Recognition, immune evasion, and exploitation of DNA viruses

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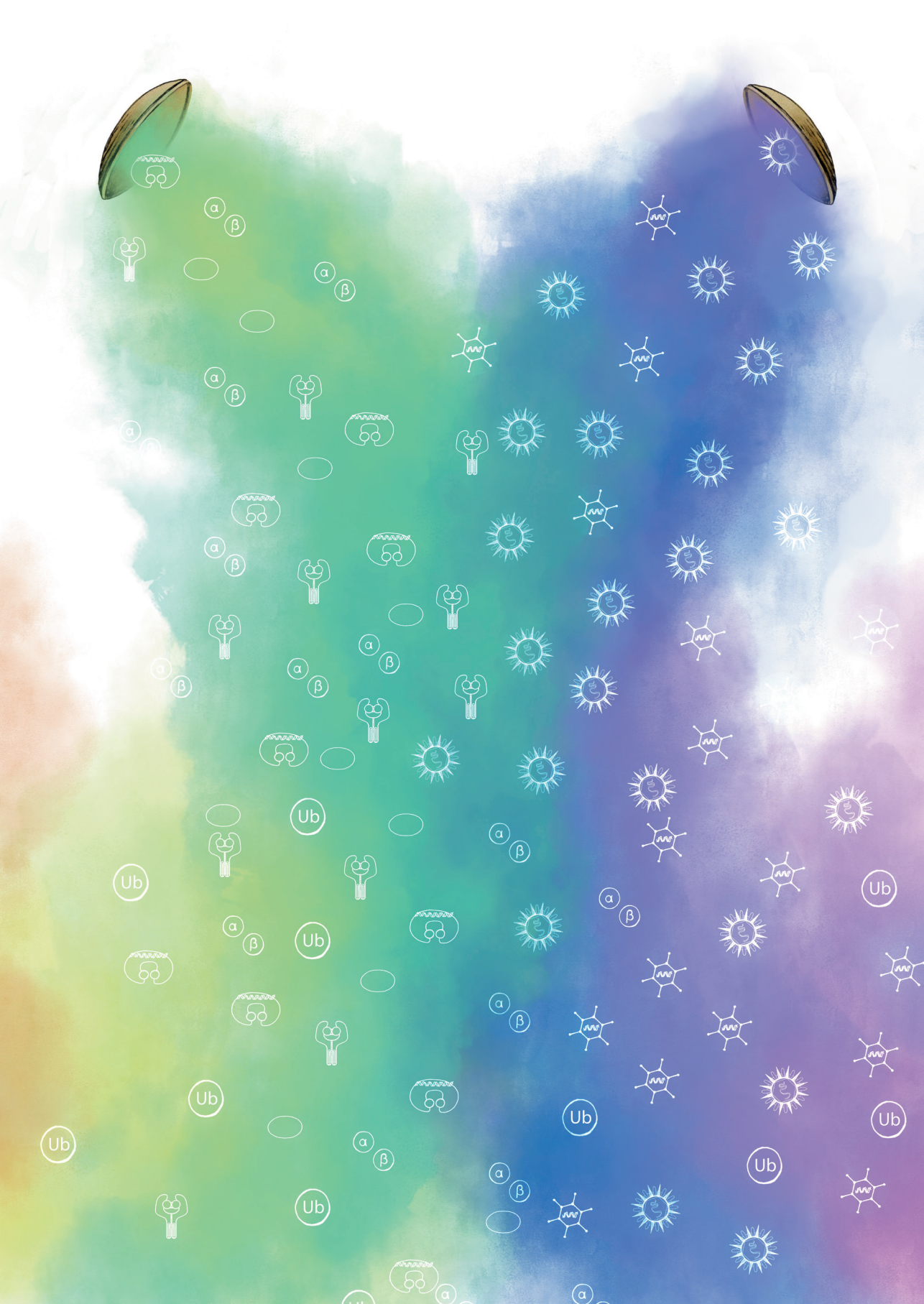
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General introduction

Viruses are infectious agents that require living cells of a host for their replication. They are classified into different families based on factors such as phenotypic characteristics, host organisms and associated diseases. In this thesis, we focus on two virus families: *herpesviridae* and *adenoviridae*. Both viruses infect mammals, birds, reptiles, and fish and are primarily host specific. To protect themselves against viral infections, cells possess an intricate network of antiviral immune pathways. In turn, viruses have evolved equally complex methods to evade detection by the immune system. Understanding how viruses activate and evade various immune responses provides us with the tools to tip the balance in our favor. In addition, the knowledge gained in this thesis is of value for the combat against immunosuppressive tumors, as viral factors can expose these cancer cells to the immune system.

