Chapter 6

Summarizing discussion

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Herpesviruses establish lifelong persistent infections in immunocompetent hosts. The herpesvirus Epstein-Barr virus (EBV) has evolved numerous immune evasion strategies that reduce immune activation and recognition of latently as well as productively infected cells. In this thesis, we defined new cellular targets of known and newly identified EBV evasion molecules acting during the lytic cycle and unraveled their underlying molecular mechanisms. In the first part, the findings of each chapter are summarized and discussed. In the second part, overarching points related to the research topic are addressed.

In **Chapter 2**, an shRNA-based approach was employed to reduce translation of the virus-encoded shutoff protein BGLF5 in productively EBV-infected B cells. The list of cellular molecules whose level is decreased by BGLF5-mediated RNA destabilization was extended by the innate immune molecules CD1d and TLR2. In lytically infected B cells, CD1d surface expression was reduced compared to latently infected cells. This phenotype was substantially rescued upon knock-down of the BGLF5 transcript. Expression of the *BGLF5* gene in EBV-negative cells decreased CD1d and TLR2 surface levels, thereby confirming the involvement of BGLF5. In addition to CD1d, a panel of cell surface markers was tested in lytically infected BGLF5-silenced B cells and compared to control cells. Some of these molecules displayed a limited downregulation, whereas certain molecules were strongly downregulated. Overall, the effect of BGLF5-mediated shutoff appeared to be rather limited, although the efficient shutoff by BGLF5 had been demonstrated using pulse-chase analysis in an earlier study [1]. Our findings on the limited effect of BGLF5 are supported by a study, in which the contribution of BGLF5 on the downregulation of HLA I in lytically infected B cells was found to be marginal [2]. Similar to our study, a shRNA approach was used to investigate the extent of BGLF5-mediated HLA I downregulation. In both studies BGLF5 transcript and protein levels were reduced up to 75%. The remaining levels of BGLF5 protein could contribute to downregulation of the molecules tested; thereby the effect of BGLF5 might be underestimated. Our preliminary pulse-chase analysis of BGLF5-silenced cells suggests that remaining BGLF5 protein was sufficient to induce efficient shutoff. Viral proteins are known to combine distinct functions. Also, BGLF5 has a dual function in EBV infection. The BGLF5 protein has RNase and DNase activity [3,4]. The RNase activity is responsible for shutoff, while the DNase function contributes to genome processing [1,5]. Therefore, silencing BGLF5 may also affect other processes of EBV replication. In BGLF5-silenced cells, the late phase of lytic replication was delayed. To exclude that this is due to insufficient genome processing, it might be interesting to selectively inhibit RNase function, without interfering with DNase activity. However, BGLF5 has one catalytic site for both activities making it challenging to generate a mutant that lacks only RNase activity. Two studies attempted to find such a mutant, but none of the BGLF5 mutants fulfilled these requirements entirely [6,7]. The best candidate obtained from these studies has a single mutation (K231M) in the so-called

"bridge", a structural feature of BGLF5 [4]. This mutant displayed severely impaired shutoff function as determined by rescued GFP reduction, HLA I cell surface expression and T cell recognition as well as cytosolic poly(A) binding protein relocalization compared to wild-type protein. Its DNase activity was reduced as well, but not absent. The maintained DNase activity determined differed significantly amongst the two studies, therefore, it remains unclear how suitable the BGLF5 mutant would be for complementation studies. Moreover, introduction of such a mutation into the BGLF5 gene in EBV-infected B cells is, however, laborious, if done in EBV-infected B cells (s. section on *Genetic modifications*). Still, this might be an elegant solution to yet another problem intrinsic to shRNA-based BGLF5 knockdown: the BGLF5-coding transcript also encodes BGLF4. Hence, shRNAs targeting the BGLF5 transcript simultaneously reduce BGLF4 levels. Knockdown of the BGLF4 transcript, coding for the EBV protein kinase, decreases expression of 31 late viral genes [8]. Indeed, we observed decreased levels of late protein expression on the cell surface of BGLF5-silenced cells during the lytic cycle. Therefore, the strong effect of the early gene product BGLF5 on CD1d observed in EBV-infected B cells may also be accounted to late viral proteins.

