their receptors IFNs initiate a signal cascade through the proteins Janus kinase (JAK) and signal transducer and activator of transcription (STAT). Signalling activated by either type I or III IFNs result in the formation of the ISGF3 complex containing; STAT1, STAT2 and IRF9 (Horvath et al., 1996, Zhou et al., 2007). When formed ISGF3 binds to IFN-stimulated response elements (ISRE) and induces the transcription of these genes (Takaoka and Yanai, 2006). In contrast, signalling of type II IFNs occurs via the STAT1 homodimer that binds to gamma-activated sequences (GAS) and promotes gene transcription (Takaoka and Yanai, 2006). The process of IFN production and IFN action is summarised in **Figure 4**.

There are several hundred interferon stimulated genes and the action of IRF and STAT signalling ensures a coordinated expression in response to viral infection. These proteins produced via IFN signalling possess potent antiviral properties. The protein kinase PKR is an important effector of IFN stimulation, effectively controlling viral replication. Active PKR is able to phosphorylate the eukaryotic translation initiator factor eIF-2α and inhibit translation as well as mediating apoptosis (Saelens et al., 2001, Srivastava et al., 1998). Another protein produced by IFN signalling is the protective protein ubiquitin-like protein ISG15, which is able to form conjugates with many proteins via ISGylation. ISGylation displays potent antiviral properties by conjugating with viral proteins preventing their function or by forming a complex with host proteins preventing their degradation (Morales and Lenschow, 2013).

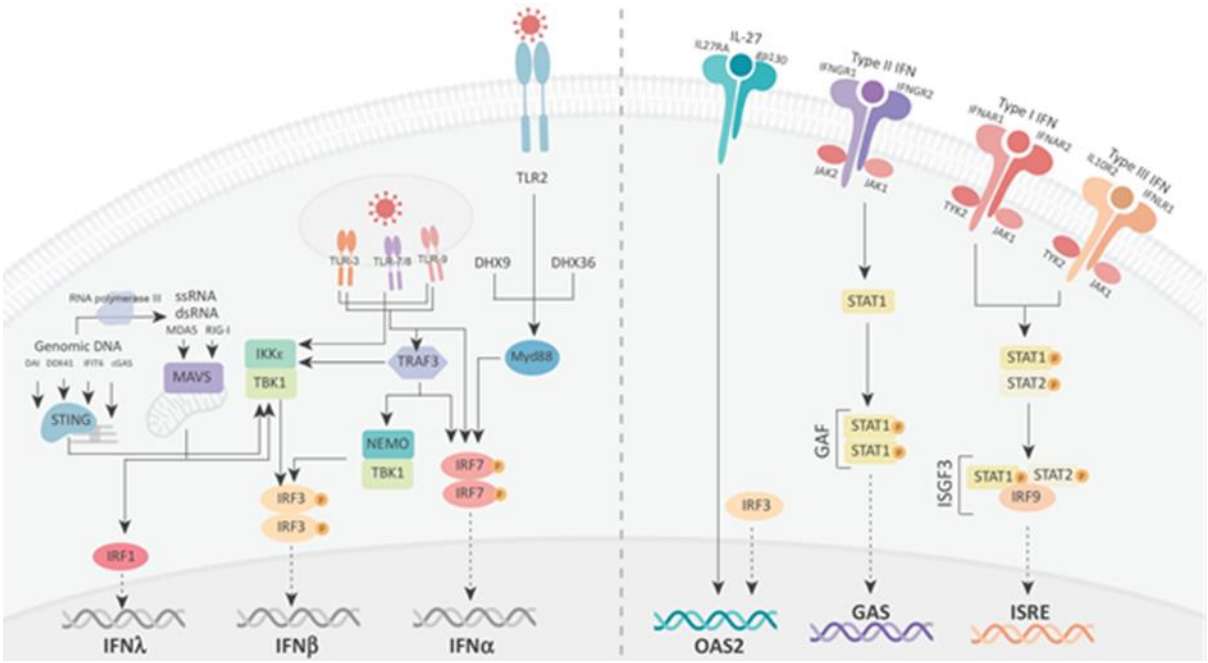


**Figure 4: The role of interferon regulatory factors and the Interferons they produce in antiviral gene expression**

A. *Interferon regulatory factors (IRFs) are key transcriptional factors that work alongside NF-κB to promote IFN gene expression. This process is initiated by PRR activation and subsequent downstream proteins. Signalling via TLR2 results in Myd88 activation, which in turn phosphorylates IRF7 promoting IFNα transcription. Other TLRs are able to activate either; IRF3 via TBK1, or IRF7 via TRAF3 leading to the transcription of IFNβ and IFNα respectively. TLRs are not the only activators of IRF3 and 7 as cytoplasmic nucleic acid sensors are able to promote TBK1 activation and subsequent IRF phosphorylation. Finally IRF1 promotes the transcription of the type III interferons (IFNλ), via RNA sensing and MAVS activation (Handfield et al., 2018).*

B. *Once produced IFNs exit the cell and bind to their respective IFN receptors. Binding of either type I or III interferons to their receptors resulting in the phosphorylation of STAT1 and STAT2, which complex with IRF9 to transcribe ISRE containing genes. Whereas, binding of type II interferons to their receptor resulting the formation of the STAT1 homodimer GAF, which is able to bind GAS elements to promote gene transcription. IL-27 signalling through IRF3 is also shown (Handfield et al., 2018).*

## 1.5 Intracellular DNA sensing

The endosomal TLR9 is the only TLR that is able to sense DNA by specifically binding to unmethylated cytosine-guanosine containing (CpG) motifs within DNA and trigger IFN production, a feature commonly found in bacterial and viral genomes but rare within the human genome (Hemmi et al., 2000). Despite this, TLR9 independent activation of the innate immune system by DNA has been observed. Studies in TLR9 deficient mice still exhibited IFN-β production in response to DNA, suggesting a novel TLR9 independent DNA sensing mechanism (Okabe et al., 2005). Before any potential TLR independent DNA sensors were discovered it was clear that signalling via TANK-binding kinase 1 (TBK1) and IRF3 was pivotal to the DNA response that resulted in induction of type I IFNs (Stetson and Medzhitov, 2006, Ishii et al., 2008). A major breakthrough for understanding intracellular DNA sensing occurred with the discovery of the endoplasmic reticulum (ER) bound stimulator of IFN genes (STING) (Ishikawa and Barber, 2008). STING was identified as a crucial upstream adaptor of TBK1 and IRF3 in the DNA response pathway (Zhong et al., 2008, Tanaka and Chen, 2012). However, how DNA activates STING signalling has been studied intensely and yielded a surprising number of upstream sensors (see **Figure 7** for a summary of these pathways).

### 1.5.1 The cGAS-STING pathway

Hailed as one of the most important discoveries with regards to DNA sensing, cyclic GMP-AMP synthase (cGAS) has been shown to be a central regulator of cytosolic DNA sensing. Discovered in 2013, cGAS is unique amongst DNA sensor as, when DNA bound, it is able to produce a potent second messenger to activate type I IFN production (Sun et al., 2013). A summary of this process is shown in **Figure 5**.

An activation loop within the cGAS molecule is able to bind DNA in a sequence independent manner by interacting with the sugar-phosphate backbone, but not to any of the bases (Civril et al., 2013). cGAS can also bind to single stranded DNA (ssDNA), under specific circumstances. Y-Shaped ssDNA that forms a duplex structure and contains unpaired guanosine overhangs are highly stimulatory and activate cGAS (Herzner et al., 2015). Although rare, these structures have been found to occur during early human immunodeficiency virus 1 (HIV-1) infection of macrophages (Herzner et al., 2015). Surprisingly, A-form dsRNA is also able to bind to cGAS but is unable to activate it, due to its inability to induce the necessary conformational change that results in cGAS activation (Zhang et al., 2014).

Once DNA is bound a dramatic conformational change occurs exposing Lys384, a positively charged region, allowing further DNA binding (Zhang et al., 2014). This DNA binding results in the activation loop moving inwards causing rearrangement of the catalytic site, resulting in cGAS activation. Once activated, cGAS catalyses a 2'-5' and 3'-5' phosphodiester linkage of cyclic guanosine monophosphate (GMP) and cyclic adenosine monophosphate (AMP), producing the second messenger cyclic GMP-AMP (cGAMP) (Diner et al., 2013, Sun et al., 2013). This is a twostep process starting first with the synthesis of linear 2'-5' linked dinucleotides, which then undergoes cGAS-depended cyclisation through 3'-5' linkage (Ablasser et al., 2013a). This process is also observed in multiple cell types after DNA transfection or viral infection, leading to detectable levels of cGAMP (Diner et al., 2013).

Once produced, cGAMP is then able to bind to the endoplasmic reticulum (ER) associated adaptor stimulator of interferon genes (STING) with higher affinity than bacterial cyclic dinucleotides (Wu et al., 2013, Ishikawa and Barber, 2008). In its non-active state, STING exists as an ER anchored dimer with a cyclic diguanylate monophosphote binding domain (CBD) facing the cytosol (Yin et al., 2012). As well as this, the CBD and the C-terminal tail of STING interact causing autoinhibition (Yin et al., 2012). cGAMP is able to bind directly to STING, within the groove created by the association of its two monomers, causing displacement of the C-terminal tail and recruitment of the ER protein autocrine motility factor receptor (AMFR). This process occurs in an insulin-induced gene 1 (INSIG1) dependent manner (Wang et al., 2014). This AMFR-INSIG1 complex displays E3 ubiquitin ligase activity, which results in the polyubiquitination of STING. This modification allows the anchoring of the serine/threonine kinase TBK1 and results in STING dimerisation and translocation to the perinuclear region, via the Golgi (Ishikawa et al., 2009). While translocating via the Golgi, STING undergoes palmitoylation on Cys88 and Cys91, an event essential for activation of TBK1 (Mukai et al., 2016). TBK1 is then able to phosphorylate STING on Ser366, allowing STING to stimulate IRF3 phosphorylation by TBK1 (Hemmi et al., 2004, Tanaka and Chen, 2012). Phosphorylated IRF3 then forms a homodimer and translocates to the nucleus where it promotes the transcription of *IFNβ* genes (Au et al., 1995, Sato et al., 2000). STING-TBK1 also activate IκB kinase (IKK), which then phosphorylates the IκB family of inhibitors associated with the transcription factor NF-κB (Abe and Barber, 2014, Ishikawa and Barber, 2008). Phosphorylation of IκB proteins leads to their proteasomal degradation; releasing NF-κB, which enters the nucleus and cooperates with IRF3 to induce IFN production, as well as other inflammatory cytokines such as tumour necrosis factor alpha (TNFα) (Abe and Barber, 2014). A unique aspect of the cGAS-STING response is that cGAMP is able to be transferred between infected and nearby cells via gap junctions (Ablasser et al., 2013b). Furthermore, cGAMP can be incorporated during virion

assembly and transferred to newly infected cells (Bridgeman et al., 2015). This cell to cell transmission of cGAMP propagates an IFN response in neighbouring and newly infected cells, limiting the dissemination of viruses.

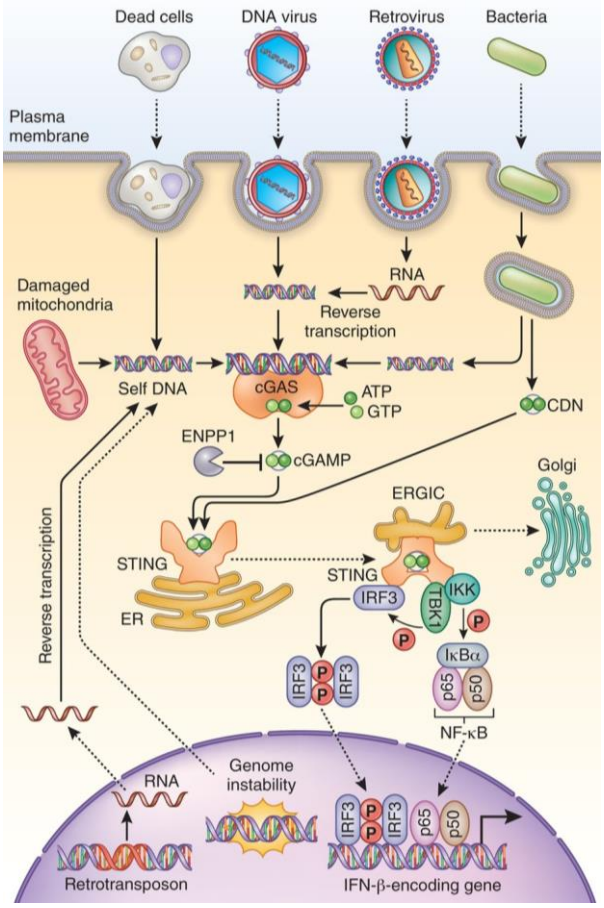


**Figure 5: The cGAS-STING pathway during cytosolic DNA sensing**

*DNA within the cytoplasm is a major PAMP that activates the innate immune response. Cytoplasmic DNA can originate from multiple sources such as DNA viruses, retroviruses, bacteria, and damaged self-DNA. Regardless of source, cytoplasmic DNA binds and activates cGAS, which catalyses the production of the second messenger cGAMP from ATP and GTP, which then binds to the ER adaptor STING. As well as cGAMP, cyclic dinucleotides produced by some bacteria can also directly bind STING causing its activation. Once bound to its ligand, STING is then trafficked to the ER-Golgi intermediate compartment (ERGIC) and finally to the Golgi. STING then activates TBK1, which phosphorylates STING causing recruitment of IRF3 to the complex. Once associated with STING, IRF3 is phosphorylated by TBK1 resulting in its dimerisation and translocation to the nucleus. STING is also able to activate the kinase IκB which phosphorylates the NF-κB inhibitor IκBα, resulting in its proteasomal degradation. Once dissociated from IκBα, NF-κB translocates to the nucleus. Both IRF3 and NF-κB cooperate to induce the expression of genes encoding IFN-β (Chen et al., 2016).*

### 1.5.2 Regulation of the cGAS-STING pathway

Stimulation of IFN production by DNA sensing via the cGAS-STING pathway is a potent antiviral mechanism. However, constant or aberrant activation of this pathway can lead to elevated type I IFN expression, which is linked to several autoimmune diseases; interferonopathies (Crow, 2015). Therefore, the cGAS-STING pathway is under tight regulation to ensure appropriate activation.

The pathway is initiated when cGAS binds to dsDNA. However many mechanisms regulate this to ensure an appropriate response to foreign DNA while remaining unresponsive to self-DNA. One of the main methods of regulation is post translational modification. The kinase Protein kinase B (PKB), also known as Akt, is able to phosphorylate human cGAS at Ser305 and inhibit its enzymatic activity (Seo et al., 2015). This suggests that pathways involved in the regulation of PKB also are able to regulate cGAS activity. cGAS can also be modified by the addition of monoglutamic or polyglutamic residues, known as glutamylation, by the tubulin tyrosine ligase-like (TTLL) proteins TTLL4 and TTLL6 (Janke et al., 2005, Xia et al., 2016a). Polyglutamylation by TTLL6 prevents DNA binding to cGAS, whereas TTLL4 monogultamylation blocks its enzymatic activity (Xia et al., 2016a). This modification can be reversed by the carboxylpeptidases CCP6 and CCP5; which can remove poly and monoglutamylation respectively, leading to cGAS activation (Xia et al., 2016a). By tightly regulating the inhibitory phosphorylation or glutamylation of cGAS, pathway initiation can occur to eradicate DNA viral infections or be supressed to prevent an excessive immune response. After STING activation, and subsequent IFN production, the activated protein is translocated from the ER and packaged in vesicles. STING is then phosphorylated by UNC-51-like kinase (ULK1), promoting STING degradation by autophagy and repressing IRF3 function (Konno et al., 2013). This process is triggered by the cyclic dinucleotide cGAMP. This suggest that cGAMP initiates STING signalling initially, but triggers negative feedback control after activation, preventing persistent transcription of *IFN* genes by IRF3. (Gonugunta et al., 2017, Konno et al., 2013)

Another mechanism for the control of the cGAS-STING pathway is by transcriptional and epigenetic regulation. Many cancer cells have lost the expression of both cGAS and STING, allowing evasion of the host immune system (Xia et al., 2016b). In many cancer cell types, it was found that both STING and cGAS were silenced by the epigenetic process hypermethylation, a process which can be reversed by demethylation (Xia et al., 2016b). In addition to cancer cells, both T-cells and hepatocytes also lack a functional cGAS-STING pathway, and therefore may contribute to infection with HIV and hepatitis B, respectively (Berg et al., 2014, Thomsen et al., 2016). This suggest a role for cGAS-STING pathway

silencing by methylation within primary cells. Finally, the intracellular secondary messenger cGAMP is regulated by its rate of synthesis and decay. The extracellular enzyme ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) is able to degrade cGAMP with high specificity within murine and porcine cells (Li et al., 2014, Wang et al., 2018b). However, the role of ENPP1 in cGAMP regulation in humans is yet to be determined.