1. Herpesviruses

Herpesviruses are enveloped viruses containing a linear double-stranded DNA genome that is tightly packed within a capsid. So far, eight human herpesvirus species have been discovered. These are the α-herpesvirus species Herpes simplex virus (HSV) 1, 2, and Varicella Zoster virus, the β-herpesvirus species Human cytomegalovirus, Human Herpesvirus 6, and 7, and the γ-herpesviruses Kaposi's sarcoma-associated herpesvirus and Epstein-Barr virus (EBV). A hallmark of herpesviruses is their ability to establish life-long infection in a host, also known as latency (1). From within the latent state, lytic reactivation is initiated frequently, leading to the production of viral progeny. Latency is achieved by the expression of latency associated genes and several noncoding RNAs, which regulate a multitude of processes such as 1) stimulation of growth, 2) partitioning of viral genomes in mitosis, 3) suppression of lytic genes, and 4) inhibition of the antiviral immune response (1, 2). The α-herpesviruses establish latency in the peripheral nervous system, β-herpesviruses latently infect monocytes, and γ-herpesviruses cause life-long infection in B-cells (1). As a result, these viruses are highly prevalent, the most common virus being EBV, which has persistently infected over 90% of the human adult population. Herpesvirus infections are associated with a range of clinical manifestations ranging from mild symptoms, such as blisters (HSV), to life-threatening diseases, i.e., Burkitt lymphoma (EBV). In addition, immunocompromised individuals have a high risk of morbidity and mortality caused by lytic reactivation. The latter poses an enormous threat to patients who receive immune suppressors when they undergo stem cell or organ transplantation (3). Although antiherpesviral agents are available, the number of resistant viruses continues to grow (4). This calls for novel antiviral agents targeting distinct viral pathways.

2. Adenoviruses

Adenoviruses (AdVs) contain a linear double-stranded DNA genome, which is protected by an icosahedral capsid. The capsid consists mainly of hexon molecules and a dozen penton bases. In addition, each penton bears a trimeric fiber, which are essential for binding of AdVs to host cells. There are seven human adenovirus species known (hAdV-A to hAdV-G), to which over 108 isolated types belong (*http://hadvwg.gmu.edu/*). Each type has a unique genomic sequence, with great variation especially in the capsid protein sequences. Depending on their fiber sequence, adenoviruses bind the Coxsackie and Adenovirus Receptor (CAR), CD46, desmoglein-2 (DSG2), glycoproteins, and/or scavenger receptors,

followed by binding of the penton-base to an integrin structure (5, 6). This permits AdVs a broad tissue tropism, which includes the eyes, kidneys, blood stream, gastrointestinal and respiratory tract. In immunocompetent individuals, infections are usually accompanied by mild symptoms (7). For immunocompromised hosts, however, an AdV infection can be life threatening (8). Upon entering cells, AdVs are taken up into early endosomes, from which they subsequently escape into the cytosol (9). Naked virions are then transported along the cytoskeletal network towards the nucleus, wherein the viral dsDNA genome is released (10). This allows for the expression of early genes that activate the cell cycle (E1A), suppress apoptosis (E1B), induce viral genome replication (E2), inhibit immune activation (E3), and regulate stability of viral RNA (E4) (11).

The dependency of AdVs on early genes for replication and survival, as well as their broad tropism, makes these viruses ideal vectors for gene and cancer therapy. Tumor specificity of AdVs can be achieved by deleting the Rb binding domain of E1A, or by deleting E1B from the viral genome (12, 13). In healthy cells, E1A binds to Rb to remove inhibition of the transcription factor E2F, a key regulator of cell cycle progression. Deletion of the Rb binding domain within E1A restricts viral replication to cells that are already rapidly dividing, i.e. cancerous cells. E1B targets p53 for degradation. Thus, deletion of E1B from the virion allows AdVs to replicate efficiently only in cells that are p53 deficient. Conveniently, p53 is the most commonly mutated gene in human cancers (14). Currently, these oncolytic adenoviruses are tested in clinical trials as immunotherapy in combination with immune checkpoint inhibitors (15, 16).

3. Antiviral immunity

To protect itself from the incoming infection, the host is equipped with an immune system. Within the immune system, two subsystems can be distinguished (17). The first type is innate immunity, which is fast, non-specific and has a short-lived memory. The second type is adaptive immunity, which is slow, pathogen-specific and provides long-term protection. Both innate and adaptive immunity contribute to the recognition, slowing down and full clearance of circulating infectious agents.

