Immune checkpoints in T cells during oncogenic γ-herpesvirus infections

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Abstract

Epstein–Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KSHV) are two persistent oncogenic γ-herpesviruses with an exclusive tropism for humans. They cause cancers of lymphocyte, epithelial and endothelial cell origin, such as Burkitt’s and Hodgkin’s lymphoma, primary effusion lymphoma, nasopharyngeal carcinoma, and Kaposi sarcoma. Mutations in immune-related genes but also adverse events during immune checkpoint inhibition in cancer patients have revealed molecular requirements for immune control of EBV and KSHV. These include costimulatory and coinhibitory receptors on T cells that are currently explored or already therapeutically targeted in tumor patients. This review discusses these co-receptors and their influence on EBV- and KSHV-associated diseases. The respective studies reveal surprising specificities of some of these receptors for immunity to these tumor viruses, benefits of their blockade for some but not other virus-associated diseases, and that EBV- and KSHV-specific immune control should be monitored during immune checkpoint inhibition to prevent adverse events that might be associated with their reactivation during treatment.

KEYWORDS

2B4, CD27, CTLA-4, Epstein–Barr virus, Kaposi sarcoma-associated herpesvirus, PD-1

1 | INTRODUCTION TO EPSTEIN–BARR VIRUS AND KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS

The Epstein–Barr virus (EBV) and the Kaposi sarcoma-associated herpesvirus (KSHV) make up the human γ-herpesviruses, EBV belonging to the genus lymphocryptoviruses and KSHV to the genus rhadinoviruses.1–3 Both are human tumor viruses but do not cause diseases in the majority of their persistently infected human hosts. Nevertheless, EBV is estimated to cause yearly around 300 000 cancers in humans, half of which are fatal.4 KSHV causes more than 40 000 cancers yearly, as has been estimated for Kaposi sarcoma alone.5 Nasopharyngeal carcinoma and the subset of gastric carcinomas that are EBV positive contribute 100 000 cases each to the worldwide tumor burden of EBV,6,5 but EBV is associated also with several lymphomas, including B cell-derived Burkitt’s and Hodgkin’s lymphomas as well as natural killer (NK)/T-cell lymphomas.6,7 In addition to Kaposi sarcoma, KSHV is associated with primary effusion lymphoma that harbors also EBV in most cases.8 Furthermore, KSHV is associated with inflammatory syndromes such as multicentric Castleman’s disease and KSHV inflammatory cytokine syndrome.8,9 Similarly, EBV can also cause hyperinflammation and associated disease during a primary infection such as infectious mononucleosis and upon persistence with poorly immune controlled
viremia such as chronic active EBV (CAEBV). These immune pathologies and their hyperinflammation might also share features with the contribution of EBV to the autoimmune disease multiple sclerosis. Therefore, EBV and KSHV cause persistent asymptomatic infections in most human hosts but are associated with tumorigenesis and immune pathologies.