### 1.5.3 Additional DNA Sensors

cGAS is not the only DNA sensor able to stimulate IFN production through STING activation. Many DNA sensors exist that are able to perform the same role as cGAS, however many are only found in specific cell types and their importance in DNA sensing is poorly understood. While the pathway from STING activation to IRF production is mostly the same, many DNA sensors have distinct mechanisms that lead to the activation of STING. As well as this, there are some distinct DNA sensors that are able to stimulate IFN production, without STING activation.

#### DNA- dependent activator of IRFs (DAI)

DAI, known also as Z-DNA binding protein 1, was the first DNA sensing receptor found to function directly upstream of STING (Takaoka et al., 2007). It contains a Z-binding domain, critical to its function, which is able to bind nucleic acids that adopt the atypical Z conformation (Maelfait et al., 2017). It was shown to bind DNA and RNA from a multitude of sources including viral, bacterial and mammalian DNA. Furthermore, artificially induced dimerisation of DAI also resulted in activation of IFN I genes (Wang et al., 2008). Alongside IFN gene activation, sensing of viral nucleic acids by DAI can also trigger the DAI-RIPK3 dependent necroptosis pathway (Nogusa et al., 2016). This has also been observed in VacV infected cells (Chan et al., 2003). Although DAI displays properties of a nucleic acid sensor, its role in the innate immune system is unclear as it displays redundancy in DNA sensing within certain cell types (Lippmann et al., 2008)

#### PYHIN Family

The PYHIN family of DNA sensors are characterised by the presence of the Pyrin and HIN2000 domains, and many of the family are inducible by IFNs (Schattgen and Fitzgerald, 2011). Absent in melanoma (AIM2) was identified as the first PYHIN family to act as a PRR for intracellular DNA, and therefore defines a sub-family of AIM2-like receptors (DeYoung et al., 1997, Schattgen and Fitzgerald, 2011). AIM2 is an intracellular dsDNA sensor which activates caspase 1 leading to IL-1β production (Fernandes-Alnemri et al., 2009). Another AIM-2 like receptor shown to act as a PRR for intracellular DNA was γ-Interferon Inducible protein (IFI16) (Unterholzner et al., 2010). IFI16 is able to directly bind double stranded DNA

in a non-sequence specific manner, mediated by the two HIN domains of the protein (Jin et al., 2012). Upon activation, IFI16 associates with STING and promotes the activation of both IRF3 and NF-κB (Unterholzner et al., 2010). Furthermore, IFI16 has also been shown to activate the inflammasome after viral infection (Ansari et al., 2013). Unlike other DNA sensors IFI16 has been shown to be vital for the IFN response to DNA in many cell types, such as human fibroblasts, monocytes and dendritic cells (Unterholzner et al., 2010, Duan et al., 2011). Its function is also unique amongst DNA sensors as it is able to shuttle between the cytoplasm and the nucleus, as well as sensing DNA in both regions (Dell'Oste et al., 2014, Kerur et al., 2011, Orzalli et al., 2012). IFI16 has also been shown to cooperatively work with other DNA sensors to induce IFN production. In human keratinocytes, cGAS and IFI16 cooperate during DNA sensing to fully activate the innate immune response and prevent spurious IFN activation (Almine et al., 2017). However, AIM2-like receptors have been shown to be dispensable in murine models and during human cytomegalovirus infection (Gray et al., 2016), casting doubt over the extent IFI16 contributes to viral DNA sensing.

**DEAD-Box Helicase 41 (DDX41)**

DDX41, a member of the DEAD-box helicase protein family, was first identified as an intracellular DNA sensor within myeloid dendritic cells. Knockdown of DDX41 blocked the activation of TBK1, NF-κB and IRF3 within dendritic cells when exposed to viral DNA (Zhang et al., 2011b). Like most DNA sensors DDX41 uses STING as an important adaptor protein to facilitate signal transduction from the activated sensor to downstream TBK1 and IRF3, resulting in production of type I IFNs (Zhang et al., 2011b). DDX41 is able to directly bind both DNA and STING via its conserved Asp-Glu-Ala-Asp (DEAD) domain (Linder et al., 1989, Zhang et al., 2011b)

Alongside viral DNA, DDX41 is also able to bind bacterial cyclic dinucleotides di-GMP and cyclic di-AMP and trigger type I interferon response in the host (Parvatiyar et al., 2012). These cyclic dinucleotides are released by certain bacterial species and used as secondary messengers, as well as triggering STING depended signalling in mammalian cells (Hengge, 2009, McWhirter et al., 2009). As with viral DNA sensing, the DEAD domain of DDX41 is essential in detecting cyclic dinucleotides thus triggering a type I interferon response (Parvatiyar et al., 2012). After binding these dinucleotides, DDX41 then promotes the binding of STING to form a dinucleotide-DDX41-STING complex, resulting in STING activation (Parvatiyar et al., 2012). Other dinucleotides have also been shown to directly bind STING, notably cyclic cGAMP, (Wu et al., 2013) although the ability of DDX41 to bind cGAMP is still undetermined.

**DNA dependent protein kinase (DNA-PK)**

DNA-PK is a heterotrimeric complex of proteins, consisting of three proteins Ku70, Ku80 and a DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Ferguson et al., 2012). Both the Ku70 and Ku 80 subunits form a basket shaped heterodimer and works alongside DNA-PKcs to directly bind DNA (Walker et al., 2001). DNA-PKcs can bind DNA in the absence of Ku subunits, but with greatly reduced affinity (Yaneva et al., 1997). These properties were first characterised when DNA-PK was identified to play a key role in double strand break repair within the nucleus (Lieber et al., 2003). In addition to this, there is evidence that DNA-PK plays a role as a cytoplasmic DNA sensor. Firstly, the protein has been detected in the cytoplasm where it may function in the innate immune response (Huston et al., 2008). It has been shown that during VacV infection, DNA-PK co-localises at the sites of *Vaccinia* DNA replication. As well as this co-localisation, DNA-PK acts upstream of IRF-3 and TBK1 leading to a type I interferon response (Ferguson et al., 2012). The subunit Ku70 has also been show to act as a cytosolic DNA sensor that induces a type III interferon response, rather than the well characterised type I response to intracellular DNA (Zhang et al., 2011a). Transfection of DNA, from various sources including viral, induces the activation of *IFNL1* and production of IFN λ mediated by the activation of both IRF1 and IRF7 (Zhang et al., 2011a). This shows that DNA-PK as well as its subunit Ku70, can work together or individually to activate type 1 and 3 interferon responses in an IRF dependent manner.

**Meiotic Recombination 11 (Mre11)**

Meiotic Recombination 11, as with DNA-PK, is a previously well characterised DNA damage factor that has shown properties of a cytosolic DNA sensor (Kondo et al., 2013). Mre11 is commonly found within the MRN complex which also contains nijmegen breakage syndrome 1 (NBS1) and a RAD50 homolog (Stracker and Petrini, 2011). Mre11, similar to DNA-PK, is widely recognised for its role DNA double strand break repair and genomic stability (Buis et al., 2008). However, Mre11 has been shown to be required for the DNA stimulated IFN response in dendritic cells from mouse bone marrow origin (Kondo et al., 2013). Components of the MRN complex such as Mre11 and RAD50 were shown to be crucial for DNA induced IFN response, whereas other components such as NBS1 had little or no effect on cytoplasmic DNA sensing (Kondo et al., 2013). Furthermore, Mre11 driven IFN response only occurred in response to transfected DNA and not with intracellular pathogens. Cells infected with either HSV-1 or *Listeria monocytogenes* did not exhibit an Mre11 depended IFN response (Kondo et al., 2013). Therefore the role of Mre11 depended DNA sensing during infection is debateable.

**RNA polymerase III (Pol III)**

As stated previously, RIG-I and MDA5 have an important role in RNA sensing and IFN production, especially during viral infection. However, even though RIG-I is a cytosolic RNA receptor, studies have shown that it is capable of acting as an indirect DNA sensor (Chiu et al., 2009). RNA polymerase III is a specialised transcription protein important for the production of non-protein coding RNA transcripts, such as tRNAs, snRNAs, 5S rRNA as well as other essential RNA (Dieci et al., 2013). Alongside this, RNA polymerase III has been shown to transcribe synthetic poly(dA-dT) DNA when transfected into cells (Chiu et al., 2009). Cytosolic B-form DNA such as poly(dA-dT) is converted into 5' triphosphate double stranded RNA, which is then able to induce IFNβ production through the RIG-I pathway and culminating in the activation of IRF3 and NF-kB (Chiu et al., 2009, Valentine and Smith, 2010). An example for the importance of RNA polymerase III activation of RIG-I is in *Varicella zoster* (VZV) infections. In adults with severe VZV central nervous system infections, mutations within the Pol III gene were identified in a quarter of patients (Carter-Timofte et al., 2018). Cells possessing these mutations display reduced expression of antiviral cytokines in response to poly(dA-dT) as well as an increased viral replication rate. This shows that Pol III has an important role in certain viral infections (Carter-Timofte et al., 2018). However, transfection with most other IFN stimulating DNA motifs such as oligonucleotides derived from VacV, or DNA isolates from other bacteria and viruses do not result in the production of stimulatory RNA and RIG-I activation (Unterholzner et al., 2010). Although some evidence shows Poll III to act as a DNA sensor, most is the result of synthetic DNA stimulation, whether Pol III is a true DNA sensor is still controversial.

**Extrachromosomal Histone H2B**

During the search for cytoplasmic DNA sensors many proteins were shown to have intrinsic DNA sensing properties alongside their normal function, one such protein is histone H2B. Histones are essential proteins involved in the structure and organisation of chromatin, as well as this histone modification is especially important in gene activation and silencing (Kobiyama et al., 2013). Alongside its normal function histone H2B has been shown to exist within the cytoplasm where it acts as a DNA sensor (Kobiyama et al., 2013). H2B is able to detect fragmented double stranded DNA produced from either viral infections or cellular damage. Once bound to DNA H2B then interacts with MAVS causing the induction of IFN signalling through CIAO (COOH-terminal importin 9-related adaptor organising histone H2B and IPS-1) and TBK1 (Kobiyama et al., 2010). This shows that histone have an important role in viral infections and DNA damage, this is summarised in **Figure 6**.

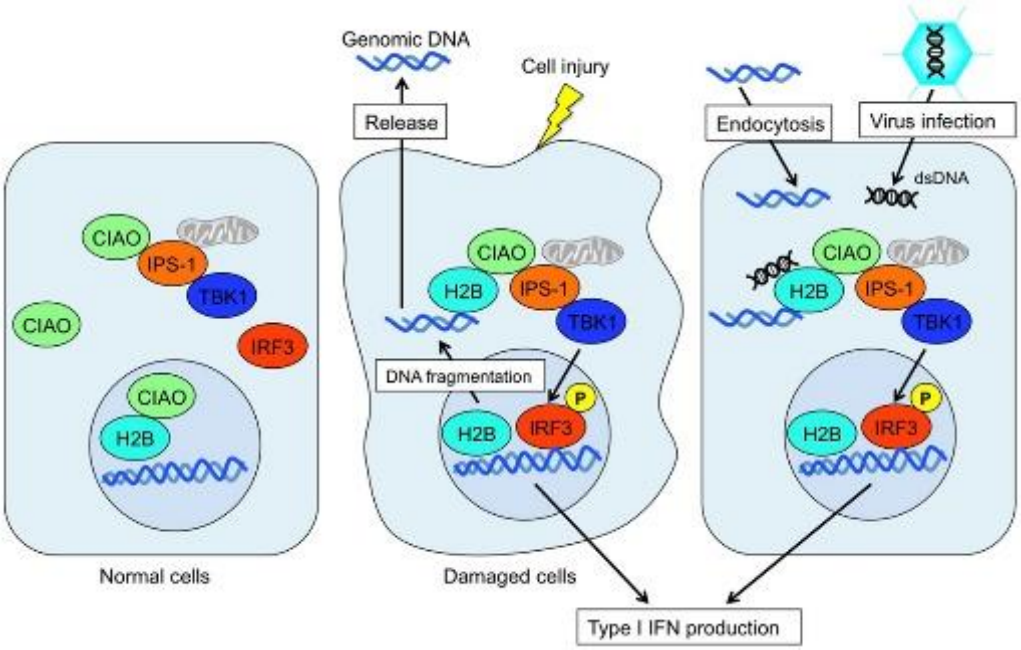


**Figure 6: DNA sensing by the histone H2B in damaged and virally infected cells.** *Under normal cellular conditions the histone H2B is localised in the nucleus where it is important for chromatin organisation and structure. During cellular damage or viral infection, DNA fragments in the nucleus are recognised by H2B. After binding DNA H2B forms a complex with CIAO and MAVS, also known as ISP-1, which induces TBK1 activation and subsequent IRF3 phosphorylation. Phosphorylated IRF3 then translocates to the nucleus where it activates type 1 interferon genes (Kobiyama et al., 2013).*

## Non-specific nucleic acid receptors

Several non-specific nucleic acid sensors have been identified that are able to recognise both DNA and RNA. These sensors enable an innate immune response towards nucleic acids in general, as opposed to targeted receptors. It has also been suggested that more general nucleic acid sensing is required for activation of more specific PRRs such as TLRs and intracellular DNA sensors (Yanai et al., 2009)

High mobility group box 1 (HMGB1) is a non-histone chromosomal protein that functions as a DNA chaperone within the nucleus (Goodwin et al., 1977). In eukaryotic cells, HMGB1 is found within the nucleus and in the cytoplasm where it has many functions. HMGB1 is mostly found in the nucleus where its main role is to associate with DNA and aid with replication, repair, and stabilisation of the genome (Lee et al., 2014). Within the cytoplasm, its main role is to maintain homeostasis by regulating autophagy and reduce protein aggregation caused by cellular stress (Lee et al., 2014). As well as this cytoplasmic HMGB1 can also act as a proinflammatory cytokine when released from cells, particularly activated macrophages (Yanai et al., 2009). Furthermore, HMGB1 also shows properties of a DNA

sensor. Within the cytoplasm HMGB1 is able to bind DNA or RNA with high affinity, once bound it promotes the activation of TLRs and cytoplasmic DNA sensors (Yanai et al., 2009)

Another non-specific nucleic acid sensor is Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1). As with other non-specific nucleic acid sensors LRRFIP1 is able to bind both DNA and RNA with high affinity. However, activation of LRRFIP1 stimulates IFN producing genes without activating IRF3. Instead, it is able to signal through the co-activator pathway containing β-catenin and p300 which can modify histones to enhance transcription of IFN genes (Yang et al., 2010).