3.1. Innate immunity

The innate immune response against pathogens is established by a multitude of processes. These include the secretion of small defense peptides, activation of the complement system and secretion of inflammatory and antiviral cytokines (18–20). Type I interferons (IFN I) comprise an essential class of cytokines consisting of 13 IFNα subtypes, IFNβ, IFNε, IFNκ and IFNω, all of which bind to the IFN I receptor (IFNAR) upon being secreted from cells. Activation of the IFNAR leads to a secondary response that induces an antiviral state within, and in the proximity of, the infected cell (21–23). IFN I are produced upon recognition of viral proteins or nucleic acids by pattern recognition receptors (PRRs), as a consequence of their downstream signaling cascades (24, 25). One of the earliest types of PRRs to be discovered are the toll-like receptors (TLRs). TLRs are transmembrane proteins that detect incoming pathogens at the plasma membrane or within endocytic vesicles (26). In addition, nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), absent in melanoma-2 (AIM2)-like receptors (ALRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and Cyclic GMP–AMP synthase (cGAS)-like receptors are

known to contribute to the detection of a broad range of pathogen-associated molecular patterns (PAMPs) (27, 28). Signal transduction downstream of these receptors is predicated on the recruitment and activation of (partially) unique combinations of downstream adaptor proteins, kinases and transcription factors, ultimately leading to the induction of an inflammatory response and the release of cytokines into the environment of the infected cell.

3.2. Adaptive immunity

The adaptive immune response is dependent on the activation of B and T lymphocytes. B cells are essential for the establishment and maintenance of humoral antiviral immunity, whereas T cells provide a cell-mediated immune response.

B cell response

B cells mature in the bone marrow and subsequently migrate to lymphoid tissues, where they await activation. Upon recognizing a specific antigen through its B-cell receptor (BCR), B cells become active plasma cells that secrete large quantities of antibodies essential for the establishment and maintenance of humoral antiviral immunity.

Neutralizing antibodies (NAb) target AdVs for degradation and enhance the innate antiviral immune response (29). These NAbs are directed primarily against the hexon hypervariable regions and the fiber knobs (30, 31). The seroprevalence against AdV serotypes differs greatly (32, 33). For hAdV species A and C, high titers of neutralizing antibodies are measured, while species B has the lowest seroprevalence. Globally, hAdV C5 has the highest seroprevalence on average (32–34). Interestingly, hAdV C5 has been used extensively in clinical trials as an oncolytic agent, albeit with moderate success (35). It is possible that high levels of pre-existing immunity create a major obstacle for the establishment of hAdV C5 as a successful oncolytic therapy (36).

NAbs against herpesviruses are predominantly targeted against the envelope glycoproteins (37). For the γ-herpesvirus EBV, NAbs are mainly directed against gp350, which is the most abundant glycoprotein (38). Other EBV surface glycoproteins, including gB, gH, gL, gp42 and BMRF2 can be targeted by NAbs as well (39–41). Due to the high global burden of the virus, scientists worldwide have been attempting to develop a prophylactic EBV vaccine directed against viral glycoproteins for four decades (42). However, these studies have been challenging due to lack of efficiency, suitable animal models, and proper vaccine delivery platforms. As an alternative, the development of therapeutics targeting latent proteins of EBV could be pursued as most EBV-associated malignancies are linked to viral latency (43).

T cell response

T cells are lymphocytes that migrate to the thymus for their development after being produced in the bone marrow. They have a broad range of effector functions essential for clearance of infected cells (44). There are two groups of T-cells – CD4+ helper T cells and CD8+ cytotoxic T cells. T cells express the T-cell receptor (TCR) on their cell surface which they use for the detection of specific peptides that are being presented on a specialized molecule called MHC complex. All nucleated cells express MHC I, which can interact with the

TCR of CD8+ T cells. In addition, specialized antigen presenting cells express MHC II, which engages the TCR of CD4+ T cells. Binding of the TCR to the matching MHC-bound peptide sequence is an essential step for the initiation of T cell effector functions.

For AdVs, CD4+ T cells are mainly directed against conserved regions of the hexon protein (45, 46). Furthermore, hexon-specific CD4+ T cells are cross-reactive between many serotypes and species, which can be attributed to the high degree of conservation among their target epitopes (47). Hexon and penton both contain immunodominant peptides that are recognized by CD8+ T cells (48, 49). Similar to hexon-specific CD4+ T cells, CD8+ T cells targeting hexon epitopes are cross-reactive between serotypes (47).