As γ-herpesviruses EBV and KSHV can either enter latent and cell proliferation driving, or lytic and infectious viral particle-producing infections. Both viruses are thought to be primarily transmitted via saliva exchange, reaching more than 90% seropositivity for EBV and more than 50% for KSHV in Sub-Saharan children. In the Northern hemisphere, EBV infection is delayed into adolescence and young adulthood in one-third of the population, and KSHV seroprevalence is below 10% in most countries, except for the Mediterranean basin and Eastern Europe where it can reach 30%. Late primary infection with EBV can lead to infectious mononucleosis, caused by a massive expansion of CD8+ T cells in response to primarily lytic EBV antigens. However, latent infection is the default program for both viruses with which they are thought to cause proliferation and apoptosis resistance of their host cells to reach a long-lived cellular compartment for persistence. This is better understood for EBV that drives infected naïve B cells in submucosal secondary lymphoid tissues like the tonsils into differentiation, rescues them in the germinal center reaction, to finally gain access to the memory B cell pool, where they lie dormant. This latent replication via cellular proliferation is achieved by both EBV and KSHV via latent viral gene products, the six EBV nuclear antigens (EBNAs) and two latent membrane proteins (LMPs) of EBV and latency-associated nuclear antigen (LANA), viral FLICE inhibitory protein (vFLIP), and viral β-type cyclin of KSHV. For this purpose, both EBV LMP1 and KSHV vFLIP mediate nuclear factor-kB activation as cell survival and pro-proliferative program. Furthermore, EBV EBNA3A, EBNA3C, and LMP2 as well as KSHV vFLIP block apoptosis. Although five latent EBV gene expression patterns (0, I, IIA, IIB, and III) with decreasing gene expression from latency III to 0 can be distinguished in B cells of healthy virus carriers and EBV-associated malignancies, the KSHV latent genes seem to be expressed together with a variable number of lytic KSHV genes, some of which like viral interleukin-6 (IL-6) and viral G protein-coupled receptor, have also oncogenic functions. EBV and KSHV can reactivate from latency to produce infectious virus particles during lytic infection. For EBV, this can occur from latency 0 and I during plasma cell differentiation. Additional lytic replication in the mucosal epithelium might further increase the shedding of EBV and KSHV into saliva for further transmission. Therefore, both B cell and epithelial cell infections are probably part of the life cycle of both EBV and KSHV, and malignancies emerge from both host cell populations for EBV and B cells for KSHV. However, it remains unclear what role endothelial cell infection provides for KSHV, even so, Kaposi sarcoma emerges from this cellular source. Nevertheless, all gene expression programs of EBV- and KSHV-associated malignancies are also expressed in healthy carriers of the viruses, and it is important to understand what goes wrong when these transition into diseases.

2 | DIFFERENCES IN THE CELL-MEDIATED IMMUNE CONTROL OF EBV AND KSHV

EBV- and KSHV-associated lymphomas, as well as Kaposi sarcoma, are AIDS-defining malignancies in human immunodeficiency virus-infected individuals. This already indicates that T-cell responses are essential for the immune control of persistent EBV and KSHV infection. Furthermore, cytotoxicity or interferon-γ (IFN-γ) signaling is required for the immune control of EBV and KSHV, respectively. Mutations in genes that affect these lymphocyte effector functions have been identified in individuals that suffer from EBV- and KSHV-associated diseases as the basis of their primary immunodeficiencies (PIDs). A prominent source of these effector functions that control EBV and KSHV are T cells, and indeed mutations in T-cell receptor signaling, such as in ZAP70 (HGNC: 12858) and STIM1 (HGNC: 11386), predispose for EBV- and KSHV-associated disease, respectively. EBV-associated lymphoproliferation can also be observed in patients with immune-suppressive treatment after organ transplantsations, so-called posttransplant lymphoproliferative disease. These can be treated by the adoptive transfer of EBV-specific T cell lines. Vice versa depletion of predominantly CD8+ T cells compromises immune control of EBV infection and its lymphomagenesis in mice with reconstituted human immune system components (humanized mice). The protective function of T cells against KSHV is less clear but spontaneous regression of Kaposi sarcoma has been associated with increased KSHV-specific T-cell responses. Therefore, T cells killing EBV-infected B cells and possibly primarily producing IFN-γ to suppress KSHV infection seem to be essential for the immune control of these two oncogenic γ-herpesviruses (Figures 1 and 2).

However, these T-cell responses are quite different in their composition and magnitude. Even when individuals are infected with both KSHV and EBV, KSHV-specific T-cell responses constitute a smaller proportion of the total peripheral blood T-cell repertoire than those directed against EBV. In addition, some of the EBV proteins are immunodominant and elicit T-cell responses in nearly all healthy virus carriers, such as EBNA1 for CD4+ T cells and EBNA3A-C as well as LMP2 for CD8+ T cells. In contrast, KSHV-specific T-cell responses vary widely in their viral antigen recognition, with the most frequently recognized antigens, LANA (ORF73) and K8.1, only being recognized by T cells in less than half of healthy KSHV carriers. Responses against LANA and K8.1 contain both CD4+ and CD8+ T cells. Interestingly, KSHV-specific CD4+ T cells, such as the LANA-specific responses, were able to recognize KSHV-infected B cells by IFN-γ production, but not able to kill them. In contrast, EBV-specific CD4+ T cells, including EBNA1- and late lytic antigen-specific responses, are able to kill EBV transformed B cells. Possibly these EBV-specific cytotoxic CD4+ T cells are particularly
well expanded by LMP1 activated B cells.\textsuperscript{61,62} Thus, evidence exists that T cells prevent both EBV-\textsuperscript{and KSHV-driven diseases, possibly primarily by cytotoxicity for EBV and IFN-γ production for KSHV. Nevertheless, differences exist in this T cell-mediated immune control with respect to magnitude, antigen immunodominance, and CD4\textsuperscript{+} T-cell effector function. In the following chapters, differences in costimulation and coinhibition of these T-cell responses will be discussed. These might identify immune checkpoints that could be blocked or stimulated as treatment avenues against malignancies and immune pathologies that are caused by these oncogenic γ-herpesviruses.\textsuperscript{31,63–65}