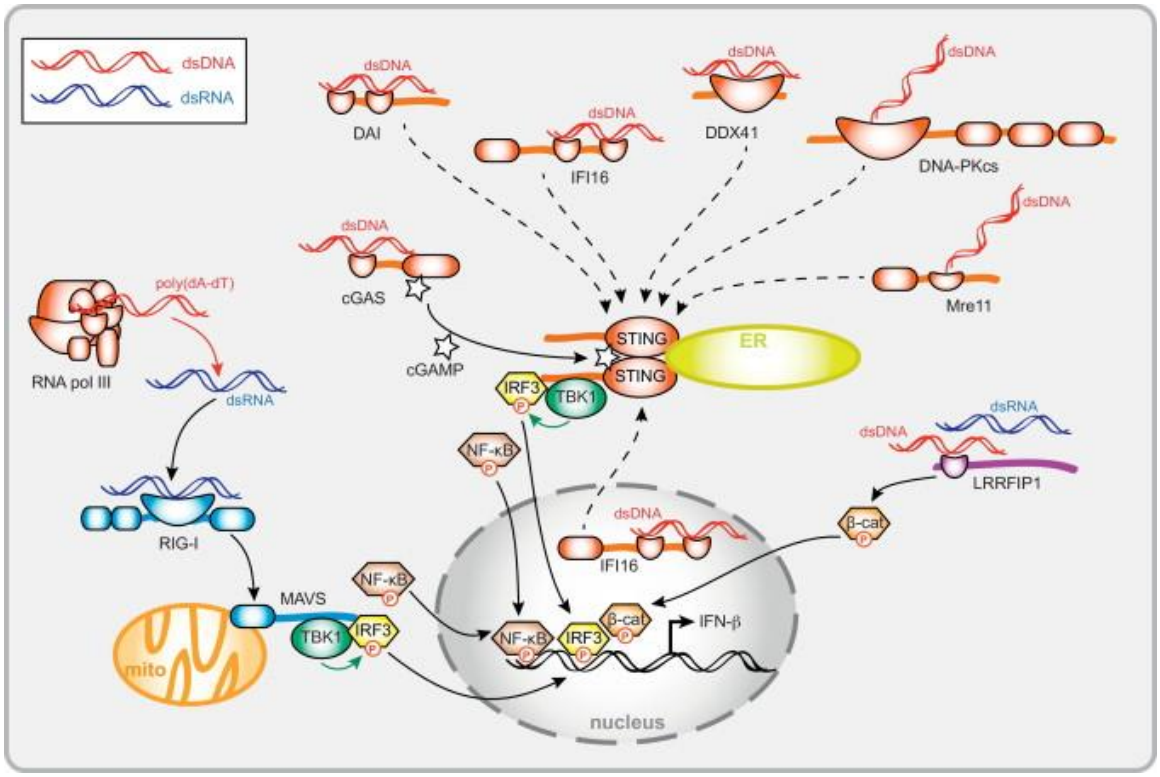


**Figure 7: The activation of type I IFN by cytoplasmic DNA and RNA sensing**

*There are many intracellular DNA sensors, each with a distinct mechanism of action. Most of the DNA sensors converge on the common signalling pathway through STING and IRF3, with the exception of RNA pol III and LRRFIP1. RNA pol III is an RNA polymerase that is able to convert viral DNA into RNA that is then sensed by RIG-I and MAVS and leads to IRF3 dependent IFN production. LRRFIP1 is a non-conventional sensor as, instead of signalling via STING, it is able to activate β-catenin and modify histones to enhance IFN transcription. Regardless of activation method, all intracellular DNA sensor result in the activation of transcription factors NF-κB or IRF3 and IFN gene transcription (Unterholzner, 2013).*

## 1.6 *Vaccinia* Immune evasion

The innate immune system possesses a wide array of mechanisms and factors that limit and destroy invading pathogens. Despite this poxviruses, including *Vaccinia*, are able to effectively infect and replicate within a human host. This is due to their large repertoire of innate immune evasion strategies (Smith et al., 2013). As it is the most studied member of the family, most *Poxviridae* innate immune evasion strategies were discovered in VacV. The collective research into VacV immune evasion has shown that it is able to inhibit or counteract almost all parts of the innate immune response, particularly disrupting the actions of IFNs (Smith et al., 2013). These are summarised in **Table 1**

| Protein | Function | Mechanism | *Reference* |
|---|---|---|---|
| **A46** | Disruption of TLR signalling | Interaction with TIR domains | (Stack et al., 2005) |
| **A49** | Prevents IκBα degradation | Mimics IκBα and binds β-TrCP | (Mansur et al., 2013) |
| **A52** | Stimulates IL-10 production | Induction p38 MAP kinase pathway | (Maloney et al., 2005) |
| **A56** | Reduces NK cell activity | NKp30 and NKp46 ligand | (Jarahian et al., 2011) |
| **B14** | Prevents IκBα from dissociating with NF-κB | Binds IKKβ subunit preventing phosphorylation of IκBα | (Chen et al., 2008) |
| **B18** | Prevents IFN reaching their receptor | Type I IFN binding protein | (Smith et al., 2013) |
| **B8** | Prevents IFNs reaching their receptor | Soluble IFN receptor | (Smith et al., 2013) |
| **C16** | DNA sensor blockade | Binds to Ku70 blocking DNA-PK/DNA interaction | (Peters et al., 2013) |
| **C6** | Prevents IRF activation | Interacts with TBK1 adaptor proteins | (Unterholzner et al., 2011) |
| **D9/D10** | Degradation of host and viral DNA | mRNA decapping | (Liu et al., 2015) |
| **E3** | Sequesters DNA and RNA | Contains a RNA and DNA binding domain | (Koehler et al., 2017, Valentine and Smith, 2010) |
| **F17** | cGAS degradation | Dysregulates mTOR causing proteosomal degradation of cGAS | (Meade et al., 2018) |
| **K1** | Prevents NF-κB activation | Prevents acetylation of NF-κB subunit p65 | (Bravo Cruz and Shisler, 2016) |
| **K7** | Disrupt TLR signalling<br>Disrupt TBK1 activity | Binds TRAF6 and DDX3 disrupting normal function | (Kvansakul et al., 2017) |
| **N1** | Prevents NF-κB activation | Interactions with IKK complex | (DiPerna et al., 2004) |
| **N2** | Blocks IRF activity | Translocates to nucleus where it interferes with IRFs | (Ferguson et al., 2013) |
| **SPI-2 CrmA** | Prevents TBK1 phosphorylation of STING and IRF | Interacts with TBK1 and IKKε preventing normal function | (Qin et al., 2017) |
| **Unknown** | Supress cGAS activity | Akt inhibition by phosphorylation | (Seo et al., 2015) |
| **Unknown** | Prevent STING activation | Inhibition of phosphorylation and dimerisation | (Georgana et al., 2018) |
| **VCP** | Blocks Complement activation | Cleavage of C3b and C4b | (Jha and Kotwal, 2003) |
| **VH1** | Disrupts IFN signalling | STAT phosphatase | (Smith et al., 2013) |

**Table 1: Proteins produced by VacV and their function on the innate immune system.**

*VacV is able to produce a wide range of proteins that disrupt the immune system and allow replication. These proteins, produced mostly in early infection, effect all parts of the innate immune system including complement system, NK cells, TLR and RLRs, and IFN signalling. Shown in the table is the majority of the known VacV proteins, their function on the innate immune system and mechanism of action. Each protein is also discussed in detail in the text.*

### 1.6.1 Complement

The complement system is an important early host defence system, able to destroy viruses and virally infected cells before significant replication can occur. The main process behind this is virion opsonisation by antibodies and promoting destruction by phagocytosis (Dunkelberger and Song, 2010). To counteract this VacV produces and secretes the protein *Vaccinia* virus complement control protein (VCP). VCP is a 35kDa protein that is structurally similar to the potent complement inhibitor C4b binding protein (Jha and Kotwal, 2003). VCP is able to directly bind to both C3b and C4b and, with the aid of factor I, promotes cleavage of both proteins. The result of this is the blockage of both the classical and alternate complement pathways. This is an important extracellular immune control protein as VacV strains lacking the gene encoding VCP, *C21L*, are attenuated *in vivo* (McKenzie et al., 1992)

### 1.6.2 Cell mediated innate immunity

Cell mediated innate immunity plays an important role in viral infection by preventing further spread of the pathogen as well as activating the adaptive immune response. One of the main innate cellular components that play an important role in VacV infection are NK cells (Caligiuri, 2008). VacV is able to modulate NK cell activation by interfering with IL-18 dependent activation of NK cells (Born et al., 2000) as well as directly infecting NK cells at the site of infection (Kirwan et al., 2006). Furthermore, The VacV protein A56 is abundantly present on a cell surface when infected with VacV, which is a ligand for the NK cell receptors NKp30 and NKp46 (Jarahian et al., 2011). These interactions block NKp30 triggered activation of NK cells and stimulate NKp46 depended activation. This results in a subset of activated NK cells with reduced cellular activity and a decreased susceptibility of infected cells to NK mediated lysis (Jarahian et al., 2011)

### 1.6.3 Nucleic Acid Sensors

One of the simplest ways VacV is able to avoid innate immune system is to prevent viral PAMP recognition by PRRs. Most PRRs are able to sense viral nucleic acids, therefore VacV has developed mechanisms to prevent this. VacV genes located at the terminal regions of the genome can only be transcribed outwards, towards the terminus, and from only one DNA strand (Smith et al., 1998). This limits the formation of immunostimulatory dsRNA. As well as this, early replication of VacV genome occurs in ER-enclosed cytoplasmic “viral factories”. These may isolate the VacV genome from any cytoplasmic DNA sensor (Tolonen et al., 2001).

Toll-like receptors have an important role in innate defence against viruses as they are able to recognise viral DNA and stimulate IFN production. TLR signalling is dependent of the

interactions of the TIR domains of the receptor with specialised adaptor molecules, specifically MyD88 and TRIF (Botos et al., 2011). *Vaccinia* virus is able to exploit this common signalling pathway with the protein A46. The viral protein A46 is a *Vaccinia* produced TIR domain containing protein that specifically counteracts multiple TLR signalling pathways (Stack et al., 2005). These interactions interfere with the downstream activation of both NF-κB and IRF3 hindering the host cytokine and interferon response. Alongside A46 production, VacV also produces the protein A52 that stimulates cytokine production (Maloney et al., 2005). The VacV protein A52 interacts with tumour necrosis factor receptor-associated factor 6 (TRAF6) resulting in the polyubiquitination of TRAF6 and induction p38 MAP kinase pathway. This results in the production of the immunoregulatory cytokine IL-10 (Maloney et al., 2005). As well as this, A52 is also able to block the activation of NF-κB and therefore limit cytokine production (Harte et al., 2003).

As well as TLR inhibition VacV also produces proteins that inhibit or prevent RLR activation by dsRNA. VacV has an AT rich genome, making it particularly prone to sensing via Pol III (Valentine and Smith, 2010). However, VacV is able to limit this with the protein E3, a bifunctional *Vaccinia* protein with both DNA and RNA binding domains (Marq et al., 2009). By binding dsRNA, E3 is able to prevent the interaction of viral RNA with PRRs, thus limiting an intracellular immune response. As well as this, E3 has been shown to bind to immunostimulatory RNA produced by Pol III activation and prevent activation of the RIG-I pathway (Valentine and Smith, 2010).

Other nucleic acid sensors alongside TLR are targeted by VacV for immune evasion. The protein E3 contains both a C-terminal RNA and N-terminal DNA binding domain and therefore prevents either from triggering PRRs (Marq et al., 2009). The N-terminal DNA binding domain shares homology with the DNA sensor DAI. This allows E3 to sequester any free viral DNA and prevent any potential interaction with cytoplasmic DNA sensors (Marq et al., 2009). Furthermore, E3 is able to competitively bind VacV DNA and prevent DAI activation and the resultant necroptosis (Koehler et al., 2017). VacV is also able to limit sensing of nucleic acids by other DNA sensors. The viral protein C16 is able to bind to the Ku70 subunit of DNA-PK blocking DNA interaction and preventing the activation of IRF3, therefore blocking IRF-dependent IFN signalling (Peters et al., 2013)

### 1.6.4 The cGAS-STING pathway

The cGAS-STING pathway is one of the most important sensing pathways for VacV detection, due to its entirely cytoplasmic replication cycle. Because of this VacV utilises a number of mechanisms to disrupt the normal function of this pathway, although many

proteins involved are currently unknown. One of the mechanisms of inhibition utilised by VacV is to interfere with the normal function of the key adaptor protein STING. During infection VacV is able to prevent STING activation by blocking its phosphorylation and therefore dimerisation (Georgana et al., 2018). This inhibition of STING dimerisation by VacV prevents IRF3 activation even when cells are stimulated with DNA or cGAMP, although cGAMP degradation may also occur (Georgana et al., 2018). During normal cellular function, PKB is able to phosphorylate cGAS and supress its activity (Seo et al., 2015). This mechanism could potentially be exploited by VacV. Within *Vaccinia* infected cells PKB is activated, this is due to the virus utilising the pathway for its own replication (Soares et al., 2009). Furthermore, PKB activation also leads to a reduction in cellular cGAMP and IFN-β production. This could be due to activated PKB phosphorylating and supressing cGAS activity, although this has yet to be directly observed with VacV infections (Seo et al., 2015). As well as inhibiton of key proteins in the cGAS-STING pathway, VacV has been shown to promote degradation of key proteins (Meade et al., 2018). The VacV core protein F17 is able to sequester key regulators of mammalian target of rapamycin complexes (mTORC); Regulatory-Associated Protein of mTOR (RAPTOR) and Rapamycin-Insensitive Companion of mTOR (RICTOR) (Meade et al., 2018). By removing regulatory proteins VacV infection results in the dysregulation of mTOR pathway, resulting in cGAS localisation in the golgi and driving its proteasomal degradation (Meade et al., 2018). Interestingly, this was observed fully in dermal fibroblasts but only partial cGAS degradation occurred in the human monocyte cells THP-1, suggesting F17 only targets a sub population of cGAS (Meade et al., 2018). Furthermore, F17 is a late VacV protein so the aformentioned mechanism may only occur after replication of the viral genome.

### 1.6.5 Interferons

The interferon response is the most important antiviral innate immune response as it activates cellular components of both the innate and adaptive immune systems, stimulates nearby cells to produce antiviral proteins, and facilitate apoptosis of infected cells. Due to their many functions interferons are an integral part of the hosts antiviral response, because of this VacV has developed a wide range of proteins to counteract IFNs. VacV is able to interfere with every step of IFN signalling (Smith et al., 2013).

One of the simplest ways VacV is able to prevent IFN production is to inhibit host protein synthesis. This is achieved by the viral decapping enzymes D9 and D10 (Parrish et al., 2007). These proteins are able to bind to mRNAs and remove their 5’ methylated cap, resulting in their degradation. As well as this, D9 and D10 also target and degrades *Vaccinia* mRNA, preventing accumulation in the cytoplasm, and limiting an antiviral response by RNA

sensors, such as RIG-I (Liu et al., 2015). As well as preventing PAMP recognition by PRRs, VacV is also able to block parts of the signalling pathway leading to NF-κB or IRF3 activation and subsequent IFN production.

VacV produces many proteins during early infection that are able to inhibit both IRF3 and NF-κB activation . Although all these are expressed at the same time during early infection, each protein is able to inhibt different stages of each signalling pathway. Many of these proteins share homology as they all contain the conserved B-cell lynphoma-2 (Bcl-2) fold (Kvansakul et al., 2017).

## Inhibition of IRF3 activation

In addition to NF-κB *Vaccinia* also targets IRF3 in its immune evasion stratergies (**Figure 8**). IRF3 depended IFN signalling is initiated by several receptors such as TLRs, RLRs and intracellular DNA sensors; however, all these receptors share a common signalling pathway that converges on TBK1. Therefore unsurprisingly, VacV targets this in an attempt to disrupt IRF3 signalling. Firstly, the viral protein C6 prevents IRF3 activation by interacting with several key TBK1 complex scaffold proteins; TRAF family member-associated NF-κB activator (TANK), NAK-associated protein 1 (NAP1), and similar to NAP1 TBK1 adaptor (SINTBAD) (Unterholzner et al., 2011). These interactions inhibit the downstream activation of TBK1 and IRFs without effecting NF-κB (Unterholzner et al., 2011). Another protein able to interact with TBK1 scaffold proteins is K7. During early infection K7 is able to bind to DDX3, an important for the TBK1-mediated activation of IRF3 and *ifnb* promoter induction (Schröder et al., 2008). This results in the inhibition of IRF activation via TBK1/IKKε. As well as this, the viral prtoteins SPI-2 and CrmA are also able to inhibit activation of IRF3 (Qin et al., 2017). Both viral proteins are able to associate with TBK1 and IKKε and disrupt the phosphorylation, therefore activation, of STING and IRF3. As with C6, SPI-2 and CrmA disrupt IRF3 activation without effecting NF-κB activation (Qin et al., 2017). A more unusual example of IRF3 signalling disruption is by the viral protein N2. Unlike most VacV proteins, N2 is localised within the host cells nucleus were it is able to inhibit the transcriptional activity of IRF3 (Ferguson et al., 2013).