EBV specific CD4+ and CD8+ T cells recognize both lytic and latent EBV antigens, albeit with unique immunodominance (50). In patients with infectious mononucleosis (IM), CD4+ T cells are swiftly activated upon primary infection, followed by a rapid decline in the response (51, 52). During infection, there is no strong increase in CD4+ T cell (50). In contrast, the CD8+ T cell population directed against lytic antigens expands rapidly to remain high in the weeks following acute infection. CD8+ T cells directed against lytic antigens can even reach up to 40% of the total CD8+ T cell pool in IM patients (53). In contrast, in asymptomatic individuals EBV-specific CD8+ T cells were detectable, but no increase in the total CD8+ T cell population was observed (54).

3.3. Shaping of adaptive immune signaling through IFN I

In response to viral infection, secreted IFN I stimulates clonal expansion and memory formation of CD8+ T cells (55–57). In addition, IFN I can enhance the antibody response against soluble proteins (57). However, the effect of IFN I on CD4+ T cells is less clear (58). Some have reported that IFN I is important for clonal expansion of CD4+ T cells upon viral infection, whereas others have shown that CD4+ T cells can only clear a persistent viral infection when IFNAR signaling is blocked (59, 60). The latter could be related to the observation that IFN I signaling upregulates the expression of coinhibitory receptors, such as PD-1, on the cell surface of CD4+ T cells (61). In addition, IFN I promotes the survival of immunosuppressive regulatory T cells by enhancing the expression of Foxp3 and the anti-apoptotic genes Bcl-2 and Mcl-1 (62, 63).

4. Control of immune activation

While virus-induced IFN I upregulation protects a host from disastrous infection-related outcomes, persistently high levels of IFN I in the absence of infection can have detrimental effects on the human body. Patients with excess levels of IFN I develop systemic chronic inflammation causing a variety of symptoms, including painful chilblains and interstitial lung disease (64). This heterogeneous group of diseases characterized by uncontrolled IFN I was brought to clinical attention in 2011 by Yanick Crow under the term ‘type I interferonopathies’ (65). A genotype is considered as type I interferonopathy when an inborn error of immunity results in uncontrolled immune signaling involving persistent IFN I upregulation (66). In addition, dysregulated IFN I signaling seems to enhance progression of several autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis (67–69).

In healthy individuals IFN I induction is tightly regulated. To achieve this, various post-translational modifications (PTMs) are employed. Immune modifying PTMs range from the incorporation of small chemical groups, including phosphorylation, methylation, acetylation, palmitoylation, and succinylation to the attachment of small proteins, such as ubiquitin and ubiquitin-like modifiers SUMO and ISG15, to PRRs and signaling intermediates (70–72). These modifications can alter the activation state, localization and protein-protein interactions of innate immune signaling components.

4.1. Ubiquitination

Ubiquitination is one of the most versatile PTMs. It involves the covalent attachment of a ubiquitin moiety to a protein through a three-step process performed by E1 activating, E2 conjugating and E3 ligating enzymes. Monoubiquitination has been reported to play a role in DNA-based processes, such as replication, transcription and repair (73). In addition to monoubiquitination, proteins can be polyubiquitinated through the linkage of lysine K6, K11, K27, K29, K33, K48, K63 or methionine M1 of the attached ubiquitin to the carboxyl terminus of the next ubiquitin moiety (74). These different linkages can alter the fate of their target proteins in various ways, exemplified by K48-linked polyubiquitination, which targets proteins for 26S proteasomal degradation. To reverse the effects of ubiquitination, cells express ubiquitin specific proteases known as deubiquitinating enzymes (DUBs). Cells encode over 600 E3 ligases and close to 100 DUBs to dynamically target a wide variety of protein substrates (75, 76). This way, ubiquitination can be used to regulate a broad range of signaling pathways.

4.2. Ubiquitination in immune signaling

Production of IFN I is initiated upon recognition of PAMPs by PRRs, a step that is highly controlled by ubiquitination (77). For example, the PRR cGAS alone can be targeted by monoubiquitination (78, 79), K27-, K48-, or K63-linked polyubiquitination (80–82), as well as deubiquitination (83–85), at multiple sites. The PRR RIG-I is predominantly tagged with K63-linked polyubiquitin chains at either the N- or C-terminus by four different E3 ligases (86–88). While the modification on either terminus is the same, both are required for proper downstream signaling, as their impact on RIG-I is unique (88). Ubiquitination of its N-terminal domain enables binding of RIG-I to its downstream adapter MAVS, whereas ubiquitination of its C-terminus seems to induce conformational changes in RIG-I to allow its assembly along a dsRNA strand. The importance of ubiquitination for the control of PRR signaling is further emphasized by the observation that viruses have evolved strategies to use the ubiquitin system for their escape of immune recognition (89).