These patients are often identified by the development of EBV-driven immune pathologies, such as hemophagocytic lymphohistiocytosis (HLH), or EBV-associated tumors, mostly lymphomas. For KSHV, primarily predisposition for the development of Kaposi sarcoma has been characterized. Quite different costimulatory requirements for EBV- or KSHV-specific immune control were revealed by these PIDs.

EBV infection causes disease in individuals that are deficient for CD27 or its ligand CD70\textsuperscript{66} (Figure 1). EBV-driven lymphoproliferation and HLH were the most prominent disease manifestations in 33 CD27\textsuperscript{−}deficient patients, while many CD70-deficient patients suffered from EBV associate Hodgkin’s lymphoma. Despite CD27 being considered an essential tumor necrosis factor (TNF) receptor superfamily member in the priming of T-cell responses\textsuperscript{67} CD27- and CD70-deficient patients are primarily susceptible to disease after EBV infection. Moreover, they maintain some EBV-specific CD8\textsuperscript{+} T-cell responses,\textsuperscript{66} and most EBV-specific CD8\textsuperscript{+} T cells of healthy virus carriers express CD27,\textsuperscript{58} suggesting that they are able to continue to receive costimulation from CD70-expressing cells, such as EBV-infected B cells.\textsuperscript{69} Recently, it was shown that blocking CD27 with antibodies compromises EBV-specific immune control in humanized mice.\textsuperscript{70} However, in humanized mice, EBV infection

\textbf{3 | COSTIMULATORY RECEPTORS OF CYTOTOXIC LYMPHOCYTES REQUIRED TO CONTROL EBV AND KSHV}

Costimulatory receptors that are essential to control EBV and KSHV infection have been identified in PID patients that suffer from diseases upon the first encounter with these human tumor

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\caption{Immune checkpoints of EBV-specific immune control. EBV-infected B cells are mainly targeted by cytotoxic lymphocytes, such as CD8\textsuperscript{+} T cells. These recognize EBV antigens via MHC class I-restricted antigen presentation via their T cell receptor (TCR) and use the costimulatory molecules CD27, NKG2D, 2B4, and 4-1BB that interact with their ligands CD70, ULBP1, CD48, and 4-1BBL, respectively, on EBV-infected B cells. The coinhibitory receptors PD-1 and CTLA-4 attenuate CD8\textsuperscript{+} T cell function as well as immune-suppressive functions of regulatory CD4\textsuperscript{+} T cells (CD4\textsuperscript{+} Treg) that recognize presumably mostly self-antigen presentation on MHC Class II molecules via their TCR. EBV, Epstein-Barr virus; MHC, major histocompatibility complex.}
\end{figure}
expands CD8+ T cells that maintain CD27 expression and their antibody-mediated depletion compromises immune control leading to lymphomagenesis. Moreover, not all CD8+ T-cell specificities are equally affected by CD27 blocking. Latent EBV antigen-specific CD8+ T cells seemed to be activated normally, but lytic EBV antigen-specific CD8+ T cells were severely compromised in their expansion and cytotoxicity by antibody-mediated CD27 blocking. Accordingly, immune control of infection with wild-type but to a much lower extent infection with BZLF1-deficient EBV that cannot switch into lytic replication was compromised by CD27 blocking. These findings in both PID patients and humanized mice suggest that CD27 or CD70 absence affects primarily a subset of EBV-specific CD8+ T cells, mainly those directed to control lytic EBV infection, which is nevertheless essential to control this persistent tumor virus infection. Although CD27 is constitutively expressed on naïve T cells another TNF receptor superfamly member 4-1BB is upregulated upon T-cell receptor signaling and often used to isolate antigen-specific T cells, preferentially CD8+ T cells.71 Patients with 4-1BB deficiency present also with susceptibility to EBV-associated disease72-75 (Figure 1). Even so only a handful of patients have been identified with this deficiency many of them suffered from EBV-driven lymphoproliferation or lymphomas. However, for some of them, also healthy siblings with 4-1BB/TNFRSF9 (HGNC: 11924) loss-of-function mutations were identified,73,74 arguing that 4-1BB is not absolutely required to control EBV. Furthermore, in one patient, EBV infection spread to T cells which is sometimes observed during CAEBV.76,77 Thus, a 4-1BB deficiency might be less detrimental for EBV-specific immune control than CD27 or CD70 deficiency.