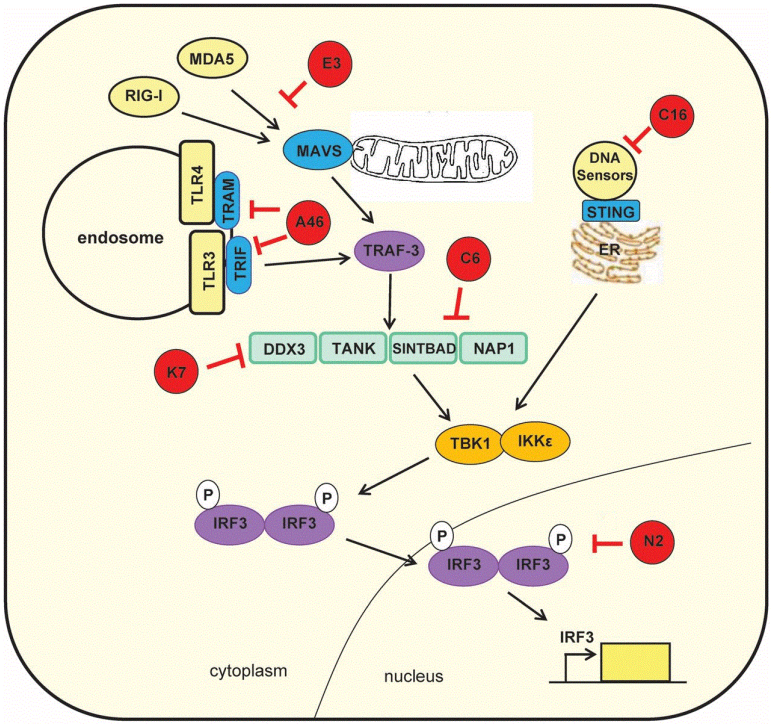


**Figure 8: Inhibition of the IRF3 signalling pathway by VacV**

*Pathway activation occurs when viral nucleic acids interact with PRRs in the cytoplasm or endosome. There are two main activation pathways, either via TRAF3 or STING, both converging on a common signalling pathway via TBK1 and IRF3. Once IRF3 is phosphorylated it forms a dimer where it translocates to the nucleus and acts as a transcription factor. VacV proteins are able to disrupt this at multiple points, these are shown in the figure and discussed further in the text (Smith et al., 2013).*

## Inhibition of NF-κB activation

Activation of NF-κB is an important step in viral innate immunty as it leads to the production IFNs and many proinflammatory cytokines, able to limit viral replication. Therefoe VacV produces multiple proteins that limit its activation (**Figure 9**). Many VacV proteins are able to directly bind to NF-κB signalling pathway components and disrupt their function, such as the viral protein K1. When expressed K1 is able to prevent the acetylation of the NF-κB subunit p65 preventing its activation (Bravo Cruz and Shisler, 2016). Other VacV Bcl-2 proteins disrupt NF-κB signalling further downstream, by interrupting the normal function of inhibitor of κB kinase (IKK). The VacV proteins N1 and B14 have both been shown inhibit NF-κB activation by drisupting IKK. B14 is able to bind the the IKK subunit IKKβ, preventing its phosphorylation and therefore preventing the phophorylation of the inhibitor of κB (IκBα) (Chen et al., 2008). By preventing the phosphrylation of IκBα, NF-κB remains bound to IκB and therfore inactive. Similarly, N1 is also able to bind to the IKK complex and disrupt NF-κB activation (DiPerna et al., 2004). Alongside direct interactions with IKK, VacV produces the

protein A49 which mimics a stable phophorylated IκBα (Mansur et al., 2013). Under normal signalling conditions, IκBα is phosporylated by IKKβ and then ubiquinated by the E3 ligase β-TrCP resulting in IκBα degradation and NF-κB activation, A49 is able to bind β-TrCP preventing this (Mansur et al., 2013).

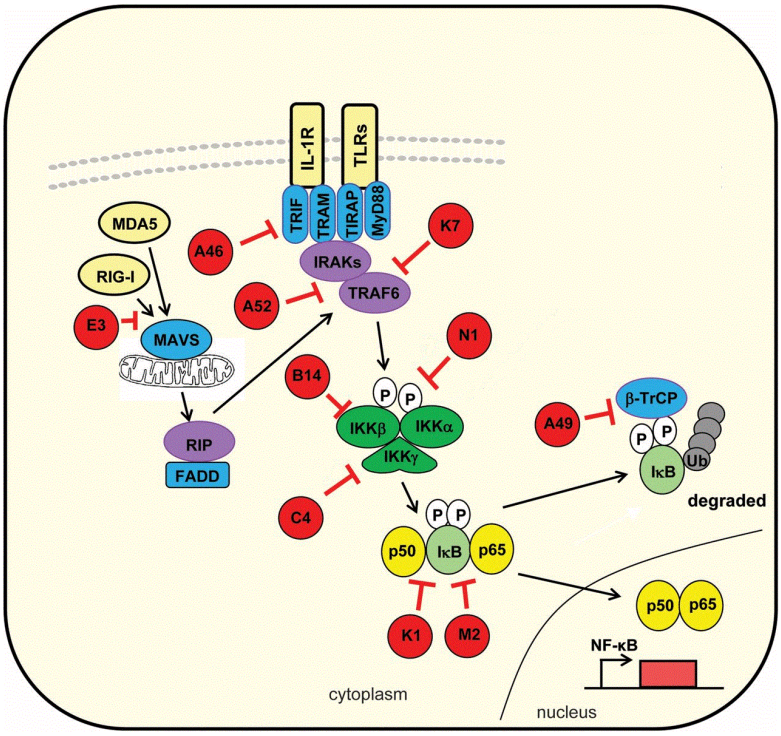


**Figure 9: Inhibition of the NF-κB signalling pathway by VacV**

*The pathway leading to NF-κB can be activated by both the intracellular and extracellular binding of nucleic acids to PRRs. Also shown is the activation by IL-1 binding to its receptor. Once PRRs bind to nucleic acids signalling occurs that activates TRAF6, resulting the phosphorylation and activation of IKK. Active IKK is able to dissociate IκB from NF-κB by targeting for proteosomal degradation. The action of VacV preventing this are shown as discussed further in the text (Smith et al., 2013).*

## Disruption of IFN signalling

As well as preventing IFN production, VacV is also able to prevent the dissemination and downstream signalling of IFNs. After IFNs are produced and released from cells, VacV is able to prevent IFNs from reaching their receptors by producing a number of soluble extracellular proteins including B18; a type I IFN binding protein(Xu et al., 2008) and B8; a soluble IFN receptor (Alcamí and Smith, 1996). Signal transduction from IFN signalling is also prevent in infected cells, due to the STAT phosphatase VH1 (Koksal et al., 2009). Despite VacV producing a wide array of proteins enabling multi-step inhibition of IFN signalling, most are required for effective replication. Mutations or loss of one or several of the immune evasion proteins result in reduced virulence and potentially ineffective replication (Smith et al., 2013). Suggesting that these immune evasion strategies do not display redundancy.

## 1.7 Aims

In this project, the ability of VacV to inhibit DNA sensing by the innate immune system, during early infection, is being investigated. The aim of this project are:

- To determine if VacV can inhibit the normal function of intracellular DNA sensors, with a focus on cGAS.
- To determine mechanisms and proteins involved in the inhibition of DNA sensing by VacV

This will be achieved by using molecular techniques such as western blotting, qPCR and cloning to detect cGAS changes during VacV infection and identify proteins involved. By achieving these aims, we hope to uncover a mechanism employed by VacV, during infection, which limits the cellular IFN response by disrupting the cGAS-STING pathway.

# 2 Materials and Methods

## 2.1 Cell culture

Immortalised human keratinocytes (HaCaTs) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) with the addition of 10% (v/v) Fetal Calf Serum (FCS) (Sigma) and 50µg/ml Gentamicin (Life Technologies). HEK293T cells (Thermo, HCL2517) were grown in DMEM with the addition 10% (v/v) Foetal Calf Serum (FCS) (Sigma) and 50µg/ml Gentamicin (Life Technologies). All cells were grown at 37°C with 5% C02
For viral work; BS-C-1, BHK21, and RK13 cells were used. These cells were grown in DMEM with the addition 10% (v/v) Foetal Calf Serum (FCS) (Sigma) and 50µg/ml Gentamicin (Life Technologies).

## 2.2 Buffers Used

| | |
|---|---|
| 10X Tris Buffered Saline (TBS) pH 7.6 | 0.2M Tris base<br>1.5M NaCl<br>H20 |
| Mammalian Cell Lysis Buffer | 50mM Tris-HCl (pH 7.5)<br>1mM EDTA<br>1mM EGTA<br>1% (v/v) Triton X-100<br>1mM Sodium Orthovanadate<br>50mM Sodium Fluoride<br>5mM Sodium pyrophosphate<br>10mM Sodium β-glycerophosphate<br>0.27M sucrose<br>0.1% (v/v) β-mercaptoethanol<br>0.1mM PMSF<br>10µl/ml Aprotinin |
| 3x SDS Sample Buffer | 62.5mM Tris-HCl (pH 6.8)<br>2% (w/v) SDS<br>10% (v/v) Glycerol<br>0.1% (w/v) Bromophenol blue<br>H2O |
| 50X TAE | 2M Tris Base<br>5.7% (v/v) Glacial acetic acid<br>50mM EDTA (pH 8)<br>H2O |

| | |
|---|---|
| Resolving Gel Buffer (pH 8.8) | 1.5M Tris Base<br>H20 |
| Stacking Gel Buffer (pH 6.8) | 0.5M Tris Base<br>H20 |
| 6x DNA Loading Buffer | 30% (v/v) Glycerol<br>0.025% (w/v) Bromophenol blue<br>0.025% (w/v) Xylene cyanol<br>H2O |

**Table 2: Buffers used throughout this project.**

## 2.3 DNA transfection

DNA transfections in HaCaT cells were performed using Lipofectamine 2000 (Invitrogen) to the manufacturer's instructions, by using 1µg DNA: 1µl of Lipofectamine 2000 per transfection. Treatment of cells with Lipofectamine 2000 alone was used as a control. DNA transfection in HEK293T cells were performed using GeneJuice (Novagen) to the manufacturer's instructions, by using 1µg DNA: 3µl GeneJuice. For double stranded DNA stimulation, Deoxyribonucleic acid sodium salt from herring testes (HT-DNA) (Sigma) was used.

## 2.4 Inhibitors

All inhibitors were dissolved in dimethyl sulfoxide (DMSO; Agilent) and applied to cells 1 hour before stimulation or infection. DMSO was used as a control for all inhibitors. The proteasome inhibitor MG132 (Sigma) was used at 10µM. The autophagy inhibitor Bafilomycin A1 (Sigma Aldrich) was used at 500nM. The general caspase inhibitor Q-VD-Oph (R&D systems) was used at 10µM.

## 2.5 Immunoblotting

Proteins were separated by size using sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

1.5mm 12% polyacrylamide gels were produced using the following:

| | dH2O | 40% Acrylamide | 1.5M Tris | 10% SDS | 10% APS | TEMED |
|---|---|---|---|---|---|---|
| Resolving Gel (12%) | 4.4ml | 3.0ml | 2.5ml | 0.1ml | 0.05ml | 0.005ml |

A higher percentage gel was used to seal the plates before addition of the resolving gel to prevent leaking. The plug was produced as follows:

250µl Resolving gel mix (without APS or TEMED)

5.0µl 10% APS

0.5µl TEMED

| | dH2O | 40% Acrylamide | 0.5M Tris | 10% SDS | 10% APS | TEMED |
|---|---|---|---|---|---|---|
| Stacking Gel (4%) | 1.9ml | 0.3ml | 0.75ml | 0.03ml | 0.03ml | 0.003ml |

**Table 3: Production of 12% polyacrylamide gels.**

Cultured cells were lysed in Mammalian cell lysis buffer on ice for 30 minutes. Lysate was scraped into clean Eppendorfs (Eppendorf) and centrifuged at 12,000 rpm on a benchtop centrifuge for 10 minutes and supernatant moved to a fresh tube. Samples were denatured using SDS sample buffer at 99°C for 10 minutes before loading onto SDS-PAGE gels

SDS-PAGE gels were run in a Mini-Protean Tetra Cell tank (Biorad) at 120V for 1.5Hours or until the dye reached the bottom of the gel. SDS-PAGE gels were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon), which was activated in 100% methanol before use. The transfer occurred in a semi-wet transfer pack for 1 hour at 0.1Amp per SDS-PAGE gel. Membranes were blocked using either 5% BSA (Sigma)/TBS/0.1% Tween or 5%non-fat milk (Marvel)/TBS/0.1% Tween depending on antibody compatibility with the buffer. After blocking the membrane was incubated with antibodies (see antibody section) at dilution 1:1000 in either 5% BSA/TBS/0.1% Tween or 5%non-fat Milk (Marvel)/TBS/0.1% Tween at 4°C overnight. Blots were then washed 3 X 5 minutes in TBS-0.1%Tween then incubated with secondary antibody, either anti-Rabbit-IgG-HRP (Horse Radish Peroxidase) or anti-Mouse-IgG-HRP (Horse Radish Peroxidase) at dilution 1:3000, for 3 hours at room temperature. The blots were then developed with ECL (Bio-Rad) by UV exposure with Chemidoc (Bio-Rad)

## 2.6 Co-Immunoprecipitation

Cells were lysed in mammalian cell lysis buffer. After lysis cells were centrifuged at 1000g for 3 min to pellet cell debris. Samples were pre cleared using Protein G agarose beads (Thermo Scientific) for 1 hour at 4°C. Samples were then centrifuged at 1000 X g for 5 minutes and supernatant collected. This supernatant was incubated with 1:2000 FLAG antibody overnight, followed by addition of fresh Protein G Spharose 4 Fast Flow beads (GE healthcare) for 3 hours at 4°C. After incubation, beads were washed 3 times with Mammalian cell lysis buffer, proteins bound to beads were eluted with 100ug/ml FLAG peptide/TBS (Sigma) for 1 hour. After Flag peptide elution, beads were washed 3 times with Mammalian cell lysis buffer, the resultant samples were analysed by Mass Spectrometry.

Small aliquots were taken from each step and mixed with 3X SDS sample buffer + DTT (150nM), then boiled at 99°C for Western blot analysis. These were used as an input control and for quality assurance.

