Recognition, immune evasion, and exploitation of DNA viruses

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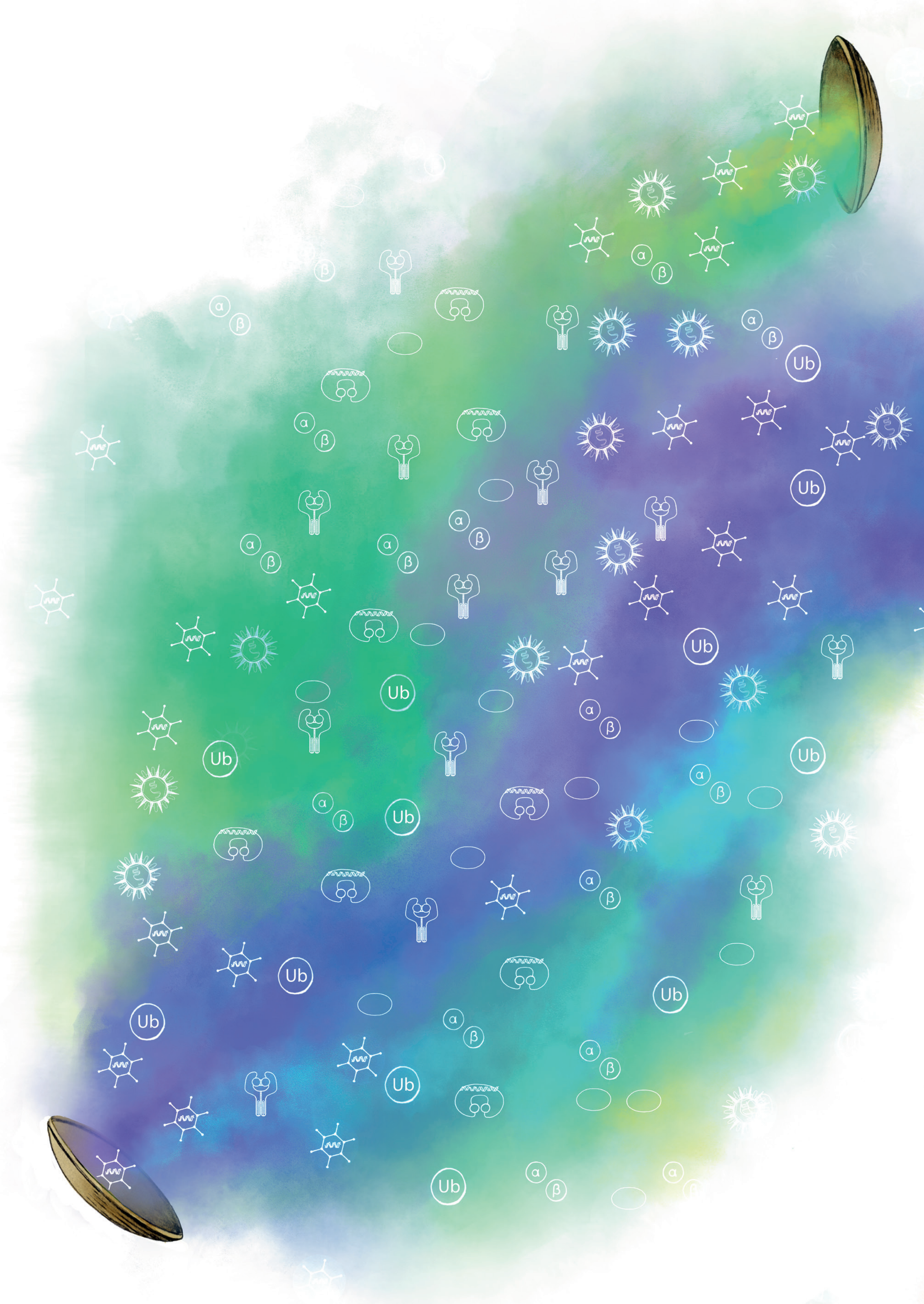
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General discussion

Herpes- and adenoviruses (AdVs) are common pathogens in vertebrates. Both virus types contain a double-stranded DNA core protected by capsid proteins and have a broad tropism in terms of host species (1). There are seven adenovirus species and eight herpesviruses known to infect humans (2–4). To counter viral replication and spread, cells possess intrinsic mechanisms of antiviral defence (5). One key defence mechanism is the production of type I interferons (IFN I), which is initiated upon pathogen-associated molecular pattern (PAMP) detection by pattern recognition receptors (PPRs). Secreted IFN I in turn induces antiviral responses through autocrine and paracrine signaling, culminating in the establishment of an antiviral state (6). The interplay between the virus and the host's immunity constitutes a delicate balance. Whereas too little antiviral response enables viruses to thrive with detrimental consequences for the host, too much IFN I expression can lead to the induction of severe immunopathology.