In the adaptive immune response, ubiquitination plays an important role in the regulation of signaling pathways downstream of BCRs and TCRs (90). A well-studied downstream signaling molecule in this context is the transcription factor NF-κB (91). In the cytoplasm, NF-κB is kept inactive by members of the inhibitory IκB protein family. Upon viral infection, PRR signaling leads to downstream recruitment of the IκB kinase (IKK) complex to NF-κB and subsequent phosphorylation of IκB (92). Phosphorylated IκB is then polyubiquitinated by the large ubiquitin E3 complex SCF and targeted for degradation by the proteasome. Upon being released from IκB, NFκB is translocated to the nucleus, where it initiates transcription of various proinflammatory cytokines and chemokines (93). An

essential step in the recruitment of the IKK complex to NF-κB is K63-linked polyubiquitination of its subunit NEMO (94). Ubiquitination of NEMO is in turn reversed by the DUB CYLD, rendering CYLD a negative regulator of NFκB signaling (95). Viruses also interfere with NF-κB pathways through (de)ubiquitination of NF-κB signaling intermediates (96). For instance, HSV-1 encodes the ubiquitin-specific protease UL36 to reverse K48-linked ubiquitination of the IκB family member IκBα, thereby blocking its degradation and antagonizing activation of NF-κB (97). BPLF1, a DUB encoded by EBV also prohibited IκBα degradation and is likely involved in the removal of K63-linked polyubiquitination of NEMO (98).

