Cancer Immunology Research

Modification of Cell Differentiation, One of the Mechanisms in the Surveillance of Malignancy

Eva Klein, Noemi Nagy, and Eahsan Rasul

Abstract

Most humans carry the potentially life-endangering Epstein–Barr virus (EBV). The immediate danger after infection is imposed by proliferation of the B cells that carry the viral genome. Although a number of different cell types can be infected with EBV, B lymphocytes are exceptionally sensitive; they express a set of virus-encoded proteins, which collaborate with host proteins to induce proliferation. This phenomenon can be demonstrated *in vitro* with experimentally infected B cells. These viral genes are expressed only in B lymphocytes and are restricted to a defined differentiation stage. This limitation is of high importance for the maintenance of the controlled EBV-carrier state of humans. The emergence of EBV-induced B-cell malignancies is counteracted by highly efficient immunologic mechanisms. Recognition of EBV-transformed immunoblasts in an MHC class I–restricted manner by cytotoxic CD8 T cells and, to a lesser extent, by CD4 T cells, is thought to play the major role. The *in vitro* experimental results are in accordance with the emergence of EBV^+ B-cell malignancies in immunosuppressive conditions. In this Masters primer, we emphasize that in addition to eliminating B cells that carry the virus genome, the regulatory circuit of the immune response also operates in surveillance, particularly in the early phase of infection. This mechanism involves T-cell–mediated regulation of B-cell differentiation. Because of the strict dependence of the viral growth program on the expression of host cell factors, altering the differentiation state can curb the proliferation of B cells that harbor the viral genome. *Cancer Immunol Res; 3(2); 97–102. ©2015 AACR.*

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Learning Objectives

Most humans are Epstein–Barr virus (EBV) carriers. The emergence of EBV-induced malignancies is counteracted by highly efficient host immunologic surveillance mechanisms. EBV can infect many cell types, but only B lymphocytes in a defined differentiation stage express the set of viral proteins that can induce proliferation without additional contributing factors. This set of EBV-encoded proteins is referred to as the growth program. Upon completion of this activity, the participant should gain a basic knowledge of the host's mechanism for restricting the growth program and thus the proliferation of EBV-infected B cells.

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Introduction

The Epstein–Barr virus (EBV)–carrier state in humans is almost universal. It reflects a long evolutionary history that has resulted in a finely tuned coexistence of virus and host. The constant control is essential because once infection occurs, the virus remains for the lifetime of the host; it cannot be eliminated. EBV infection is potentially life threatening by inducing the proliferation of B cells carrying the viral genome. However, both the primary infection and the permanent carrier state are tightly controlled by immunologic mechanisms (1, 2). When these control mechanisms are compromised, the B-cell–resident viral genome can manifest its proliferation-inducing function (3). Although a number of different cell types can be infected with the virus, B lymphocytes are exceptionally sensitive. EBV binds to the complement receptor CD21 that is a marker of mature B cells (Fig. 1; ref. 4).

The first encounter of the virus can, although not always, induce the syndrome of infectious mononucleosis (IM; ref. 5), which is initiated by virus-induced activation of the B cells. With very rare exceptions, the infection does not cause the immediate danger that would be expected from an efficient transforming agent. In this Masters of Immunology article, we focus on the virus–B-cell

Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Stockholm, Sweden.

Corresponding Author: Eva Klein, Karolinska Institute, Nobels väg 16, S-171 77 Stockholm, Sweden. Phone: 46-8-524-86761; Fax: 46-8-330498; E-mail: Eva.Klein@ki.se