1. Tight regulation of STING activation is essential to prevent autoinflammation

When DNA viruses enter a cell, they risk exposure of their genome to PRRs, such as the cyclic GMP-AMP (cGAMP) synthase (cGAS) (7). Upon recognizing viral DNA, cGAS produces the second messenger cGAMP, which in turn activates the stimulator of interferon genes (STING), leading to downstream activation of TBK1, IRF3 and the IFNβ promoter. Excess activation of STING is an underlying factor in autoinflammatory diseases, hence controlled activation is crucial to retain proper balance. In **Chapter 2** we describe how activation of STING is tightly regulated through localization, degradation and post-translational modifications. Despite these measures, single nucleotide polymorphisms (SNPs) in STING can drastically increase its activity, giving rise to severe vascular inflammation and tissue damage (8). Full elucidation of STINGs regulatory network may inspire the development of novel clinical interventions for the treatment of autoinflammatory diseases.

2. Defunct STING pathway as a precaution?

Certain cell types harbour an unresponsive STING signaling pathway. For instance, in hepatocytes and primary human B cells (**Chapter 3**), impaired responses can be attributed to lack of STING protein expression (9). In other cases, the molecular reasons for lack of responsiveness remain unclear. In **Chapter 3**, we show that B-lymphoblasts – B cells that have been infected and transformed by the human herpesvirus Epstein-Barr virus (EBV) – despite expressing sufficient levels of STING, as well as cGAS and their downstream signaling intermediates, are nevertheless unable to produce IFN I upon stimulation. Similarly, activated CD4+ T-cells have been characterized as unable to induce a strong IFN response, despite expressing all key molecules involved in cGAS-STING signaling (10). However, both B- and T-cells produce IFN I upon cytosolic RNA exposure, indicating that signaling inhibition takes place upstream of TBK1 activation. Furthermore, in our hands, the human B-lymphoblastoid cell line (B-LCL) JY failed to produce IFN I upon cGAMP stimulation (**Chapter 3**), suggesting that inhibition takes place at the level of STING. This could mean that additional, yet to be identified regulators of STING activity may be at play. Interestingly, naïve CD4+ T-cells were found to produce detectable levels of IFN I when stimulated with both cGAMP and T-cell receptor activating αCD3/CD28 coated beads, but not with either stimulation alone, implying a need for co-stimulation prior to STING activation in lymphocytes (11).

Currently, it still comes down to speculation as to why the STING signaling pathway is defective in B cells. Considering their high proliferation rates, these cells may shut down cGAS/STING signaling to avoid recognition of self-DNA. It is known that cGAS cannot discriminate self-DNA from non-self, and for a decade since its discovery as a DNA sensor, the potential consequences of cGAS binding to chromatin, such as during mitosis, remained unknown (12–14). This changed recently, when cryo-EM structures revealed how cGAS activity is inhibited by nucleosomes (15–19). Rapidly dividing B cells may still accumulate high amounts of misplaced DNA in micronuclei or in the cytoplasm, from where cGAS can actively signal (20). It has become apparent that both cGAS and STING have cellular functions beyond the induction of an interferon response (21). Due to their defect DNA sensing pathway, primary B cells and B-lymphoblasts may be the ideal cell types to further investigate these IFN I independent functions.

3. Shutdown of interferon induction by viral hijacking

Viruses have evolved many strategies to manipulate host immune responses (22). Therefore, we hypothesized that impaired induction of IFN I in the presence of STING in EBV-infected B-LCLs may be due to the presence of viral inhibitory molecules. In **Chapter 4**, we assayed whether EBV encodes latency-associated genes that interfere with IFN I induction and identified the EBV nuclear antigen 3A (EBNA3A) as a novel inhibitor. Further analysis revealed that EBNA3A binds to histone acetyltransferase P300 and IRF3, both of which are components of the IFN I enhanceosome. EBNA3A interferes with IFNβ transcription activation by hampering binding of nuclear IRF3 to the IFNβ promotor region. It is the first latent gene of EBV to be identified as an inhibitor of IRF3 signaling. The γ-herpesvirus family member KSHV encodes four viral IRFs (vIRFs) of which two, vIRF1 and vIRF3, are expressed during latency (23). Both vIRF1 and vIRF3 bind to P300 in the nucleus of infected cells (24). vIRF1 binds to the IRF3 binding and interaction domain (IBID) of P300, which blocks the association between P300 and IRF3 (25). In contrast, vIRF3 binds to P300 through a LXXLL motif, which enables vIRF3 to inhibit T-cell factor (TCF)-dependent transcriptional activity (26). Interestingly, EBNA3A also contains two LXXLL motifs, suggesting its mechanism of transcriptional inhibition may be similar to that of vIRF3. Both EBV and KSHV cause lymphoproliferative diseases in individuals who have a dysregulated immune system (27, 28). The commonality between EBNA3A and vIRF3 in the context of immune evasion may therefore be of key interest in the development of antiviral therapies for the treatment of lymphoproliferative disorders.