For analysis of samples by Mass Spectrometry, the sample was treated with Trichloroacetic acid (TCA) to precipitate proteins. TCA was added to sample in a 1:4 (TCA:Sample) ratio, vortexed and incubated at 4°C for 10 minutes. Samples were then centrifuged at 13000 x g for 5 minutes to pellet the precipitate. Pellet was washed with ice cold acetone and air dried. Once air dried, pellet was resuspended in PBS overnight at 4°C. Resuspended samples were then mixed with 4X NuPAGE LDS Sample Buffer (Life technologies), 10X NuPAGE Reducing Agent (Life technologies) and deionised $H_20$. Samples were heated at 70°C for 10 minutes then loaded onto a precast NuPAGE 4-12% Bis-Tris Mini Gel (Life technologies). The gel was run following manufacturer's instructions by using MES buffer at 200V for 30 minutes or until the dye reached the bottom of the gel. The resultant gel was sectioned into equal parts and analysis by peptide mass fingerprinting at the FingerPrint Proteomics Facility (University of Dundee)

## 2.7 qRT-PCR

RNA was extracted from cells using RTK lysis buffer and ethanol. Extracted RNA was filtered through High Filter tubes using RNA Isolation Kit (Roche Applied Science), including a DNase step using 10µl DNase to 90µl DNase incubation buffer (Roche). RNA concentration was measured by using a Nanodrop 2000c spectrophotometer (Thermo Scientific) and equal amounts of RNA were taken from each sample.

cDNA was synthesised by reverse transcription of RNA by using iScript cDNA Synthesis Kit (Bio-Rad); using 0.5µl iScript reverse transcriptase and 2µl 5X iScript reaction mix, samples were made up to 10µl with nuclease-free H2O in 8-tube PCR strips (Brand). iScript samples were then run on Eppendorf Master Cycler for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, then cooled to 4°C.

qRT-PCR was performed using 2X Fast SYBR green mastermix (Roche; SYBR Green I dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP Passive Reference, and optimised buffer components). The master mix also contained Taq polymerase which, with specific oligonucleotide probes, amplifies target genes by PCR (polymerase chain reaction). The mastermix also contains SYBR Green I; a dsDNA specific fluorescent dye, used to detect and quantify amplified PCR products. The qRT-PCR was performed on an Applied Biosystems StepPlus One Real-time PCR machine, with the following programme;
Holding Stage (1 cycle): 1 minute at 90°C

Cycling Stage (35 cycles): 15 seconds at 95°C, 1 minute at 60°C
Melt Curve Stage (1 cycle): 15 seconds at 95°C, 1 minute at 60°C, 15 seconds at 95°C, 15 seconds 60°C

Primers were designed through NCBI Primer Blast and synthesised by MWG-Biotech. Primers were designed to be specific only for the gene of interest, and span multiple introns to avoid amplification of genomic DNA. The following primers were used at the final concentration of 500nM.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| B-actin (Human) | CGCGAGAGAAGATGACCCAGATC | GCCAGAGGCGTACAGGGATA |
| RANTES/CCL5 (Human) | CTGCTTTGCCTACATTGCCC | TCGGGTGACAAAGACGACTG |
| IFNβ | ACGCCGCATTGACCATCTAT | GTCTCATTCCAGCCAGTGCT |
| cGAS | GGGATGGTGAAAGGGGTTGT | GCAGAAATCTTCACGTGCTCA |

**Table 4: Primers used in this project.**

## 2.8 Bacterial transformation and Maxiprep

NovaBlue competent *E.coli* cells (Novagen) were incubated with DNA for 5 minutes on ice, then heat shocked at 42°C for 30 seconds before cooling on ice for a further 5 minutes. 100µl of SOC (Super Optimal Broth with Catabolite repression) media (Novagen) was added to the mixtures and plated onto LB agar plates with added Ampicillin. Plates were produced from LB agar powder (Novagen; 5g yeast extract, 10g peptone, 10g NaCL, 12g agar) dissolved in 1L of distilled water and heated in a microwave until boiling for 2 minutes. Once cooled, Ampicillin (Formedium) was added at 150µg/ml. Plates were then incubated overnight at 37°C. Single colonies were then picked and incubated in 3ml of Ampicillin containing LB broth on a shaking incubator for 6 hours. LB broth was made from LB Broth powder (Novagen; 5g yeast extract, 10g peptone, 10g NaCL) dissolved in 1L of distilled water and heated by microwave until boiling. After cooling Ampicillin (Formedium) was added at 150µg/ml. After incubation, the bacterial suspension was moved into 100ml of Ampicillin LB broth and incubated in a shaking incubator at 37°C overnight. The bacterial suspension was centrifuged at 15,000 x g for 10 minutes in the Avanti J-26 XP centrifuge using the J-Lite Series Rotor JLA-16.250 (Beckman Coulter). Bacterial pellets were lysed and plasmids were purified using the Maxiprep kit (Qiagen).

## 2.9 Plasmids used

pcDNA3.1(+) EV was purchased from Clontech. pcDNA3.1(+):cGAS-FLAG was generated by J. Almine (University of Lancaster).

## 2.10 Agarose Gel Electrophoresis

1% Agarose gels were produced using Ultrapure Agarose (Invitrogen) dissolved in 1xTris-Acetate-EDTA (Ethylenediaminetetraacetic acid) buffer (TAE; 2M Tris Base, 5.7% (v/v) glacial acetic acid. 50mM EDTA (pH 8), then heated in a microwave until boiling. After cooling, 1X SYBR safe stain was added and gently mixed. Agarose was then poured into a gel tray with well combs in place (VWR) and allowed to set at room temperature. Once set, the gels were placed in a gel tank and submerged in 1XTAE.

DNA samples were mixed with 3x DNA loading buffer. To provide size markers, a 2-log DNA ladder (Peqlab) was also mixed with DNA loading buffer. Both the samples and the DNA ladder were then loaded onto the gel, which was run at 5-7V/cm using a power pack (VWR) for 1.5 hours or until the dye had moved over half-way down the gel. Gels were then imaged on a GelDoc EZ Imager (Bio-Rad) using Image Lab Software (Bio-Rad)

## 2.11 Antibodies Used

| Target | Species | Company | Catalogue number |
|---|---|---|---|
| B-actin | Mouse | Sigma Aldrich | A2228 |
| IFI16 | Mouse | Santa Cruz | 8023 |
| cGAS | Rabbit | Cell Signaling | 15102 |
| STING | Rabbit | Cell Signaling | 13647 |
| IκBα | Mouse | Cell Signaling | 4814 |
| FLAG-tag | Mouse | Sigma | F3165 |
| Anti-mouse-HRP | Goat | Cell Signaling | 4414 |
| Anti-rabbit-HRP | Goat | Cell Signaling | 8889 |

**Table 5: Antibodies used in this project.**

## 2.12 Subcloning of cGAS

cGAS gene sequences was obtained using NCBI Gene database and restriction sites were checked using NED Cutter to identify 0 cut restriction enzymes. The restriction enzymes selected were Xhol (for forward primer and Not1 for reverse primer. By using the high fidelity polymerase, Herculase II, amplification of the cGAS insert by PCR occurred. This was performed using the following programme:

Holding Stage (1 cycle): 2 minute at 95°C
Amplification Stage (35 cycles): 20 seconds at 95°C, 20 seconds at 52°C, 1 minute at 72°C
Final Extension Step (1 cycle): 3 minutes at 72°C.

| Target sequence | Forward | Reverse |
|---|---|---|
| cGAS | AACTCGAGACCATGCAGC CTTGGCACGGA | AAGCGGCCGCAAATTCATCAAAAACTG GAAACTC |

**Table 6: Primers used for subcloning of Cgas**

## 2.13 Virus work

All viruses were stored at -80°C, defrosted thoroughly and mixed before use.
The viruses used throughout this project were: VacV-A5-EGFP, a VacV with GFP bound A5 protein and MVA

### 2.13.1 Virus propagation and purification

Flasks of either RK13 (VacV) or BHK21 (MVA), in DMEM + 10% FBS and gentamicin 50µg/ml, were infected with their respective viruses for 3 days (VacV) or 5 days (MVA). Cells were then scraped into media and collected in Falcon tubes (Falcon). The suspension was then centrifuged at 2000rpm in a benchtop centrifuge, the pellet washed, then re-centrifuged at 2000rpm for 10 minutes. The resultant pellet was then resuspended in 10mM Tris-HCL (pH9) and incubated on ice for 15 minutes. After incubation the cells were Dounce homogenised with 25 strokes before centrifugation at 1000rpm for 5 minutes. The supernatant was then layered above a 36% (w/v) sucrose/10mM Tris-HCL (pH9) solution in a polycarbonate ultracentrifuge tube. Further 10mM Tris-HCL (pH9) was added to the tube to ensure over half full and balanced. The tubes were then centrifuged in the Avanti J-26 XP centrifuge at 16500rpm for 70 minutes at 4°C using the JA-25.50 Fixed-Angle Rotor (Beckman Coulter). The supernatant was aspirated and the pellet resuspended in 10mM tris-HCL and stored at -80C before a plaque assay was performed to determine PFU.

### 2.13.2 Plaque assay

BS-C-1 cells were seeded in 6 well plates and grown in DMEM + 10% FCS until a confluent monolayer was achieved. A serial dilution of the virus into serum free DMEM was performed to obtain dilutions from $10^{-2}$ to $10^{-8}$, which was then added to each well. The virus was allowed to infect the cells for 1 hour, with gentile rocking every 15 minutes. After incubation the virus was removed and the cells were covered with 1:1 ratio of 3% Carboxymethyl cellulose and 2X DMEM (Milipore; with additional 100nM sodium pyruvate, 7.5% (w/v)

sodium bicarbonate). Cells were then incubated until visible plaque could be seen; 3 days (VacV), 5 days (MVA). The cell overlay was then removed and wells were washed 3 times with sterile PBS, before staining with Crystal violet + Methanol. Cells were stained for 30 minutes before removing the stain, then plates were air dried at room temperature. Once dry, visible plaques were counted and multiplied by the dilution factor to obtain plaque forming units per ml (PFU/ml).

### 2.13.3 Viral Infection

VacV and MVA were added at specified Multiplicity of Infection (MOI) to cells, in serum free DMEM, for one hour at 37C with 5% $CO_2$. During this hour the plate was rocked gently every 15 minutes. After one hour infection the media along with the virus was removed, the plate was washed with PBS then fresh DMEM + 10% FBS was added. The plate was then incubated for 4 hours, after which the cells were lysed on ice with mammalian cell lysis buffer.

## 2.14 Statistics

Results from RT-PCR analysis are presented as averages of biological triplicate samples with standard deviations displayed as Error bars. Statistical testing was performed using GraphPad QuickCalcs software. Statistical significance was determined using unpaired two sample Student t test (***= $P<0.001$)

# 3 Results

## 3.1 Cytosolic DNA activates the cGAS-STING pathway leading to IFNβ production

Cytoplasmic DNA is known to be recognised by the cGAS-STING pathway and promote IFN production (Sun et al., 2013). Transfection of HT DNA into wild type keratinocytes cells (HaCaT) was performed to show this. Cells transfected with DNA showed activation of STING (**Figure 10.A**), seen by the change in the ratio of phosphorylated (higher band) and non-phosphorylated STING (lower band). The total STING levels in the cells also reduced significantly, most likely due to the degradation of STING after activation (Konno et al., 2013). This shows that DNA promotes STING activation in HaCaT cells. To determine the effect STING activation has on IFNβ production, HaCaT cells were stimulated with DNA for 6 hours and mRNA levels were measured by RT-PCR using IFNβ specific primers. After DNA stimulation a clear 140 fold induction of IFNβ could be seen in the HaCaT cells (**Figure 10.B**). These result show that when HaCaT cells are stimulated with DNA STING is activated and IFNβ production is induced, suggesting cytoplasmic DNA sensor activation. These results were used as the baseline data for IFN induction with DNA when comparing to DNA virus infection.

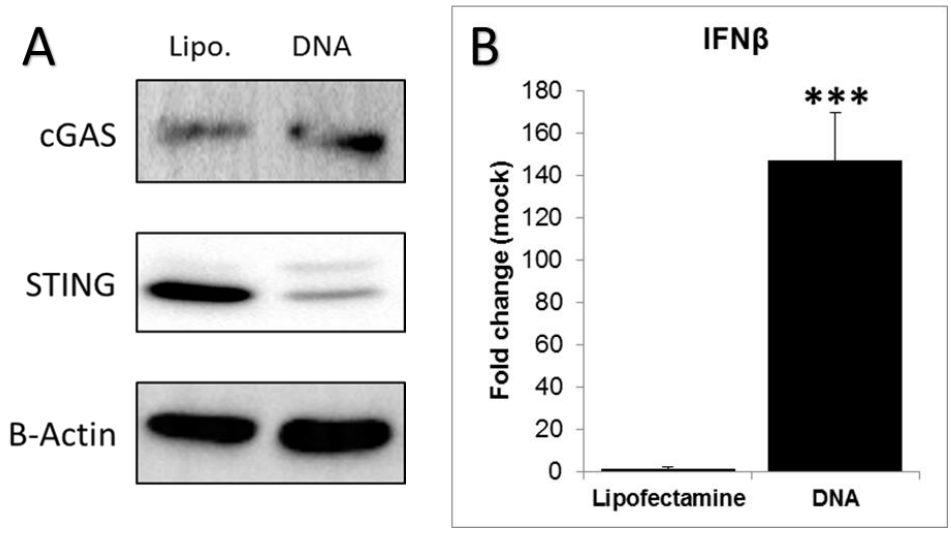


**Figure 10: DNA stimulation promotes IFNβ production and STING activation in wild type HaCaT cells.**

**A.** WT *HaCaT cells were either stimulated with 5µg/ml Lipofectamine 2000 (Lipo) or DNA, as indicated, for 4 hours in complete DMEM after which cells were lysed in mammalian cell lysis buffer. Cell lysates were separated by SDS-PAGE and analysed by immunoblotting with indicated antibodies.*

**B.** WT *HaCaT cells were stimulated with 5µg/ml Lipofectamine 2000 or DNA for 6 hours, as indicated. Cells were then lysed in RNA lysis buffer and analysed by RT-PCR. The results shown are averages of three biological triplicates. Error bars displayed represent standard deviation. A two-tailed unpaired T-test was performed and is displayed as *** (<0.001).*

## 3.2 Growth and purification of MVA stocks

Before infections could occur a stock of MVA with a known concentration would need to be generated. First MVA was grown in baby hamster kidney cells (BHK21) for 5 days, after which the cells were lysed by Dounce homogenisation and virus purified using sucrose cushion centrifugation. To determine the concentration of the virus sample obtained a plaque assay was performed (**Figure 11**). Monolayers of BS-C-1 cells were infected with MVA at dilutions $10^{-5}$ to $10^{-10}$ and allowed to infect cells for 1 hour in serum free DMEM. Virus was then removed and a semi-solid overlay media, complete DMEM + 3% carboxymethyl cellulose (CMC), was added to cells to inhibit viral dissemination and cell-to-cell transmission. After 5 days cells were stained and fixed with a crystal violet methanol solution. The visible plaques of dead cells were then counted (**Figure 11**). This gave the titre of MVA ($4x10^8$ pfu/ml) which was then used in subsequent infections.

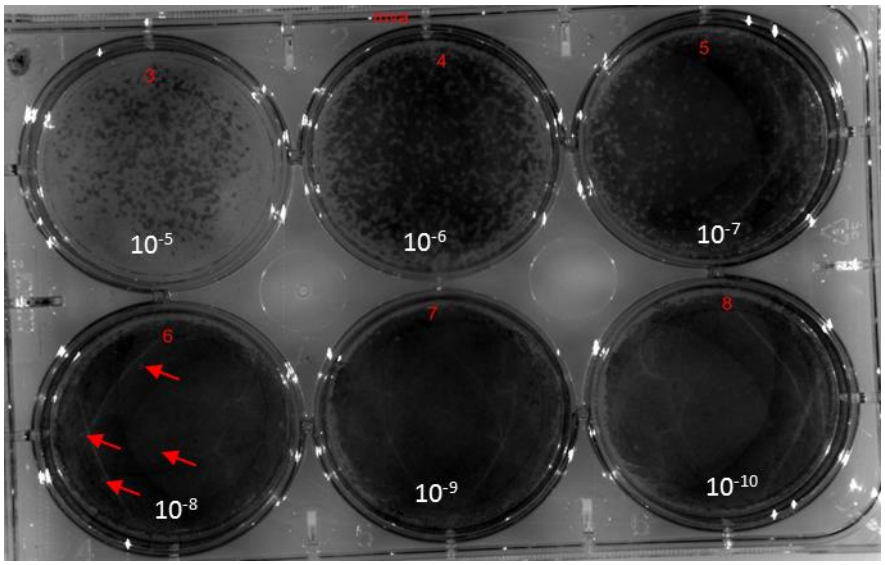


**Figure 11: Modified *Vaccinia Ankara* plaque assay.**

*A monolayer of BS-C-1 cells were infected with MVA at the dilutions shown, allowed to infect cells, and then immobilised with CMC. After 5 days cells were stained and fixed with a crystal violet/methanol mixture. After staining, areas of dead cells (plaques) were counted, the result was multiplied by the dilution factor to determine plaque forming units (pfu). Red arrows indicate visible plaques.*

## 3.3 VacV infection limits IFNβ mRNA upregulation when compared to DNA stimulation.

To determine if DNA viruses could induce the same levels of IFNβ mRNA as direct DNA transfection, VacV and the attenuated DNA virus MAV were used. MVA is an attenuated cytoplasmic DNA poxvirus closely related to VacV. MVA was produced by multiple passages through chicken fibroblasts, resulting in a 10% loss of genetic material compared to VacV. MVA lacks several key immune evasion genes found in VacV which contributes to its attenuated phenotype (Antoine et al., 1998). This results in MVA being unable to limit the cellular IFN response during infection.