Another family of costimulatory molecules whose activating signaling seems to be required for EBV-specific immune control is the signaling lymphocyte activation molecule (SLAM) receptors.78,79 Loss of the SLAM-associated protein (SAP) is the molecular basis for X-linked lymphoproliferative disease type 1 (XLP1, Duncan Disease – OMIM: #308240). Mostly boys with this PID develop HLH after primary EBV infection and one-third of them lymphomas.78 Among other receptors, the SLAM molecule 2B4 is required to support the killing of EBV-infected B cells by CD8+ T cells80,81 (Figure 1). Furthermore, 2B4 blocking with antibodies compromises CD8+ T cell-mediated immune control of EBV in humanized mice.37 Haploinsufficiency of the expression of the 2B4 ligand CD48 leads also to HLH, even so in the sole so far described patient no involvement of EBV has been described so far.82 The requirement of SAP for CD8+ T-cell recognition of EBV-infected B cells is also documented by findings that in female SAP/SH2D1A (HGNC: 10820) mutation carriers preferentially the defective X chromosome is condensed in EBV-specific CD8+ T cells.83 Furthermore, in XLP1 patients that manage to control EBV infection somatically SAP gene reverted CD8+ T cells specific for this tumor virus can be found.84 Thus, SAP is essential to control EBV infection via cytotoxic CD8+ T cells.

2B4 is also expressed by NK cells but CD8+ T cell depletion together with 2B4 blocking did not further compromise EBV-specific immune control in humanized mice.37 However, NK cell depletion leads to increased EBV viral loads and lymphomagenesis in humanized mice.85 NK cells primarily restrict lytic EBV replication, because infection with the lytic cycle-deficient BZLF1 knockout virus is not influenced by NK cell depletion in humanized mice. This preferential recognition of lytically EBV-replicating B cells can also be observed for the NK cell subset that expands during infectious mononucleosis.86 Activation of NK cells by EBV-infected B cells is mainly mediated by the activating NK cell receptors NKG2D (Figure 1) and DNAM1.87 Along these lines, it was also noted that patients with a genetic deficiency in the magnesium transporter MAGT1 lead to X-linked immunodeficiency with magnesium defect (XMEM, OMIM: #300853) present with defective N-linked glycosylation and decreased NKG2D expression on NK and CD8+ T cells, culminating in increased susceptibility to EBV-associated diseases.88-91 Forty percent of these patients present with EBV-associated lymphoproliferation and lymphomas.89 Accordingly, NK and CD8+ T cell recognition of EBV transformed B cells is severely impaired.88 Magnesium supplementation in vitro increases NKG2D expression and restores recognition of EBV-infected B cells, but in vivo, this might be difficult to achieve or work only in a subgroup of patients after prolonged magnesium supplementation.88,92,93 Nevertheless, these studies indicate that NKG2D is an important activating receptor for the recognition of EBV-infected B cells undergoing lytic virus replication.