4. Ubiquitin deconjugases in AdV transduction

EBV is considered to be an oncogenic virus. In contrast, AdVs are recognized as highly potent oncolytic vectors. AdVs exhibit broad tropism, high transduction efficiency, and are easily modifiable, making them ideal therapeutic vectors. There are seven AdV species (A-G) and many different genotypes, of which the human AdV 5, belonging to the species C (hAdV-C5), is most studied. In **Chapter 7**, we add to the current knowledge of this virus by demonstrating that hAdV-C5 transduction depends on the presence of the deubiquitinating enzyme USP32. Prior work has shown that USP32 regulates proteolytic organelles by modulating their transport, membrane recycling and luminal degradation via its substrate, the small GTPase Rab7 (29). We now find that, contrary to loss of USP32, depletion of Rab7 increases viral transduction efficiency of hAdV-C5, suggesting that the effect of USP32 on AdV transduction may be Rab7-independent. For instance, proteins encoded by AdVs are subject to various post-translational modifications (PTMs) (30–32). In particular, capsid proteins of Adeno-Associated Viruses can be targeted by ubiquitination through a process taking place after the endosomal processing of viral particles has occurred (33). These considerations substantiate a possible role for USP32 in the removal of ubiquitin from viral capsid proteins during, or directly following, endosomal transport of AdVs. To investigate this, one could look at the ubiquitination profile of viral proteins by mass spectrometry in the presence and absence of USP32 DUB activity (34). Moreover, USP32 is a large protein that harbours several non-catalytic domains and may therefore exhibit deubiquitination-independent scaffolding functions in complex formation and beyond (35). It would therefore be interesting to reproduce the current experiments in the presence of an USP32 specific DUB inhibitor to distinguish between catalytic and non-catalytic functions of USP32 on AdV transduction. Overall, our findings demonstrate once more that the ubiquitin system, including its DUBs, is a highly potential target for the achievement of global health as manipulation of one component only can alter tumour progression, autoinflammation and viral transduction (36, 37).

5. The challenges of pre-existing immunity in AdV-based therapies

Despite the extensive knowledge base on hAdV-C5, clinical use of this genotype is hampered by the existence of widespread pre-existing immunity (38). To circumvent this issue, vaccine development has been initiated using different serotypes with low seroprevalence isolated from either humans or non-human primates (NHPs) (39, 40). This approach has proven successful for vaccines directed against SARS-CoV-2, where hAdV-D26 and a chimpanzee-derived AdV (belonging to the simian AdV species E) were used as vehicles for SARS-CoV-2 spike protein delivery for immunization (41). Interestingly, neither pre-existing immunity nor acquired immunity upon immunization against Ad26 exhibited significant effects on the humoral response directed against the SARS-CoV-2 spike protein (42, 43). Surprisingly, similar observations were made for hAdV-C5 vector-based vaccines, suggesting that the same AdV-based vector can be used for repeat vaccination of the same individual (44, 45).

Aside from being attractive vaccine vehicles, AdVs are highly potent oncolytic agents. In **Chapter 6**, we describe the use of oncolytic viruses (OVs) for the treatment of Pancreatic Ductal AdenoCarcinoma (PDAC). PDAC is a highly aggressive malignancy for which the average 5-year survival rate is less than 10% (46). This tumour type is usually diagnosed at a late stage and is extremely difficult to treat due to genetic heterogeneity, tumour architecture and other factors (47). For targeting both the primary tumour and its distal metastases, intravenous injection offers an advantageous route of administration (48). However, this would require circumvention of early clearance of AdVs before they have reached the tumour (49, 50). Despite its proven safety, intravenous treatment of PDAC with hAdV-C5 may not prove broadly effective, due to high seroprevalence in some populations (51, 52). Conversely, hAdV-D26, which is characterized by low seroprevalence, is not suitable for oncolytic therapy due to its limited oncolytic activity (51, 53). Thus, the search for the optimal oncolytic adenovirus continues.