We describe late viral proteins to interfere with CD1d surface detection in **Chapter 3**. EBV gp150, the viral protein displaying the strongest effect on CD1d of all late viral glycoproteins, appeared to interfere with detection of several additional cell surface molecules including the antigen-presenting molecules HLA I and HLA II. Decreasing HLA I and II reduced the $CD8^+$ and $CD4^+$ T cell response to gp150-expressing cells by about 40% and 60%, respectively.. This has been confirmed by the Rowe lab [9] and is the first known function of EBV gp150. Importantly, comparing lytically infected B cells harboring gp150-deficient EBV to wild-type EBV revealed that in the absence of gp150 B cells displayed higher levels of antigen-presenting molecules. This suggests that gp150 contributes to immune evasion of several T cell subsets during the late phase of lytic replication. It has been postulated earlier that besides the early inhibitors BNLF2a, BGLF5, and BILF1, additional HLA I immune evasion molecules act during the late lytic phase [2]. It is currently unclear whether there are more late proteins targeting HLA I, but none of the late EBV glycoproteins showed substantial downregulation. We identified two additional glycoproteins, gp350 and BMRF2, interfering with CD1d surface expression, but the mechanisms underlying their action remains to be established. We focused on the elucidation of the mechanism of EBV gp150. Microscopy analyses of gp150-expressing cells led to the conclusion that gp150 acts on the cell surface, as antibody-based detection of GFP-tagged HLA molecules was impaired. In contrast, these HLA molecules were still present on the cell surface as indicated by GFP fluorescence. Supporting the hypothesis that the heavily glycosylated protein gp150 acts at the cell surface, inhibition or absence of sialoglycosidases or enzymatic removal of sialic acids from surface glycoproteins increased the antibody-mediated detection of surface molecules. This suggested that gp150 shields antigen-presenting molecules by means of its glycans. In sharp contrast, Quinn *et al.* claimed that EBV gp150 enhances ubiquitin-dependent internalization of HLA from the cell

surface, although a reduction in total HLA levels was not detected [9]. Furthermore, they report that proteasome inhibition completely rescued the gp150-mediated downregulation of HLA molecules, which is opposing to our findings. They observed only an effect of gp150 on HLA I molecules, but not on other molecules [9]. This discrepancy may be accounted to lower expression levels of gp150. We find that decreasing doses of gp150-coding lentivirus resulted in a reduced phenotype. Future studies should address the question whether gp150 acts in both suggested ways on antigen-presenting molecules and whether the expression levels of gp150 influence the mode of action. The Ebola glycoprotein is the only viral protein reported to act in a similar way [10]. It seems plausible that other heavily glycosylated (herpes)virus proteins shield cellular surface molecules. A homologue of the *BDLF3* gene is present in the genome of rhesus LCV, but displays the lowest degree of conservation among all glycoproteins [11]. As the glycans present on gp150 shield surface molecules, it may be assumed the exact amino acid sequence is less relevant to its function providing glycosylation is supported. Given that BDLF3 of rhesus LCV is predicted to be heavily glycosylated, it seems likely that it has immune evasive properties similar to EBV gp150. BDLF3 is absent from marmoset LCV [12]. This may suggest that *BDLF3* appeared rather late during evolution of the lymphocryptoviruses.