For KSHV-specific immune control, the requirements of costimulation are less clear due to the lower prevalence of this virus in the Western world. One XMEM patient has been described with Kaposi sarcoma.94 Otherwise, the TNF receptor superfamly member OX40 has been implicated in KSHV-specific immune control95 (Figure 2). OX40 is upregulated on T cells upon activation, preferentially on CD4+ T cells.67 Accordingly, the one patient with OX40/TNFRSF4 (HGNC: 11918) loss-of-function mutations that suffered from Kaposi sarcoma had a diminished CD4+ T-cell memory compartment.95 Thus, especially CD4+ T cells and their interaction with endothelial cells via OX40 and its ligand OX40L that is expressed in Kaposi sarcoma seem to be important to control KSHV infection in this cellular compartment. Overall, the above studies describe the requirements for CD27, 4-1BB, 2B4, NKG2D, and OX40 for the immune control of oncogenic herpesvirus by lymphocytes. Further mechanistic studies on how they orchestrate T-cell-mediated immune control are required to identify conditions that could benefit from stimulating these receptors with agonistic antibodies.

4 | REGULATION OF EBV- AND KSHV-SPECIFIC IMMUNE CONTROL BY COINHIBITORY RECEPTORS

In addition to the above discussed costimulatory molecules, PD-1 and CTLA-4 coinhibitory receptors get upregulated during T-cell activation and some of these regulate EBV- and KSHV-specific immune control.96 Haploinsufficiency of the inhibitory counterpart of CD28, CTLA-4, which engages CD80 and CD86 leads to elevated EBV loads and associated malignancies in more than half of the affected
patients (Figure 1). CD86 is upregulated on B cells after EBV infection. Immunologically these patients present with lymphopenia and expanded regulatory T-cell (Treg) populations. Therefore, loss of CTLA-4-mediated inhibition might lead to the expansion of Treg cells and increased stimulation of effector T cells that then succumb to activation-induced cell death resulting in lymphopenia. Both mechanisms might lead to compromised EBV-specific immune control.

A second inhibitory receptor that might be required for the immune control of EBV infection is PD-1 whose ligands PD-L1 and PD-L2 are upregulated on B cells after EBV infection (Figure 1). PD-1 is upregulated on T cells during symptomatic primary EBV infection in children and humanized mice. This is observed on both latent and lytic EBV antigen-specific CD8+ T cells. Despite their coinhibitory receptor expression, these PD-1 positive CD8+ T cells are similarly capable to secrete cytokines and even superior in degranulating as a measure of cytotoxicity compared with their PD-1 negative counterpart. Blocking PD-1 with antibodies increases viral loads and lymphomagenesis after EBV infection of humanized mice. This is particularly evident for infection with the MB1 viral strain which displays elevated lytic EBV infection. Associated with this decreased EBV-specific immune control after PD-1 blockade is the expansion of FoxP3+CD25+ regulatory CD4+ T cells and elevated production of the immune-suppressive cytokine IL-10. Thus, PD-1 inhibition similar to CTLA-4 haploinsufficiency seems to expand Treg cells that might compromise EBV-specific immune control.

Under conditions with already compromised EBV-specific immune control and CD4+ T-cell populations that actually support EBV transformed lymphoma growth in humanized mice PD-1 and CTLA-4 blockade, however, improves immune control. Even so all mice that are injected with EBV-infected cord blood develop lymphomas, the lymphoma weight could be reduced by simultaneously blocking CTLA-4 and PD-1. This effect was due to T cells because CD3-directed antibody depletion restored tumor weight. CTLA-4 and PD-1 blockade also led to increased T cell infiltrates into the smaller lymphomas. These findings suggest that some EBV-associated tumors regress after blocking the inhibitory PD-1 and CTLA-4 coreceptors while this treatment might be detrimental to the immune control of EBV infection in general, possibly mainly lytic EBV infection.