As DNA transfection stimulates IFNβ upregulation, the DNA viruses VacV and MVA were investigated to determine if they were able to induce IFNβ. HaCaT cells were infected with MVA or VacV using a multiplicity of infection of 1 (MOI 1), for 1 hour in serum free DMEM. After this, media was removed and replaced with DMEM + 10% FBS and incubated for 6 hours. This was performed alongside a 6 hour stimulation of HaCaT cells with HT DNA. After incubation, cells were lysed and mRNA levels were measured by RT-PCR using IFNβ specific primers. When HaCaT cells were stimulated with HT DNA, there was a 160 fold increase of IFNβ compared to the mock. MVA infection also resulted in elevated IFNβ levels, around 40 fold increase, but did not induce IFNβ by the amounts seen in DNA transfection

(**Figure 12**). A two tailed T-test showed that the increase in IFNβ observed in MVA was not statistically significant (P= 0.149, *due to a large standard deviation*). VacV infection has a minor effect on IFNβ production, as infection only lead to a 20 fold increase. This suggest that VacV possesses a mechanism able to limit IFNβ production that MVA lacks.

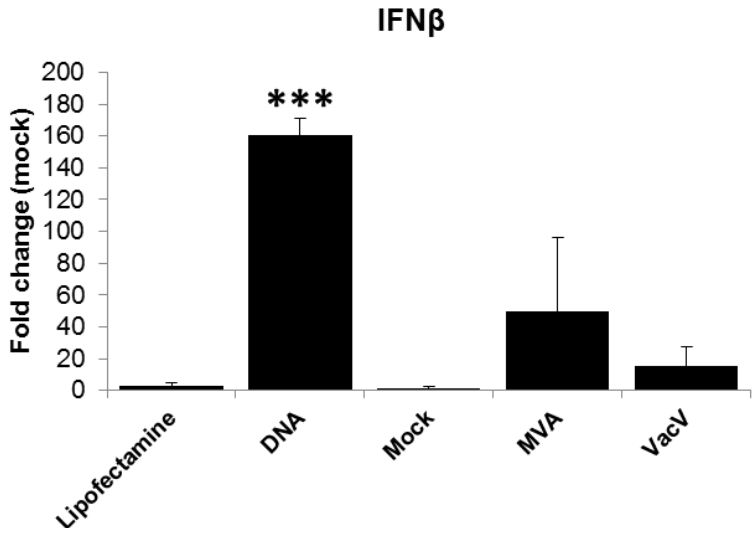


**Figure 12: VacV infection limits IFNβ mRNA upregulation in HaCaT cells.**

WT *HaCaTs were either stimulated with 5μg/ml DNA or infected with MVA or VAcV (MOI 1) for 6 hours, as indicated. Cells were then lysed in RNA lysis buffer and analysed by RT-PCR. The results shown are averages of three biological triplicates. Error bars displayed represent standard deviation. A two-tailed unpaired T-test was performed and is displayed as *** (<0.001). The T-test showed that MVA was not statistically significant compared to mock infection (P= 0.149, due to a large standard deviation.*

## 3.4 Both MVA and VacV have minimal effect on CCL5 mRNA upregulation compared to DNA transfection.

When cells are infected with viruses many proinflammatory cytokines are produced in an attempt to combat the infection. To determine the effect of MVA and VacV infection on cytokine production, CCL5, a chemokine upregulated during DNA stimulation (Levy, 2009), was investigated. HaCaT cells were infected with MVA or VacV (MOI 1), for 1 hour in serum free DMEM. After that, the media was removed and replaced with DMEM + 10% FBS and incubated for 6 hours. This was performed alongside a 6 hour stimulation of HaCaT cells with HT DNA. After incubation cells were lysed and mRNA levels were measured by RT-PCR using CCL5 specific primers. HT DNA stimulated cells show a 90 fold increase in CCL5 mRNA compared to the lipofectamine control **(Figure 13)**. However, both MVA and VacV did not show a significant increase of CCL5 mRNA. This shows that DNA transfection stimulates

CCL5 production, but the DNA viruses MVA and VacV did not result in significant CCL5 upregulation in HaCaT cells.

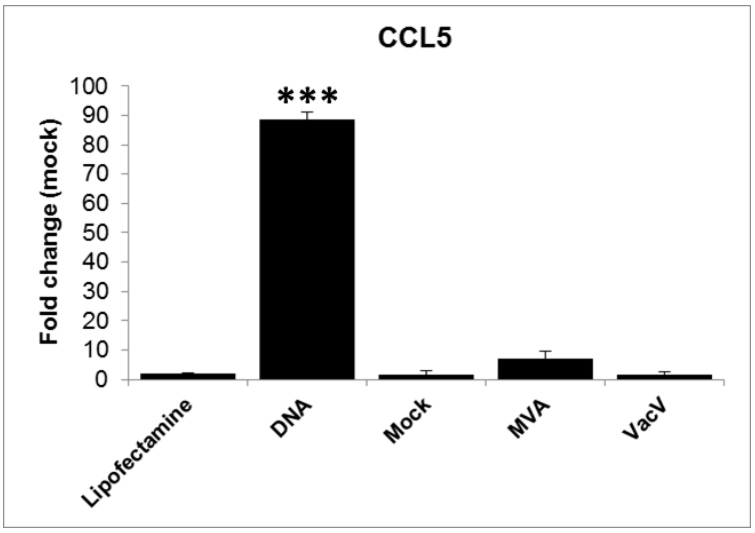


**Figure 13: MVA and VacV infections cause reduced CCL5 production in HaCaT cells, when compared to DNA transfection.**

*Wild type HaCaTs were either stimulated with 5µg/ml DNA or infected with MVA or VacV (MOI 1) for 6 hours, as indicated. Cells were then lysed in RNA lysis buffer and analysed by RT-PCR using CCL5 specific primers. The results shown are averages of three biological triplicates. Error bars displayed represent standard deviation. A two-tailed unpaired T-test was performed and is displayed as *** (<0.001).*

## 3.5 *Vaccinia* targets the cGAS-STING pathway to prevent IFN production

Transfection of HaCaT cells with DNA results in a significant upregulation of IFNβ and CCL5 however, this upregulation was limited in VacV infections. Suggesting VacV possesses mechanisms to limit cytokine production. VacV has been shown to produce multiple proteins that interfere with the innate immune response. The cGAS-STING pathway has been shown to be the main pathway that detects VacV in infected cells, and activation of this pathway leads to IFNβ production (Sun et al., 2013). To investigate the ability of VacV to limit IFNβ production during HaCaT infection, the cGAS-STING pathway was observed during infection. HaCaT cells were transfected for 4 hours with HT DNA, this was then compared to HaCaT cells infected with either MVA or VacV. Cells were inoculated with either MVA or VacV (MOI 1) for 1 hour in serum free DMEM, after which was replaced with DMEM +10% FBS. The cells were then incubated for 4 hours, after which cells were lysed by mammalian cell lysis buffer and analysed by Western blotting. Both DNA and MVA resulted in the activation of STING, shown by the presence two STING bands and a reduced levels of STING **Figure 14.A**. This is because, when STING is activated it becomes phosphorylated, the higher band visible in **Figure 14.A**, and after phosphorylation it is targeted for

degradation by autophagy (Konno et al., 2013). During DNA and MVA infection cGAS remains largely unaffected, though a slight reduction during MVA infection is observed. During VacV infection STING activation is prevented and cGAS levels are reduced and become undetectable. These observations show that VacV is able to interfere with cGAS to prevent STING activation and therefore limit IFNβ production.

In human keratinocytes, cGAS and IFI16 cooperate to activate STING during DNA sensing (Almine et al., 2017). IFI16 levels were investigated in HaCaT cells infected with VacV, to determine if IFI16 is lost during infection. Cells were infected with MVA and VacV (MOI 1), or stimulated with DNA. While VacV infection induces the loss of cGAS, IFI16 levels remain stable showing that inhibition of IFNβ occurs at the cGAS-STING level.

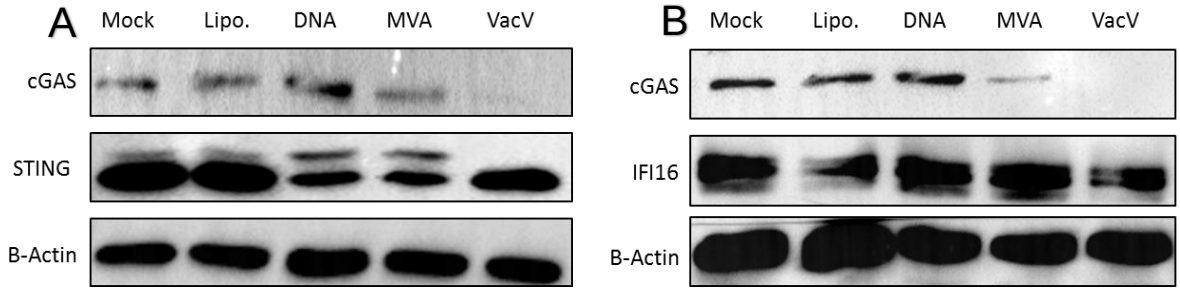


**Figure 14: VacV infection results in the loss of cGAS, but not IFI16, preventing STING activation.**

*A. Effect of DNA, MVA and VacV on cGAS levels and STING activation*

*B. Effect of DNA, MVA and VacV on cGAS and IFI16 levels*

*For both figures,* WT *HaCaT cells were either transfected with 5µg/ml HT DNA for 4 hours, or infected with MVA or VacV (MOI 1), as indicated. Cells were infected for 1h in serum free DMEM, after which the media was replaced with DMEM + 10% FBS and incubated for 4h.* Cells were lysed in mammalian cell lysis buffer and cell lysates were separated by SDS-PAGE. Gels were then analysed by immunoblotting with indicated antibodies.

## 3.6 *Vaccinia* targets cGAS directly for degradation during infection

Infection of HaCaT cells with VacV results in the loss of cGAS. To determine how VacV causes this, cGAS expression at the mRNA level was investigated. Previously VacV has been shown to directly affect host cell mRNA levels during infection (Parrish et al., 2007). To determine if VacV supresses the expression of cGAS mRNA, cellular mRNA levels were investigated in uninfected, DNA stimulated, and MVA or VacV infected HaCaT cells. Cells were treated with DNA or infected with MVA or VacV (MOI 1) for 6 hours. This time point was selected as cGAS is undetectable by Western blot from 4h, therefore any effect on mRNA should be clear by 6 hours. HaCaT stimulation with DNA and infection with both MVA

and VacV infection caused a minor (around 1.5-fold) increase in cGAS mRNA levels (**Figure 15**), although the increase was shown to be insignificant by T-test. This shows that DNA stimulation and DNA viruses have little or no effect on the expression levels of cGAS mRNA, suggesting VacV does not target cGAS at the mRNA level.

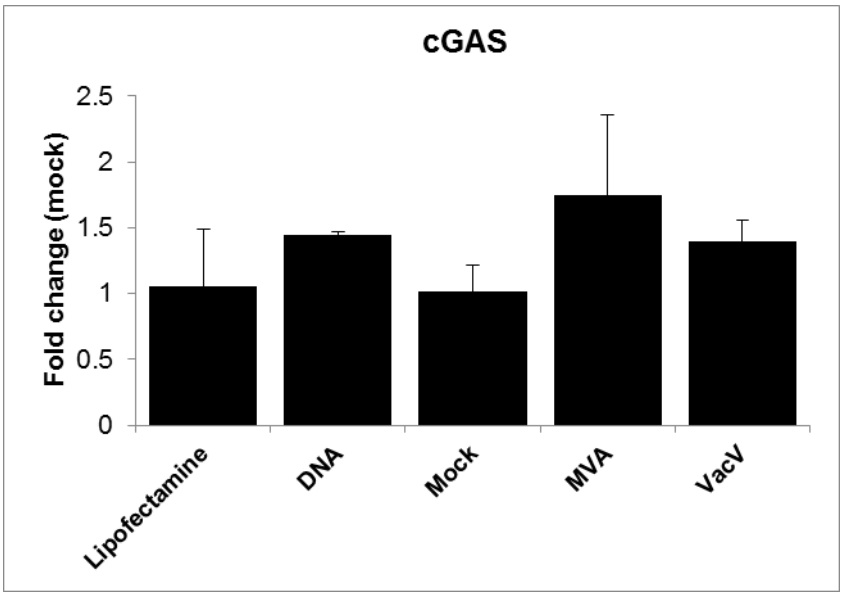


**Figure 15: cGAS mRNA remains stable during DNA stimulation and viral infection.** WT *HaCaT cells were stimulated with either 5µg/ml DNA or Lipofectamine 2000 and infected with with either VacV or MVA, both MOI 1. Cells were treated for 6 hours then were lysed in RNA lysis buffer. Lysates were analyses by RT-PCR in triplicate, then normalised to mock infection. The results shown are averages of three biological triplicates. Error bars displayed represent standard deviation. A two-tailed unpaired T-test was performed and found the increase of MVA and VacV from mock to be not statistically significant, P= 0.117 and P=0.062 respectively.*

To determine if VacV is able to directly target the cGAS protein for degradation, HEK293T cells were transfected with a plasmid containing the FLAG tagged protein cGAS-FLAG. These are an ideal cell type to study VacV/cGAS interactions as HEK293T cells lack many proteins in the DNA sensing pathway, including cGAS. Cells were transfected with a range of cGAS-FLAG concentrations to determine which levels mimic normal cellular levels. After allowing plasmid expression overnight, cells were infected with VacV (MOI 1) for 1 hour in serum free DMEM, after which they were incubated for 4 hours in DMEM + 10%FBS. These conditions are the same for HaCaT infections to allow direct comparison. **Figure 16** shows that levels of cGAS-FLAG higher than 0.5 µg/ml saturate the virus during infection and no significant cGAS loss can be seen. When lower concentrations of cGAS-FLAG are used (less than 0.5 µg/ml) results similar to HaCaT infection can be seen, as cGAS is lost after 4 hours. This suggests that during infection VacV is able to directly target and reduce cGAS

levels, although higher than physiological levels hinder the ability of the virus to reduce the protein fully.