Another immune evasion molecule of EBV is the constitutively active orphan GPCR BILF1 [13,14]. BILF1 had been reported to interfere with HLA I surface expression by reducing the exocytic trafficking of HLA I to the cell surface and enhancing endocytosis resulting in degradation of this antigen-presenting molecule [14,15]. Reduction of HLA I impaired CD8+ T cell recognition of BILF1-expressing cells [14]. BILF1 appeared to interfere predominantly with presentation of late viral antigens [2]. In **Chapter 4**, we report that EBV BILF1 displays specificity towards certain HLA molecules as HLA-A, -B and, -E were targeted for downregulation, but HLA-C appeared virtually resistant to BILF1-mediated downregulation. To downregulate HLA I molecules, BILF1 required its C-terminal cytoplasmic tail. Yet, the cytoplasmic tail of EBV BILF1 appeared not to be sufficient to render marmoset LCV BILF1, which is unable to downregulate HLA I or LCV MHC I, capable of downregulating HLA I molecules. This suggests that an additional feature in BILF1 is required to allow it to act as immune evasion molecule. HLA I molecules lacking their cytoplasmic tail were not targeted for downregulation. Therefore, the cytoplasmic part of HLA I molecules was the determinant of BILF1 sensitivity. We identified three amino acid residues in the cytoplasmic tail of HLA-C that were not present in the cytoplasmic tail of BILF1-sensitive HLA I molecules. Changing the three residues into those of the HLA-C molecule rendered an otherwise sensitive HLA-B molecule resistant to downregulation. The molecular mechanism underlying BILF1-mediated downregulation is largely unclear, but BILF1 is reported to co-immunoprecipitate with HLA I [14]. To assess how the identified amino acids render HLA-C molecules resistant to BILF1-mediated downregulation, it may be interesting to determine whether BILF1 can interact with HLA-C, despite its inability to substantially downregulate this HLA molecule. This could

facilitate further elucidation of the mechanism of action of BILF1 and assist in identification of potential intracellular adaptor proteins involved in sorting or internalization of HLA I molecules.

Lastly, we aimed to learn more about evasion of innate immune pathways present in B cells by EBV. Herpesviruses have been shown to be recognized by the cytosolic DNA sensing pathway resulting in type I IFN production or inflammasome formation (reviewed in [16]). The genome of EBV and KSHV is sensed by the DNA sensor IFI16 resulting in inflammasome formation in B cells [17,18]. Several viral evasion molecules, including EBV BLRF2, interfering with DNA sensing pathway have been identified [19,20,21] supporting the notion that this immune pathway plays a role in the immune response to herpesviruses. The cytosolic DNA sensing pathway in human B cells was analysed in **Chapter 5,** as B lymphocytes are target cells for the DNA viruses EBV and KSHV. Neither primary B lymphocytes, nor the B cell lines examined, produced type I IFNs upon exposure to cytoplasmic DNA. A similar phenomenon is reported for T lymphocytes [22]. This raises the following question: Why do lymphocytes have the DNA sensors cGAS and IFI16, but are unresponsive to cytoplasmic DNA? Most B lymphocytes lacked the STING protein, an essential adaptor protein of the cytoplasmic DNA sensing pathway. Interestingly, B cells producing STING also did not mount a type I IFN response upon stimulation with DNA or cGAMP suggesting that STING expression was not sufficient to reconstitute the pathway in B cells. This resembles the situation in T lymphocytes: The DNA sensors, STING and the downstream molecules are present, but T lymphocytes do not respond to cytoplasmic DNA, although they sense the DNA [22]. Presence of the sensors in lymphocytes suggests that these cells are responsive under certain conditions that remain to be determined. It is tempting to speculate that lymphocytes possess a safeguard mechanism that regulates responsiveness to cytoplasmic DNA. Therefore, these cell types may serve as a study field to unravel regulatory pathways of cytoplasmic DNA sensing. Unresponsiveness to cytosolic DNA may make lymphocytes attractive target cells for DNA viruses such as herpesviruses. It remains to be determined whether other target cells of EBV sense the viral genome and initiate a type I IFN response.

Conclusions and future directions

It is well established that patients lacking functional T cells are prone to developing EBV-associated diseases or malignancies (**Chapter 1**). The EBV proteins studied in this thesis (**Chapter 2, 3, and 4**) interfere with generation and recognition of antigen-presenting molecules on the cell surface resulting in reduced T cell activation during the productive cycle of EBV infection. Investigation of EBV immune evasion molecules to understand their molecular mechanism of action and to assess their cellular targets significantly contributes to our understanding of EBV biology. Considering that EBV has even more T cell evasion molecules, this demonstrates that T cell activation is a major threat to EBV infection. To counteract efficient T cell recognition, this virus evolved several ways of interference acting at

different phases of the lytic cycle as well as during latency. Yet, we are far from comprehending how and when individual EBV proteins contribute to EBV infection and how this impacts pathogenesis on an organismic level. Studying the innate immune DNA sensing pathways of B cells (**Chapter 5**) expands our knowledge on immune pathways that may limit EBV infection. In conclusion, insights are gained on the cellular pathways that are involved in antiviral defense.