Similarly, in nasopharyngeal carcinoma, which is associated with EBV, PD-1 blocking monotherapy achieved clinical responses in one-third of the treated patients. In combination with chemo- or adoptive T-cell therapies, clinical responses were even achieved in the majority of treated individuals with complete remission of metastatic disease in some patients. However, PD-1 and CTLA-4 directed antibody therapies also affect T cells that suppress immune responses via IL-2 consumption, inhibition of costimulation via CTLA-4, immune-suppressive cytokines, ATP hydrolysis, and direct effector T cell killing. CTLA-4 is constitutively expressed on Tregs and PD-1 is induced upon activation. Blocking of PD-1 enhances their immune regulation and can lead to increased cancer aggressiveness. Treg activation during immune checkpoint blockade of PD-1 could also be responsible for some of the neurological symptoms that are observed in up to 5% of treated cancer patients. Along these lines it was described in some of these patients that cytotoxic CD4+ T cells are expanded and infiltrate together with EBV-infected B cells in the CNS of these patients. These findings suggest that PD-1 blockade might compromise EBV-specific immune control possibly via Treg activation that then could drive CNS inflammation via T cell stimulation by EBV-infected B cells and cause neurological adverse events of immune checkpoint inhibition. Similar to this expansion of EBV-infected B cells, a fatal KSHV-driven B-cell lymphoproliferation was also reported in one patient with PD-1-directed immune checkpoint inhibition. Although patients with Kaposi sarcoma experienced partial responses upon PD-1 blockade, detectable KSHV viremia before treatment was associated with the development of a fatal lymphoproliferation in one of the treated patients with Kaposi sarcoma. Therefore, PD-1 blockade might compromise immune control of EBV- and KSHV-infected B cells also in patients, possibly via Treg activation.

Treg accumulation is also observed in patients with CTLA-4 haploinsufficiency that suffer from EBV-associated diseases as described above. Their symptoms can be managed by treatment with injections of a recombinant Fc fusion protein of CTLA-4, namely Abatacept, suggesting that this attenuates Treg stimulation by decreasing CD80- and CD86-mediated costimulation. Altogether, these considerations argue for monitoring of the two oncogenic herpesviruses EBV and KSHV during immune checkpoint blockade of PD-1 and CTLA-4 because they could be responsible for some of the adverse events during the respective treatments.

5 | THERAPEUTIC APPLICATION OF IMMUNE CHECKPOINTS IN THE IMMUNE CONTROL OF EBV AND KSHV

This dichotomy of beneficial or detrimental consequences of immune checkpoint blockade on the immune control of EBV and KSHV can also be observed in patients. Hodgkin’s lymphoma which is up to 50% associated with EBV can be treated by immune checkpoint blockade of PD-1 in the majority of patients. Combinations of αPD-1 antibodies with CD30 targeted toxins and CTLA-4 blockade have even achieved complete response rates of more than 70%.

6 | CONCLUSION AND OUTLOOK

PIDs and therapeutic antibody-mediated blockade of costimulatory and coinhibitory molecules start to provide us with a picture of what is required for the immune control of oncogenic herpesviruses. Due to its high prevalence in the human population, this picture is already more complete for EBV than for KSHV. Consequently, costimulation by CD27, 2B4, 4-1BB, and NKG2D and coinhibition by PD-1 and CTLA-4 seem to be required for the immune control of EBV, while
OX40, NKG2D, and PD-1 might be required for KSHV-specific lymphocyte responses. Curiously both viruses do not seem to cause diseases in patients with defects in antibody production.31.64 Thus, both seem to be primarily controlled by T cells, possibly predominantly by cytotoxic CD8+ T cells for EBV and with a strong contribution by IFN-γ-producing CD4+ T cells for KSHV.

The identified set of costimulatory and coinhibitory molecules needs to be considered for both vaccination and immunomodulatory treatments of EBV- and KSHV-associated diseases. Especially EBV-specific CD8+ T cells retain most costimulatory receptors of naïve CD8+ T cells and are therefore capable to maintain their effector functions despite upregulation of most coinhibitory molecules.68,100 Any vaccination against EBV should aim to induce a similar phenotype.317 Furthermore, immunomodulatory treatments like PD-1-specific immune checkpoint blockade seem to be beneficial for patients with EBV latency II-associated Hodgkin’s lymphoma and with Kaposi sarcoma but possibly detrimental for the immune control of EBV latency III, lytic EBV replication, and KSHV-associated lymphoproliferation. Therefore, each disease of these persistent human γ-herpesviruses should be separately evaluated for beneficial or detrimental effects of immune checkpoint blockade. Moreover, reactivation of these persistent infections could be monitored to avoid adverse events during immune checkpoint blockade in patients with infectious disease unrelated tumors.

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CONFLICT OF INTEREST
The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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