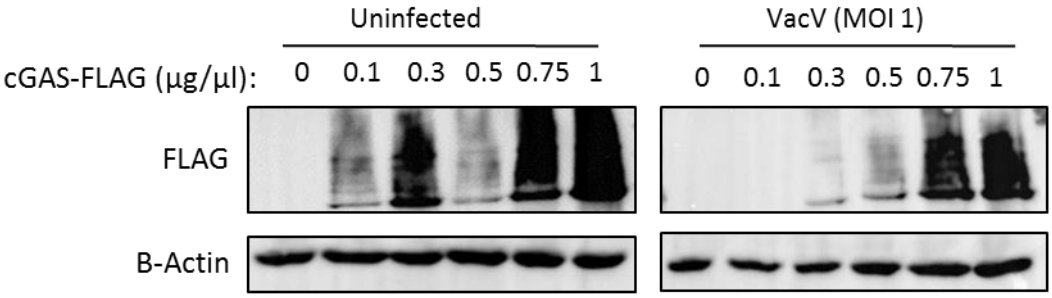


**Figure 16: Transfected cGAS-FLAG is also targeted by VacV.**

*HEK293T cells were transfected with a cGAS-FLAG containing plasmid, and allowed to express the protein overnight. A subset of these transfected cells were infected with VacV (MOI 1) for 4 hours. All cells were then lyses in mammalian cell lysis buffer, separated by SDS-PAGE then analysed by immunoblotting with an anti-FLAG antibody.*

## 3.7 *Vaccinia* does not use cellular degradation pathways to induce cGAS loss

To determine how VacV reduces cGAS levels during infection, cellular degradation pathways were investigated. The three main pathways in cells that lead to protein degradation are the proteasome, autophagy and caspases. These pathways were targeted for inhibition to investigate if VacV is drive cGAS loss by use of host degradation pathways

Autophagy is a lysosome dependent form of protein degradation within cells and is important in protein recycling. Targeted components are isolated and packaged in a cytoplasmic double membrane vesicles, autophagosomes. These autophagosomes then bind with lysosomes and target proteins are enzymatically degraded. This process is inhibited using the *Streptomyces griseus* derived Bafilomycin A1 by preventing V-ATPase dependent acidification of the autophagosome (Mauvezin and Neufeld, 2015). A control test was performed to determine efficacy before usage. DNA stimulation leads to STING activation and subsequent degradation, this degradation event uses the autophagy pathway (Konno et al., 2013). HaCaT cells were stimulated with DNA for 2 and 4 hours, with and without pre-treatment with Bafilomycin A1. **Figure 17.A** shows that DNA activates STING and after activation it is degraded, shown by the reduced band at 4h. Cells pre-treated with Bafilomycin A1 for 1h then stimulated with DNA still show STING activation, but no degradation occurs. This shows that Bafilomycin A1 treatment effectively inhibits autophagy in HaCaT cells.

Another degradation pathway targeted for inhibition was the proteasome. Proteins are targeted for degradation by the addition of ubiquitin molecules, ubiquitination, catalysed by ubiquitin ligases. Tagged proteins are then targeted by the proteasome for proteolytic cleavage (Tanaka, 2009). This process can be inhibited by carbobenzoxy-Leu-Leu-leucinal (MG132), as it blocks all proteolytic activity of the proteasome complex (Lee and Goldberg, 1998). To test this inhibitor HaCaT cells were stimulated with tumour necrosis factor (TNFα) for 15 minutes and IκBα degradation was investigated. TNFα stimulation leads to the activation of TNF receptor-associated factor 2 (TRAF2) and subsequent activation of IκB kinase, resulting in the degradation of IκBα (Wajant and Scheurich, 2001). **Figure 17.B** shows that when cells are stimulated with TNFα it results in the proteasomal degradation of IκBα. This should be prevented by MG132, but **Figure 17.B** shows MG132 has a minor effect on IκBα levels.

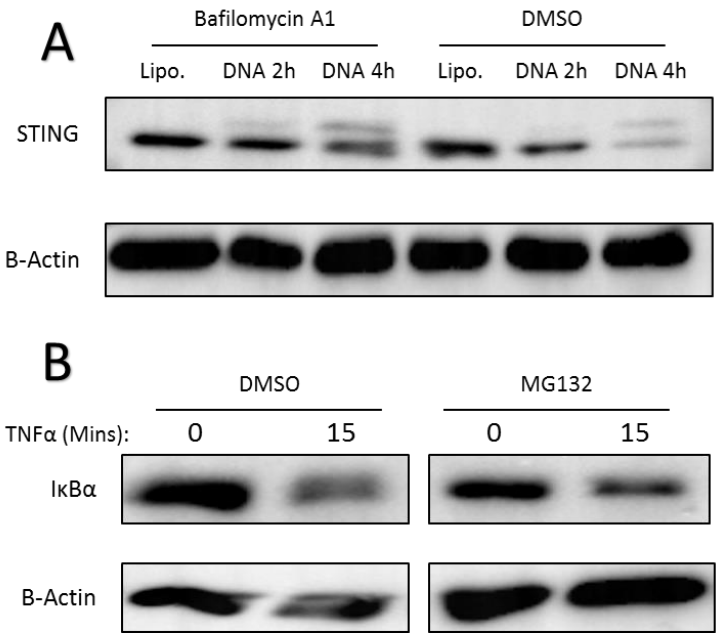


**Figure 17: Bafilomycin A1 and MG132 are able to inhibit autophagy and the proteasome, respectively.**

- **A.** WT *HaCaT cells were pre-treated with 50nM Bafilomycin A1 for 1 hour, after which they were stimulated with DNA for either 2 or 4 hours.*
- **B.** WT *HaCaT cells were pre-treated with 10μM MG132 for 1 hour, after which they were stimulated with 10μg/ml TNFα for 15 minutes.*

*All cells were lysed in mammalian cell lysis buffer, separated by SDS-PAGE and then analysed by immunoblotting with the indicated antibodies.*

After inhibitor efficacy was determined, the compounds were used alongside VacV infections to determine if host cellular degradation pathways were involved in the loss of cGAS. HaCaT cells were pre-treated with each inhibitor for 1 hour, after which they were inoculated with

VacV for 1 hour. Virus was then removed and fresh media was added, cells were left to incubate for 4 hours. When each inhibitor was used, cGAS loss caused by VacV infection was not prevented (**Figure 18**). As well as the aforementioned inhibitors, MG132 and Bafilomycin A1, the caspase inhibitor Q-VD-Oph was also used. Caspases are cellular proteases well defined for their role in apoptosis, however they also function in cell homeostasis and autophagy (Shalini et al., 2015). A Q-VD-Oph control experiment was not performed due to time constraints, although this is explored further in the discussion section. When treated with MG132 and Bafilomycin, some cGAS is still present after VacV infection as a faint band can be seen **Figure 18**. This was determined not to be significant as the majority of cGAS was still lost after VacV infection. These results show that VacV does not use the host degradation pathways to induce the loss of cGAS.

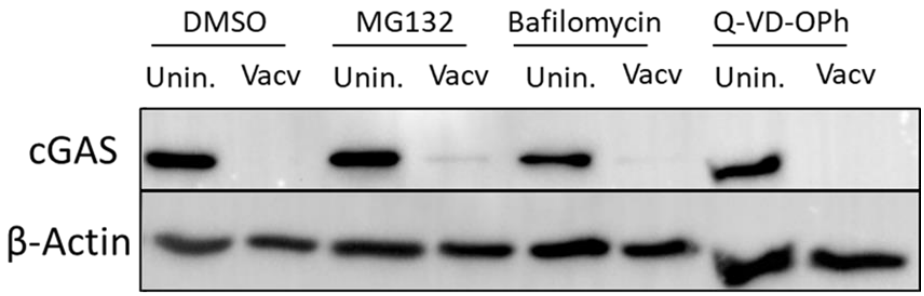


**Figure 18: Inhibition of the proteasome, autophagy, or caspase activity does not prevent cGAS degradation by VacV.**

*Wt HaCaT cells were pre-treated for 1 hour with the indicated inhibitors, after which cells were infected for 4 hours with VacV (MOI 1). Cells were then lysed in mammalian cell lysis buffer, separated by SDS-PAGE then analysed by immunoblotting with indicated antibodies.*

## 3.8 Identifying VacV proteins that bind cGAS

As VacV does not use host cellular degradation pathways to promote cGAS loss, it was determined that VacV must target cGAS directly. To identify any VacV proteins that were able to interact with cGAS an immunoprecipitation (IP) of the cGAS protein was performed. However, before performing VacV infections, it needed to be determined if cGAS could be isolated from cell lysates by IP. To test this, HEK293T cells were transfected with plasmids containing either cGAS-FLAG or IRF3-FLAG. IRF3-FLAG was used as a positive control for the IP due to previous experiments demonstrating its ability to be pulled down from lysate. Transfected cells were allowed to express proteins overnight, after which they were lysed and proteins were isolated from lysates by FLAG-IP. A FLAG elution was performed to elute proteins from the FLAG antibody and eluted proteins were analysed by Western blot. **Figure 19** shows that HEK293T cells are able to express the cGAS-FLAG containing plasmid which can then be isolated by FLAG IP.

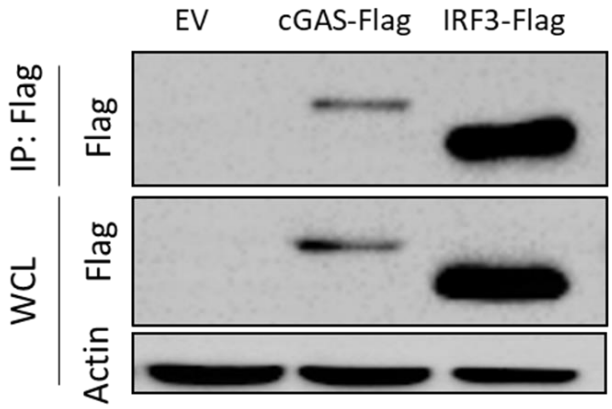


**Figure 19: cGAS-FLAG can be isolated from HEK293T cell lysate by IP.**
*HEK293T cells were transfected with plasmids containing cGAS-FLAG and IRF3-FLAG, and allowed to express these proteins overnight. After which, cells were lysed and the indicated proteins were isolated from the lysates using FLAG IP and FLAG elution. Whole cell lysate (WCL) was used as an IP control. Samples obtained from IP were separated by SDS-PAGE and analysed using FLAG antibody. Whole cell lysis is shown as an input control and was processed under the same conditions.*

Once it was determined that cGAS-FLAG could be pulled down by IP, this was used to investigate if any VacV proteins bind to cGAS during infection. However this presented a problem, as cGAS is lost within 4 hours of VacV infection. To circumvent this restriction, lysates from 2 subsets of HEK293T cells were used and then mixed. Cells were either transfected with cGAS-FLAG and allowed to express the plasmid, or infected with VacV (MOI 1), after which cells were lysed and lysates mixed. This ensured that VacV would have sufficient time to express the proteins that facilitate cGAS loss and could be mixed with cell lysate containing cGAS-FLAG to observe interactions with cGAS. To maximise the amount of VacV proteins and cGAS, this experiment was done at a large scale. HEK293T cells were grown in 15cm diameter dishes with $9x10^6$ cells per plate; 5 plates were transfected with empty pcDNA 3.1 vector, 5 plates were transfected with pcDNA 3.1 cGAS-FLAG, and 10 plates were infected with VacV (MOI 1). All transfections were performed with 20µg/mL of DNA. Cells were lysed and lysates were mixed overnight at 4°C. After mixing cGAS, was isolated from the lysate by FLAG-IP and eluted by FLAG elution. The eluted proteins were then precipitated using trichloroethanoic acid/methanol precipitation, then dissolved in PBS. Samples were separated on a 4-12% Bis-Tris gel and stained with Coomassie for 1 hour, then cut into equal parts (**Figure 20**). The samples were then sent to be analysed by peptide mass fingerprinting at the FingerPrint Proteomics Facility (Dundee). After analysis, VacV proteins were identified using the Universal Protein Resource database (Pundir et al., 2017). Viral proteins unique to the cGAS-FLAG containing samples are shown in **Table 7**. Proteins

identified were mostly VacV structural or replication proteins and many were found within the EV containing samples, suggesting the immunoprecipitation protocol was non-specific.

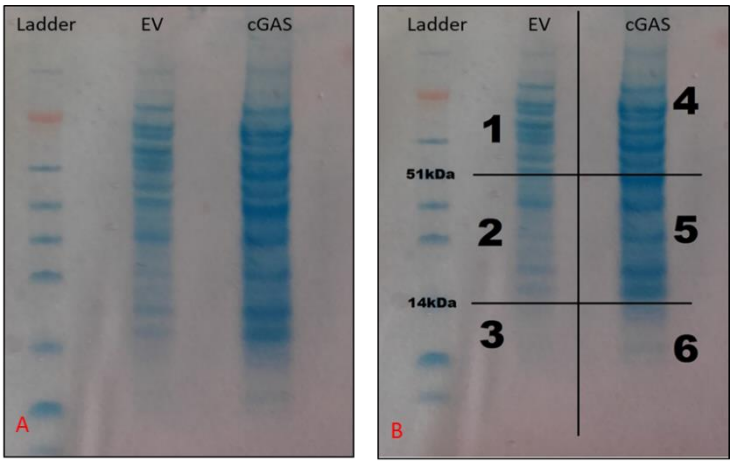


**Figure 20: Coomassie stained Bis-Tris gel of FLAG IP from HEK293T cell lysates**

*HEK293T cells were seeded into 20, 15cm diameter plates, at 9x10$^6$ cells per plate; 5 were transfected with pcDNA 3.1 EV, 5 were transfected with pcDNA cGAS-FLAG, and 10 were infected with VacV (MOI 1) for 4 hours. Cells were then lysed and each transfection lysate was mixed with an equal amount of VacV infected cell lysate, at 4°C overnight. After mixing, cGAS was isolated from the lysate by FLAG-IP and eluted by FLAG elution. The eluted proteins were then precipitated using trichloroethanoic acid/methanol precipitation, then dissolved in PBS. Samples were separated on a 4-12% Bis-Tris gel, stained with Coomassie and cut into equal sections, as shown. Samples were then sent for analysis by peptide mass fingerprinting at the FingerPrint Proteomics Facility (University of Dundee)*

| Protein | Function |
|---|---|
| ***L1*** | Role in virus fusion with host cell |
| ***B8*** | Soluble IFN-gamma receptor |
| ***VP8*** | Core Protein, virion assembly |
| ***3 beta-hydroxysteroid dehydrogenase/Delta 5>4 isomerase*** | Steroid production to inhibit host immune response |
| ***Cap-specific mRNA (nucleoside-2) methyltransferase*** | Aids viral gene transcription |
| ***Ribonucleoside-diphosphate reductase small chain*** | Aids in DNA synthesis |

**Table 7: VacV proteins identified by mass spectrometry after FLAG IP in cGAS-FLAG containing HEK293T cells.**

*Results generated from the peptide mass fingerprinting were compared against the Universal Protein Resource database (Pundir et al., 2017). Shown is the proteins only found in the cGAS-FLAG cells and their function in viral infection.*

## 3.9 Generation of pcDNA5/FRT/TO-GFP cGAS plasmid

Once VacV that associate with cGAS are identified, they would be tested for their ability to induce cGAS loss seen during infection. Potential candidate VacV proteins and genes, identified from the co-IP, would be tested for their ability to induce cGAS loss. This would be performed in a HEK293T cell line that is stably transfected and expresses cGAS. Therefore, stable HEK293T cells expressing cGAS would need to be generated. The Flp-In system was selected as it allows integration of target plasmids into the host cell genome. Firstly, the cGAS coding sequence from the plasmid pcDNA 3.1 cGAS-FLAG needed to be amplified using cGAS primers to generate the insert that would be used in the Flp-In system. Before this, restriction enzymes needed to be selected to enable insert of the cGAS sequence without causing cleavage of either the vector or insert. The Vector selected was pcDNA5/FRT/TO-GFP which contains a FRT/TO site that when used with the Flp-In system results in genome integration, also GFP is present downstream of the multiple cloning site causing any insert genes to produce a protein tagged with GPF at the C-terminus. The restriction sites present within its multiple cloning site were tested against the cGAS insert using NEBcutter (Vincze et al., 2003). The restriction enzyme sites selected were Not1 and Xhol. These restriction enzymes were tested against the pcDNA5/FRT/TO-GFP vector to ensure they do not cleave aberrantly (**Figure 21.A**). After it was determined that the restriction enzymes only cut at the desired places, they were tested against the cGAS insert (**Figure 21.B**). This showed that the selected enzymes, Not1 and Xhol, only cut the vector and insert at the desired position. Both the vector and insert were digested by the restriction enzymes and allowed to ligate. After ligation the plasmid was propagated in *E.coli* and single colonies were selected and plasmids were purified by Miniprep. The resultant plasmids underwent a restriction digest with Not1 and Xhol, to determine if ligation was successful. **Figure 21.C** shows that all samples, except one, contained the correct insert. Samples in lanes 3 and 4 were taken and purified by Maxiprep.