Animal models

Although attempts have been made to determine the contribution of individual EBV proteins to immune evasion during primary infection or lytic cycle *in vitro* [9,23,24], the role of distinct immune evasion strategies *in vivo* is largely unclear. One obstacle is the requirement for a permissive animal model that supports EBV infection. As EBV has a very narrow host range [25], the use of mice with reconstituted human immune system compartments (HIS mice) infected with EBV or infection of rhesus macaques with the closely related rhesus LCV is warranted [12,26,27]. Similar models have been informative for the investigation of the role of immune evasion *in vivo* for other human-specific viruses like HCMV. A cluster of rhesus CMV genes encoding immune evasion molecules (Rh182-189, which act homologous to the HCMV genes US2-US11) that interfere with MHC I-mediated antigen presentation to $CD8^+$ T cells are essential to establish a secondary persistent infection ('superinfection') in CMV-positive rhesus macaques [28]. The rhesus CMV-specific MHC I evasion molecule VIHCE was not required for superinfection. Depletion of $CD8^+$ T cells allowed the RhUS2-US11-deficient rhesus CMV to establish secondary persistent infection [28]. Interference with MHC I was not required for primary infection. This demonstrates that superinfection, but not primary infection relies on the presence of immune evasion molecules and that T cell control is effective, if not evaded. It is likely that the immune evasion genes are also not required for primary EBV infection in a naïve host when EBV-specific T cells are still absent. It remains to be investigated whether immune evasion plays a role during infection of uninfected cells in an EBV-experienced host or whether they rather contribute to prolonging the time span of virus production during reactivation. The EBV evasion genes *BCRF1* and *BNLF2a,* coding for vIL-10 and BNLF2a, respectively, are expressed upon primary infection of B cells *in vitro* [23]. Thereby, they may contribute to immune evasion during the pre-latent phase of infection in B cells.

To study infection with the EBV-related rhesus LCV, a specific pathogen-free colony of rhesus LCV-naïve rhesus macaques is required This animal model supports oral transmission, latent and lytic rhesus LCV infection and reproduces other key aspects of human EBV infection [12,29]. In a first study evaluating contribution of immune evasion, naïve rhesus macaques were infected with rhesus LCV lacking the innate immune evasion gene *BARF1* coding for the colony stimulating factor 1 (CSF-1) blocking protein. The viral load was reduced during lytic infection and frequency of latently infected cells was decreased [30]. This demonstrates that evasion of the innate immune system is important for efficient rhesus LCV infection.

HIS mice have hematopoietic cells of human origin and are used to study EBV infection *in vivo*. They improved our understanding of the role of NK cells early during EBV infection [31]. NK cells appear to control lytically, but not latently, infected B cells. In the absence of NK cells, CD8+ T cells expanded more dramatically and tumor incidence was higher [31]. However, the use of HIS mice is limited as not all steps of the EBV life cycle can be modeled, e.g. oral transmission, which requires infection of epithelial cells. Despite the human origin of the hematopoietic compartment, all non-hematopoietic cells including epithelial cells are of murine origin. Hence, life cycle steps involving non-hematopoietic cells cannot be investigated using this model.

These two examples show that the recent advances in establishing animal models to study EBV infection *in vivo* are promising. Further comprehension of the role of individual viral gene products, including immune evasion proteins, during infection may be expected within the coming decade. In addition, the contribution of specific immune pathways or cells to control viral infection should be studied in order to provide a complete picture of virus-host interaction.