6. NHP-derived AdVs are ideal candidates for oncolytic therapy

According to Basu and Moles, the ideal oncolytic adenovirus is non-pathogenic, exhibits no seroprevalence, is tumour-specific and is characterized by a low mutation rate (54). In our lab, we found that NHP-derived AdVs meet all of the above criteria. Similar to the observations in humans, infections with AdVs in NHPs do not appear to be associated with severe disease (55). AdVs isolated from NHPs belonging to species B had no detectable pre-existing immunity (56). In addition, two of these viruses showed high oncolytic potential in multiple tumour models. To increase tumour specificity, the E1A RB binding domain was removed from one of the NHP-derived viruses, giving rise to a mutant virus named ‘GoraVir’. Interestingly, GoraVir enters cells by binding to the cell surface receptor CD46, as opposed to hAdV-C5, which uses the entry receptor CAR (57, 58). Previously, it has been shown that hAdVs belonging to species B and D accumulate in late endosomes, rather than escaping from early endosomes, like hAdV-C5 (59). It would therefore be interesting to investigate whether GoraVir is similarly reliant on USP32 for successful transduction as hAdV-C5 (**Chapter 7**). In addition to GoraVir, we identified another interesting NHP isolate, which we termed AdV-lumc014 (**Chapter 5**). Like GoraVir, AdV-lumc014 has no detectable pre-existing immunity, making it a potential candidate for OV therapy. The genomic sequence of AdV-lumc014 is most closely related to that of hAdV-B species and, to some extent, hAdV-E. Parts of its amino acid sequence also cluster with hAdV-D, yet the phylogenetic differences exceed the value of 0.05 for most viral proteins, implying that AdV-lumc014 is a distinct and novel species in the Mastadenovirus genus.

7. Opportunities for immune activation in AdV-based therapies

Recent developments in immunotherapy offer a promising approach to cancer treatment, yet some tumours remain unresponsive due to their immunosuppressive microenvironment (60, 61). OVs have the capacity to transform ‘cold’ tumours that suppress activation of immune cells into ‘hot’ tumours, where high infiltration of lymphocytes into the tumour microenvironment (TME) can take place (62). They achieve this by inducing immunogenic cell death (ICD) of tumour cells, leading to long-term anti-tumour immune responses. An important facet of ICD is the release of danger-associated molecular patterns (DAMPs), such as heat shock proteins and IFN I (63). However, a downside to IFN I release into the TME is that it interferes with the oncolytic properties of viruses (64). Signaling of the adapter protein STING upstream of the IFN I promotor also negatively affects replication and oncolytic activity of OVs (65), although some OVs rely on STING signaling for the induction of ICD (66). This is particularly interesting because most tumours are characterized by defects in STING signaling (67). In **Chapter 6** we investigated whether the capacity to induce ICD by GoraVir and hAdV-C5 depends on STING expression levels. To this end, we tested the upregulation of ICD markers upon infection in three different PDAC cell lines (i.e. BxPC-3, HPAF-II, and MIA PaCa-2), which vary in their STING expression: HPAF-II cells exhibited the highest protein levels of STING, whereas MIA PaCa-2 cells completely lacked STING expression. Interestingly, we found no correlation between STING expression and the capacity of either GoraVir or hAdV-C5 to induce ICD. Overall, GoraVir was able to induce substantial ICD in all

PDAC cell lines, although infection with GoraVir did not induce an ISG15 response in MIA PaCa-2 or HPAF-II cells. MIA PaCa-2 cells also did not upregulate IFN I transcription upon infection with the RNA virus vesicular stomatitis virus (VSV), suggesting an overall defect in IFN I induction (68). In contrast, HPAF-II cells did induce IFN I mRNA expression upon VSV infection, suggesting these cells might be defective in STING signaling in a similar way as B-LCLs, which also respond to cytosolic RNA exposure (**Chapter 3**). Overall, the capacity of GoraVir to induce ICD irrespective of STING expression can be considered a favourable trait for future OV therapy. The combination of oncolytic viruses and immune checkpoint inhibition may prove to be a game changer in cancer treatment, providing new hope for patients facing difficult-to-treat malignancies, such as PDAC.

Concluding remarks

Viruses are extremely versatile microbes that broadly impact human health. Originating from the Latin word 'poison', viruses are considered to be harmful agents. Sensibly, cells express an expansive repertoire of PRRs and downstream immune signaling molecules for detection and interference with incoming viral infections. To avoid excess inflammation, immune activation is tightly regulated by cellular components. At the same time, viruses attempt to finetune cellular immunity to achieve an optimal outcome. A detailed understanding of viral immune evasion strategies thus promises to guide us in the development of novel antivirals, targeted against oncogenic viral agents, such as EBV. Furthermore, mapping the cellular interactions of OVs may provide insights into the improvement of oncolytic viral properties. Here, ubiquitin dynamics are of high interest in the context of viral infections, for, as the name implies, this ubiquitous modifier is a versatile regulator of cellular homeostasis. Continued elucidation of complex interactions between the virus and its host will guide us towards improved treatments for tumors and viral diseases.

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