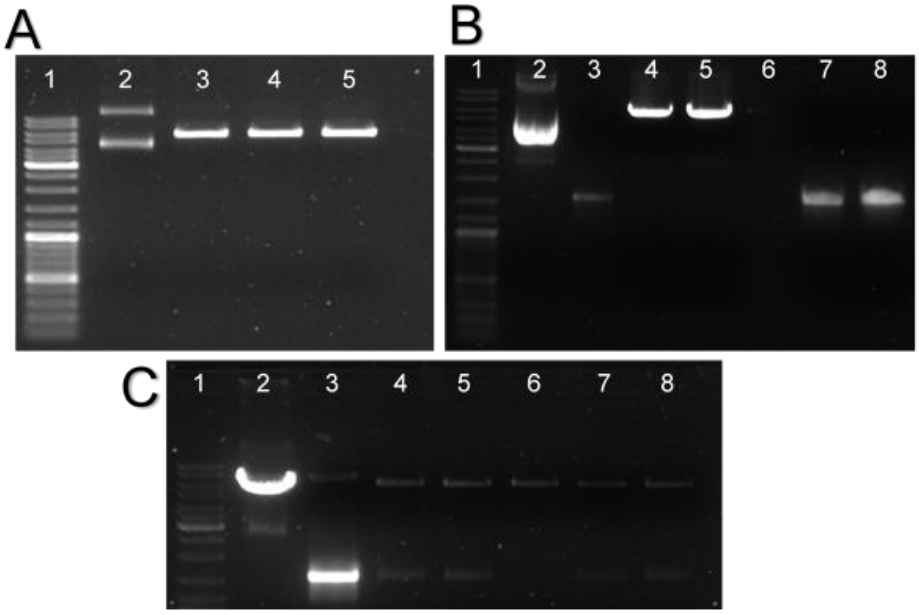


**Figure 21: Subcloning of the plasmid pcDNA3.1 cGAS-FLAG to produce the plasmid pcDNA5/FRT/TO-GFP cGAS-FLAG.**

- **A.** *The restriction enzymes Not1 and Xhol were selected. The vector pcDNA5/FRT/TO-GFP was digested with Not1 and Xhol, a double digest was also performed. Lanes represent; 1= ladder, 2=Uncut vector, 3=Not1 cut vector, 4=Xhol cut vector, 5=Not1 and Xhol cut vector*
- **B.** *To determine if Not1 or Xhol aberrantly cut the pcDNA5/FRT/TO-GFP vector or the cGAS insert a single digest was performed on both. Lanes represent; 1=Ladder, 2= Uncut vector, 3= Uncut insert, 4=Not1 cut vector, 5=Xhol cut vector, 6=blank lane, 7=Not1 cut insert, 8=Xhol cut insert.*
- **C.** *After vector and insert were ligated they were expressed in E.coli and grown on selectivity media. Single colonies were then taken and expanded in media broth. These were then purified by Miniprep and the resultant plasmids were checked for the correct insert. Lanes represent; 1=Ladder, 2= Xhol and Not1 cut vector, 3= Xhol and Not1 cut insert, 4-8= Single colony Minipreps.*

# 4 Discussion

Smallpox was once a devastating human disease, but was eventually eradicated by use of the poxvirus VacV. Long after smallpox eradication, VacV is still an area of great interest regarding the understanding of the innate immune system, as it successfully evades many antiviral processes; specifically DNA sensing and IFN production. Interactions between VacV and the innate immune system is an intensely studied area, which has discovered many VacV immune evasion strategies as well as widening our understanding of the innate immune system. One such study was the identification of the cytosolic DNA sensor cGAS and its essential role in DNA virus sensing (Sun et al., 2013). Since the discovery of the cGAS-STING pathway it has been of great interest in understanding VacV immune evasion. To date cGAS is known to be the primary DNA sensor of VacV, and the virion counters this by effecting downstream proteins such as STING and IRF3 (Georgana et al., 2018), as well as interacting with DNA sensors directly (Peters et al., 2013, Meade et al., 2018). Despite this, many questions still remain about the mechanism VacV employs to disrupt DNA sensing.

The aim of this study was to investigate the immune evasion mechanisms VacV possesses in order to counter DNA sensing in host cells. For this study it was determined that HaCaT cell lines were the most representative cell type for multiple reasons. Firstly, keratinocytes are one of the main sites of poxvirus infection, therefore HaCaT cell lines provided the most biologically relevant cell types (Boukamp et al., 1988). In addition to this, HaCaT cells have been shown to possess multiple DNA sensors which cooperate in IFN production upon DNA stimulation (Almine et al., 2017). Initial infection of HaCaT cells with VacV showed a reduction in cGAS levels by 4 hours post infection. This reduction resulted in cGAS being undetectable by Western blot, suggesting VacV is able to induce total loss of cGAS within 4 hours of infection. This provided the main focus area of this study.

During infection with VacV, the loss of cGAS prevented the activation of STING resulting in reduced IFN production. The downstream proteins IRF3 and TBK1 were not investigated during this study, though cGAS loss and lack of STING activation would likely result in no activation of IRF3 and TBK1. This could be determined by investigating the whole cGAS-STING pathway during DNA stimulation, MVA, and VacV infection. By infecting HaCaT cells and lysing at specific time points before cGAS is lost, the whole cGAS-STING pathway can be investigated during the first hours of infection. This can be performed by Western blot using antibodies specific for pathway proteins e.g. IRF3, TBK1, cGAS and STING. Surprisingly VacV did not target other DNA sensors for degradation that cooperate with cGAS to activate STING. The AIM2-like DNA sensor IFI16 has been shown to work

alongside cGAS to activate STING, especially in keratinocytes (Almine et al., 2017). This study shows that, even though VacV promotes the degradation of cGAS, IFI16 remains intact and is not targeted for degradation. Degradation of IFI16 could still occur but at a later timescale not used in this study. Conversely, VacV may produce a protein that, while it does not degrade IFI16, could potentially block interaction between IFI16 and cGAS to limit STING activation, a mechanism seen in other VacV proteins (Chen et al., 2008, Mansur et al., 2013, Peters et al., 2013). Further study would be needed to determine if VacV is able to directly target IFI16 during infection. This could be performed using confocal microscopy, by investigating VacV interactions with key DNA sensors during infection. Cells would be infected with VacV at specific time points and imaged using fluorescently labelled antibodies, able to target viral proteins expressed in early and late infection, as well as host proteins. The advantage of this would be the ability to track VacV localisation with DNA sensors at specific time points during infection. If both cGAS and the virus localise at specific locations, this could provide an insight into how VacV is able to promote loss of cGAS.

Inhibition of transcription would result in a reduction in cGAS mRNA and reduced protein levels. This mechanism could be employed by VacV during infection to reduce levels of cGAS. Therefore, the ability of VacV to influence cGAS transcription was investigated. At 6 hours post infection cGAS mRNA levels remained unchanged, suggesting VacV does not affect cGAS at the mRNA level. This lead to the hypothesis that VacV is able to facilitate the degradation of cGAS by manipulating the host protein degradation pathways. Testing with autophagy, caspase and proteasome inhibitors did not prevent cGAS degradation, showing VacV does not use host cell degradation pathways to induce cGAS loss. However, by blocking one degradation pathway VacV may be able to utilise an alternative degradation pathway to induce the loss of cGAS. To determine this, cells would be treated with all three aforementioned inhibitors and the effect on cGAS degradation by VacV would be observed. Although care would need to be taken as inhibition of multiple essential cell pathways can lead to premature cell death.

Blockage of host cell degradation pathways did not prevent the loss of cGAS during VacV infection, suggesting VacV is able to produce an enzyme that specifically targets cGAS for degradation. This has been observed in many other VacV proteins that target innate immune proteins for degradation (McKenzie et al., 1992, Jha and Kotwal, 2003). However, while enzymatic cleavage is likely, no degradation or cleavage products are seen on any of the cGAS blots. This could be because cGAS cleavage by VacV coincidentally disrupts the antibody binding site. This would result in immunoblotting yielding no results, even if degradation products were present. To identify these products, cGAS tagged with FLAG at

both the N and C terminus could be used. By transfecting cells with cGAS containing a FLAG tag at both the N and C terminus, anti-FLAG antibodies can be used to detect degradation products. This was explored superficially in this study with the plasmid pcDNA3.1 cGAS-FLAG, but no degradation product was seen.

Before using each inhibitor, a positive control was designed to ensure that the target pathway was sufficiently inhibited. However, as mentioned briefly in the results section, no positive control has been performed with Q-VD-Oph. This was because, unlike Bafilomycin and MG132, no experiment could be designed which used simple techniques such as immunoblotting. Despite this, a positive control was designed, although never performed due to time constraints. To test the ability of Q-VD-Oph to inhibit caspase activity, cells would be treated with etoposide, which is able to associate with genomic DNA of cells causing double strand breaks and inducing apoptosis. The number of apoptotic cells can be measured by flow cytometry and annexin A5 staining, a dye commonly used to detect apoptotic cells. This can be then used to measure the ability of pre-treatment with Q-VD-Oph to limit apoptosis.

A recent study has identified the VacV protein F17 that is able to target cGAS for degradation during infection (Meade et al., 2018). While this result supports the observations reported in this study, several key points differ. Firstly, F17 is a late VacV protein, that is only expressed when mature intracellular virion formation begins, around 6 hours post infection (Wickramasekera and Traktman, 2010, Wittek et al., 1984). This is shown by inhibition of viral transcription, and therefore late mRNA formation, by cytosine arabinoside resulting in no F17 production (Wittek et al., 1984). Furthermore, due to the nature of the process described; dysregulation of the mTOR pathway by (Meade et al., 2018), it was hypothesised that this is only relevant in late stage immune evasion, evident by the 24-40 hour time points used. In this study VacV was able to facilitate the complete degradation of cGAS within 4 hours, suggesting an alternative early immune evasion mechanism. These conflicting results suggest that several mechanisms may be present to facilitate the degradation of cGAS, one important in early infection the other used in late infection. Meade et al., (2018), also showed that the proteasome is essential for F17 function as MG132 prevented the mTOR dependent cGAS loss. This contradicts the results shown in this study, as treatment with MG132 did not affect VacV ability to degrade cGAS. This further supports the hypothesis that several cGAS degradation pathways exist that occur at different stages of infection. To test this hypothesis several experiments can be performed. Firstly, cGAS is undetectable by Western blot 4h post infection with VacV, therefore immunoblotting of the F17 protein can be performed 4 hours post infection to determine if VacV expresses this protein at this time point. If F17 is found to be expressed at 4 hours, co-IP can be performed to determine if RICTOR and

RAPTOR are sequestered. Furthermore, the described pathway results in mTOR dysregulation by VacV induced phosphorylation of mTORC1 substrates p70S6K and 4E-BP1 and mTORC2 substrate PKB, phosphorylated at S473 (Meade et al., 2018). These can also be identified by Western blotting at the time VacV induced cGAS loss occurs in HaCaT cells, 4h post infection.

To investigate interaction between viral proteins and cGAS a co-IP was performed. This initially presented a problem as cGAS is lost within 4h of VacV infection. This restricted cGAS pulldowns to very early infections, before many VacV proteins are expressed. To counteract this problem, two separate lysates were used. HEK293T cells were either transfected with cGAS-FLAG containing plasmids or infected with VacV, lysed then mixed. This enabled interactions between VacV proteins and cGAS, without the loss of cGAS. This allowed cGAS to be isolated by FLAG co-IP allowing investigation of VacV proteins bound to cGAS. Elution with FLAG peptide was performed after the IP to allow cGAS to separate from the bound antibody, ensuring the sample did not contain IgG heavy or light chains. IgG heavy and light chain are approximately 50kDa and 25sDa, respectively, around the size of both cGAS and VacV proteins (Janeway, 2005). Therefore, FLAG elution prevented any interference these may cause, especially when Immunoblotting.

The results gained from peptide mass fingerprinting show that optimisation is needed before useful data can be generated. These results showed that HEK293T cells were successfully transfected with cGAS-FLAG and isolated from lysates by immunoprecipitation. However, this result was not confirmed by Western blotting, due to small sample volume and a concentrated sample resulting in overloading of the gel. This resulted in unusable Western blots and little sample available to rerun. This could be avoided by reconstituting the TCA precipitated sample in a higher volume and diluting the sample before loading onto a SDS-PAGE gel. Many vaccinia proteins were identified by mass spectrometry, most of which are structural or important in viral replication, and many were also present in the empty vector control, where no VacV proteins should be isolated. This shows that the protocol is non-specific and proteins bound to cGAS are not being discriminated from general viral proteins. A way in which specificity for proteins could be increase is to introduce more stringent washing steps during the IP to remove unbound viral proteins. The expression of the cGAS-FLAG plasmid was also low when transfected into HEK293T cells. This is evident by no clear cGAS band visible in the Coomassie stained gels and low levels detected by mass spectrometry. This can be addressed by using higher amounts of plasmid DNA during transfection, allowing longer for the plasmid to be expressed or using a plasmid with stronger promoter.

During this study, the plasmid pcDNA5/FRT/TO-GFP cGAS-FLAG was produced, but was not used for its entire intended use due to time constraints. The overall objective was to create a stable GFP tagged cGAS containing HEK293T cell line by using the Flp-In system, which can then be used in viral infections. The Flp-In system allows integration and expression of genes into mammalian cells at targeted genomic locations. By introducing a FLP Recombination Target (FRT) site into the cell line genome, an expression vector containing the C-terminal GFP tagged cGAS can be integrated into the genome of HEK293T cells. This occurs via the Flp recombinase-mediated DNA recombination at the FRT site (O'Gorman et al., 1991). The cloned plasmid would contain GFP downstream of the multiple cloning site resulting in the expression of a protein tagged with GPF at the C-terminus. This would enable screening and identification to be performed via fluorescence and allowing study of VacV interaction with cGAS in isolation, due to Hek293T cells naturally lacking cGAS. After the generation of the cell line, data obtained from the co-IP identifying candidate VacV genes that target cGAS would be taken and tested against this cell line. Multiple VacV open reading frames of interest would be subsequently tested to identify candidate genes that promote the loss of cGAS. Once identified, knockout VacV could be generated to test these genes. If the identified gene was responsible for cGAS degradation, this degradation would be prevented with the KO virus. This gene sequence can also be compared against the MVA genome. MVA is a cytoplasmic DNA poxvirus closely related to VacV which, due to multiple passages through chicken fibroblasts, has lost around 10% of its genetic material. Therefore, MVA does not possesses all genes VacV has, and is attenuated *in vivo* (Antoine et al., 1998). However, during several infections with MVA, cGAS levels are reduced slightly compared to mock infection or DNA stimulation. This suggests that MVA may still possess functional genes able to target cGAS, similar to what is seen with VacV infections. However, MVA infections still resulted in STING activation suggesting DNA sensing was not affected by MVA to prevent pathway activation. While STING activation was observed, no significant IFNβ mRNA upregulation was seen with MVA infections. This could be due to not enough time between MVA infection and cell lysis to allow significant IFNβ upregulation.

Identification of VacV proteins able to induce cGAS loss and disrupt DNA sensing provides potential clinical applications. The cGAS-STING pathway is abnormally active in several diseases such as Lupus Erythematosus and Aicardi-Goutieres Syndrome, and often contributes to the disease phenotype (Gray et al., 2015, Wang et al., 2018a). Consequently the, Identification of cGAS inhibitors, derived from VacV, could lead to potential new treatments for such diseases.

# 5 Conclusion

Intracellular DNA sensing through the cGAS-STING pathway is one of the most important viral sensing pathways in the innate immune system. Activation of this pathway leads to IFNβ production and subsequent virus clearance. During infection the cytoplasmic DNA virus VacV is able to facilitate the degradation of the key DNA sensor cGAS. With the loss of this DNA sensor, activation of key downstream signalling proteins such as STING is prevented and IFNβ production is blocked. The viral protein responsible for this is expressed in early infection, within 4 hours, and appears to target cGAS directly for enzymatic cleavage, although a candidate protein remains elusive. This provides valuable information regarding intracellular DNA viral sensing and viral immune evasion.

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