Genetic manipulations

Genetic manipulation of the viral genome is a prerequisite to study the function and role of individual gene products of EBV or rhesus LCV *in vivo*. To investigate the contribution of gene products *in vitro*, transcript knock-down approaches can be employed, although often there is a preference for knock-out of viral genes. Once a gene-deficient viral genome (present in a cell line or a virus particle) is generated, many of the following steps in research become easier and clearer phenotypes can be obtained in comparison to knock-down approaches. The necessary genetic modification of the EBV genome can be achieved by the use of bacterial artificial chromosomes (BAC) or classical recombination [32]. For *in vitro* experiments, the major drawback of the BAC-based system is the necessity to infect B cells. Typically, *in vitro* infected B cells show a very low efficiency of entering the lytic cycle, thereby making it challenging to study genes expressed during the lytic cycle. Therefore, predominantly genes involved in primary infection and latency can be studied with viruses derived from the BAC system. In contrast, this system is indispensable for *in vivo* studies. The EBV BAC system was generated in the late 1990s [33], but a rhesus LCV BAC was only established recently [34] making it possible now to delete individual genes from rhesus LCV to test their function *in vitro* or *in vivo*.

As an alternative, and especially for lytic cycle-expressed genes, a classical recombination approach can be employed to delete, substitute, or introduce genes. The Akata cell line or the AKBM cell line, a derivative of the Akata cell line, allows efficient lytic replication, thereby being a suitable model to study the contribution of individual lytic phase gene products *in vitro*. As several copies of the EBV genome are present,, the classical recombination approaches to modify all genomes present are consequently very inefficient and laborious due to tedious screenings of clones, as described for the generation of the BDLF3-knock-

out Akata cell line [35]. The recently developed CRISPR/Cas9-based genome editing systems promise to facilitate fast and efficient editing of any kind of genome [36,37]. However, the genome editing systems do not prove to be a very effective tool to interrupt or introduce genes into the numerous EBV genomes present in a single cell as of yet [38]. Therefore, shRNA-based approaches as applied for BGLF5 (**Chapter 2**) or other gene products [2] may continue to be the tool of choice for studying essential, but also non-essential individual EBV proteins.

Coinfections

Endemic Burkitt's lymphoma occurring in equatorial Africa and Papua New Guinea is associated with coinfection of the malaria-causing parasite *Plasmodium falciparum* and EBV [39,40]. Although this link is known for half a century, it cannot be fully explained yet. Two recent studies provide important insights into the molecular mechanisms [41,42] (reviewed in [43]). In short, individuals chronically infected with *Plasmodium falciparum* had higher numbers of germinal center B cells that made high levels of activation-induced cytidine deaminase (AID), an enzyme involved in somatic hypermutation and class switching of immunoglobulin genes [42]. Consequently, EBV-positive germinal center B cells were more abundant in individuals infected with *Plasmodium falciparum* in comparison to uninfected ones [42]. In combination, the increased risk of AID-induced translocation and EBV-mediated survival of B cells, two factors favoring cancer cell development, may explain the higher incidence of BL in areas where *Plasmodium falciparum* –mediated malaria is holoendemic.

The example of *Plasmodium falciparum* and EBV coinfection resulting in lymphoma formation is extreme. There are more examples of microorganisms shaping their (micro)-environment, which may have deleterious or beneficial consequences. Interaction of the 'virome', defined as all viruses or virus-related sequences present in an individual, and the host influences phenotypes of health and disease (reviewed in [44]). Infection with persistent viruses such as herpesviruses, and especially with the highly disseminated EBV, might be a significant determinant. Continuous replication and shedding of EBV occurs in healthy carriers [45], hence there might be a low level of permanent immune stimulation by lytically replicating cells. Moreover, latently infected cells may release cytokines and type I IFNs as latent EBV gene products have been identified that activate and/or modulateinnate immune pathways **(Chapter 1)**. For example, LMP1 activates the several innate pathways, including the NF-κB pathway [46] and also the EBERs are sensed by different innate immune pathways [47,48]. Moreover, there are virally-encoded miRNAs present in EBV-infected cells that possibly induce transcriptional changes influencing immune-related genes ([49,50,51] and Hooykaas et al, personal communication). These different gene products are not only restricted to the virus-infected cell itself, since different RNA species including miRNA and EBERs might also be transferred by exosomes to other cells or even non-permissive cell types [52,53]. Thereby, EBV infection may shape our immune responses, but not *per se* in a negative fashion. One example of beneficial herpesvirus infection is illustrated in a report on knock-out mice lacking single immune factors such as HOIL-1, IL-6, or caspase-1, but being resistant

to lethal doses of *Listeria* due to latent infection with MHV68, a murine gamma-herpesvirus [54]. It is, therefore, exciting to consider our herpesviruses not only as pathogens, but also as "commensals" that might contribute to health and disease.