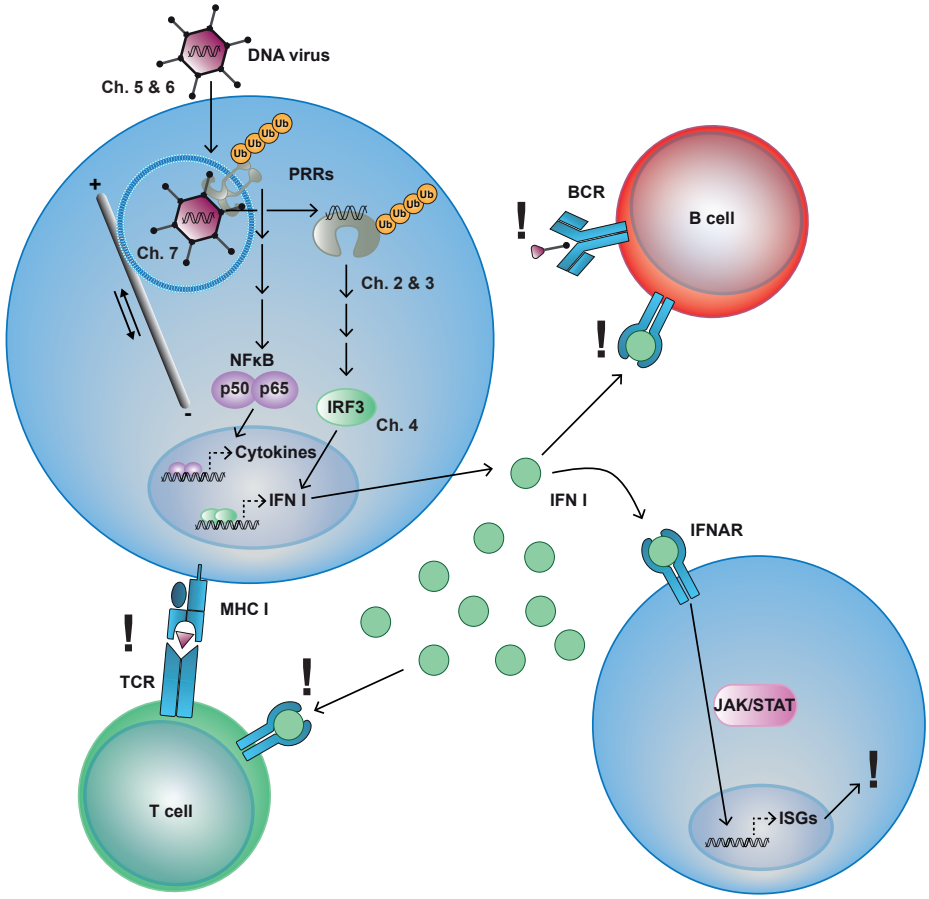

Figure 1. Schematic overview of innate and adaptive immune responses induced upon viral infection. The intracellular innate immune response against DNA viruses is initiated upon recognition of viral components by pattern recognition receptors (PRRs). Activation of PRRs, which is tightly regulated by post-translational modifications (PTMs), causes downstream signal transduction resulting in nuclear translocation of transcription factors NFκB and IRF3. Nuclear NFκB and IRF3 initiate the transcription of pro-inflammatory cytokines and type I interferons (IFN I) respectively. Secreted IFN I engages the interferon-α/β receptor (IFNAR), leading to JAK/STAT signaling and downstream production of interferon stimulated genes (ISGs). ISGs encompass proteins that are essential for restricting viral infection. In addition, adaptive immune responses, consisting of B and T lymphocytes, are essential for the control and elimination of viral infections, and this response may be enhanced in the presence of IFN I. This figure provides a schematic overview of the context of this thesis. The topics of chapters 2 to 7 are indicated in the figure.

In conclusion, infection with either herpesviruses or adenoviruses induces an extensive array of defense mechanisms in the host (figure 1). To control viral infection, the combined effort of both innate and adaptive immune responses is required. Within our cells, immune activation is tightly regulated to avoid prolonged damaging inflammation. One way cells achieve this is by regulating the expression and activity of immune signaling components through PTMs, such as ubiquitination. In turn, viruses express genes to modify immune activation to their advantage. Understanding the interactions between virus and host immunity will guide us towards better protection against harmful viruses and provide us with the knowledge on how to use viral infections as cancer immunotherapy.

Outline of this thesis

In **chapter 2** we describe a central signaling molecule of the PRR cGAS termed stimulator of interferon genes (STING). Upon its activation, STING recruits Tank binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), ultimately leading to the activation of the IFNβ receptor. Here, we review how loss of STING provides a niche for viral replication, whereas uncontrolled STING activation leads to the development of interferonopathies. We then summarize how STING activity is tightly regulated by PTMs to avoid inflammation.

In **chapter 3** we demonstrate that primary human B cells lack STING expression. EBV-infected B cells, however, express high levels of STING, yet they remain unresponsive to the presence of cytosolic dsDNA or the second messenger produced by activated cGAS, called cGAMP. We propose that absence of STING signaling may create the opportunity for EBV to persistently infect B cells.

In addition to taking advantage of favorable cellular circumstances, EBV expresses a small number of proteins to establish latency and evade antiviral immunity. In **chapter 4** we describe how the EBV nuclear antigen 3A (EBNA3A) circumvents immune activation by preventing the transcription factor IRF3 from activating the IFNβ promoter. In unstimulated cells, EBNA3A binds to the interferon-β enhanceosome complex protein P300 in the nucleus. Inhibition of IFN I induction by EBNA3A may therefore be essential for the establishment of a persistent infection in human B cells.

In **chapters 5-7** we turn out attention to adenoviruses (AdV). In **chapter 5**, we characterize a novel non-human primate (NHP)-derived AdV isolated from a gorilla that fails to sufficiently match any of the human or simian adenovirus species that are currently annotated. Due to its low level of pre-existing neutralizing immunity in the human population, this isolate, which we term AdV-lumc014, has high potential for use in a clinical setting.

In addition to the virus observed in **chapter 5**, we identify another NHP AdV from species B with low seroprevalence, which we term GoraVir. In **chapter 6** we show how GoraVir is capable of inducing immunogenic cell death in Pancreatic Ductal AdenoCarcinomas (PDAC). We further demonstrate that the efficacy of GoraVir is independent of STING expression.

In **chapter 7**, we describe the observation that the DUB USP32, but not USP54, is important for the transport of AdVs from the cytoplasm to the nucleus. Silencing of USP32 does not affect the levels of AdV entry receptors CAR or CD46 at the cell surface. However, USP32 may play a role in the escape of AdVs from endosomes. This hypothesis is further strengthened by the observation that silencing of RAB7, a marker of late endosomes and a

substrate of USP32, results in enhanced AdV transduction. This data lays the groundwork for novel insights into the mechanism of transduction of AdVs.

In **chapter 8** we summarize the content of this thesis and discuss future perspectives for herpesvirus and adenovirus research.

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