Glycosylation

Studying herpesviruses provides insights into its intriguing immune evasion mechanisms that enable the viruses to persistently infect their host, but also offer insights on fundamental molecular processes and modifications. EBV gp150 shields cell surface molecules by means of its abundant N and Olinked glycans (**Chapter 3**). A similar mechanism has been reported for Ebola glycoprotein [10]. Glycosylation of viral proteins also aids evasion of antibody-mediated neutralization (reviewed in [55]) including HCMV gN and BoHV-4 gp180 [56,57]. The glycosylation not only shielded epitopes within the glycosylated protein, but also prevented antibody-binding to other viral proteins [57]. A recent study suggests that viral glycoproteins can induce glycan-dependent immune responses [58]. The authors found that HSV-2 triggers a mucosal CXCL10 response preceding the early IFN response. The CXCL10 response appeared to be dependent on O-linked glycosylation of the viral glycoproteins. Cellular carbohydrate receptors like C-type lectins did not sense the glycosylation and activate signaling pathways. Instead, enzymatic or repeated flushing-induced disruption of the mucosal layer resulted in CXCL10 secretion. Therefore, the authors concluded that O-linked glycosylation of the virion was required to cross the mucus layer efficiently and to enter the underlying cells [58]. In summary, these studies suggest that glycosylation of viral proteins fine-tunes and adds functions to the glycoproteins. In addition, viral glycoproteins also interact with different cellular lectins (reviewed in [59]). Sialic acid–binding Ig-like lectins (Siglecs) are primarily present on immune cells and many of them trigger immune modulatory responses via intracellular signaling domains such as immunoreceptor tyrosine-based inhibitory motif (ITIM) Other Siglecs associate with a protein containing immunoreceptor tyrosine-based activating motif (ITAM). The signalling cascade initiated by sialic acid binding can dampen the outcome of other immune signalling pathways [60]. It is tempting to speculate that sialic acid-decorated glycoproteins of EBV such as gp150 may alter immune signalling events in *cis* or *trans* by activating Siglecs present on B cells or on cells interacting with B cells, respecitvely. This could be considered as immune evasion strategy. Altered glycosylation in cancer and auto-immunity is also increasingly recognized [61,62], but how and to what extent this impacts immune responses or other processes remains largely unclear. It is challenging to study the role of glycosylation of proteins without affecting the functionality of the protein. Yet, it is interesting to consider glycosylation as an additional layer of regulation similar to other post-translational modifications.

Concluding remarks

Investigation of the immune evasion strategies of the oncogenic γ-herpesvirus EBV provides essential insights in the underlying molecular working mechanisms and targets of the viral molecules. The knowledge on their different strategies might aid in designing anti-viral drugs or therapies to counteract these in order to cure EBV-associated diseases and malignancies. Currently, there is no drug available to treat infectious mononucleosis, which can be life-threatening in patients with certain immunodeficiencies. Targeting viral evasion molecules that interfere with innate or adaptive immune responses could improve immune recognition resulting in a better or faster control of the viral infection by the host. Alternatively, it is interesting to speculate whether viral evasion molecules might be exploited as tool to prevent or dampen unwanted immune responses [63,64]. Exploitation of molecules interfering with antigen presentation could be used to make transplanted or own organs such as the pancreas "invisible" to the immune system to prevent unwanted T cell attack. Viral molecules targeting innate immune responses might be exploited to reduce inflammation or auto-immunity. Of note, there are obvious obstacles such as delivery and safety issues, but also problems regarding preexisting immunity. In case of interference with antigen presentation, it needs to be considered that viral infection of the "immune-invisible" graft might not be properly controlled by the immune system. To identify the best viral target molecule to limit viral infection or the molecules with the greatest therapeutic potential, profound knowledge on the contribution and role of individual viral molecules during the replicative cycle and pathogenesis of the virus in vivo is required. Vaccination against EBV appears to be an attractive option to prevent associated diseases and malignancies. However, we should also consider that viruses that coevolved for millions of years together with their host may provide benefits, although certain individuals are at risk to develop disease or malignancies.

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