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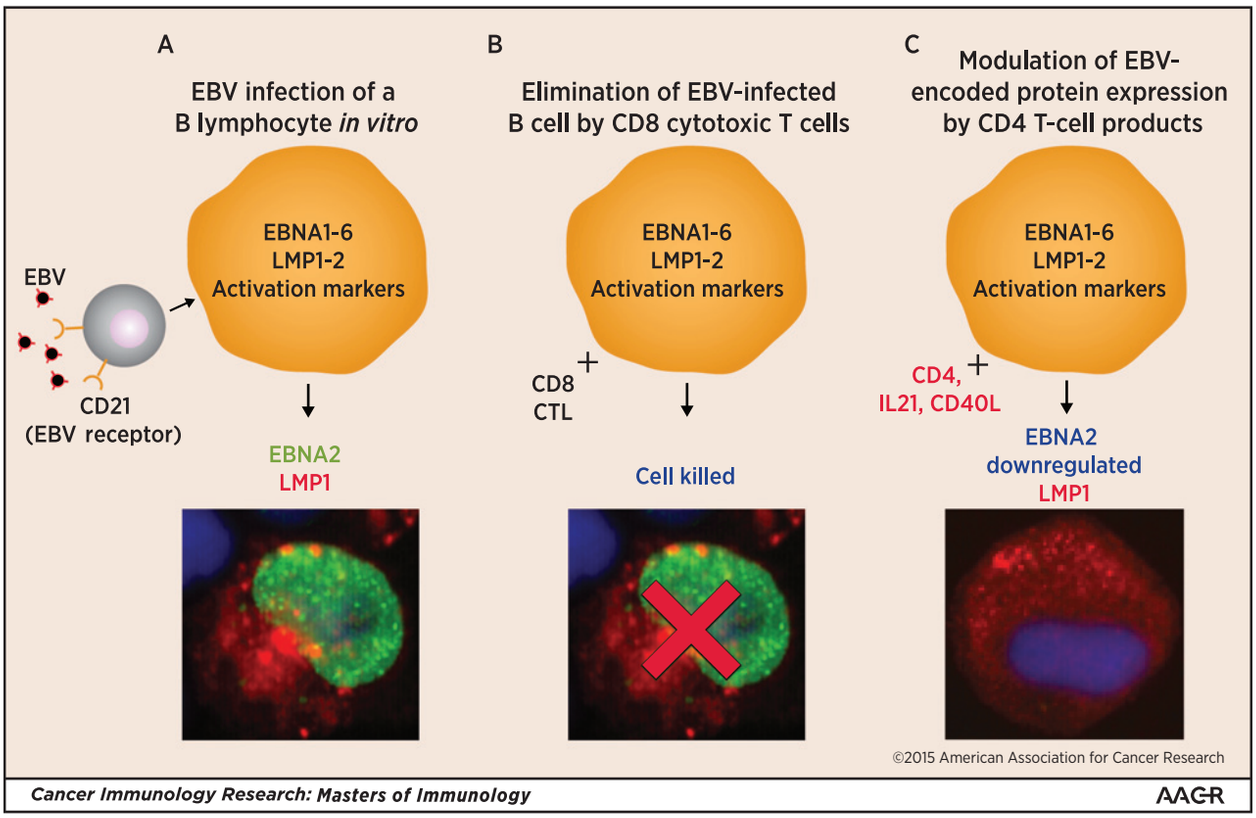

Figure 1.
Demonstration of T-cell control of EBV-infected B cells *in vitro*. EBV infects mature B cells by binding to the complement receptor CD21 on the cell surface (33). In latent infection, the viral genome exists as an episome in the infected cell. Some infected B cells are activated and express EBV-encoded proteins, EBNA-2 and LMP-1 which are pivotal for proliferation. A, the fluorescence micrograph shows an EBV-induced B blast expressing both EBNA-2 and LMP-1. B, CD8 T cells are activated upon encountering EBV-induced B blasts. Activated CD8 CTL can eliminate B blasts in a MHC class I–restricted manner. C, IL21 and CD40L from activated CD4 T cells can modify the expression of the resident EBV genome. These T cells downregulate the growth program (latency type III) and thereby stop proliferation. IL21 induces differentiation toward plasma cell (which can be seen by BLIMP-1 expression, not shown here) that does not support the expression of EBNA-2 and thus switches the latency program to type IIa. CD40L downregulates the expression of both EBNA-2 and LMP-1 by a yet unknown mechanism. The fluorescence micrograph shows downregulation of EBNA-2 by the T-cell products. Fluorescence: EBNA2 (green); LMP1 (red), DAPI (purple).

interaction and particularly the role of the differentiation state of the B cell on the outcome of infection (6).

Infectious mononucleosis

IM is the clinical manifestation of the innate immune surveillance mechanism that responds promptly to EBV infection (7). Its symptoms represent an auto-regulatory circuit within the immune system. The events of EBV infection are similar to those of the antigen response in that the T cells recognize the activated B lymphocytes. The severity and the dominating symptoms vary widely, including lymphocytosis and enlargement of lymph nodes, in which EBV genome–carrying B cells and activated T cells can be detected. Lymphokines produced by the activated T cells contribute to both the subjective and objective symptoms. Irrespective of severity, the acute phase is most often transient. The lymphokines produced by the activated CD4 T cells are particularly important for the control of EBV-infected B cells (Fig. 1). In rare individuals who carry a genetic trait that causes disturbance in the dynamics of the T-cell response, primary EBV infection can lead to a fatal disease, X-linked lymphoproliferation (XLP; ref. 8).

Adaptive immunologic mechanisms develop during the acute phase with cognate recognition of virally encoded proteins expressed by the infected cell. Adhesion molecules expressed by the blast-transformed B cells are pivotal for the interaction with T lymphocytes. The subsequent symptomless virus-carrier state is ensured by serologic and cellular immunologic memory (2, 9).

Expression of EBV-encoded proteins in B lymphocytes with latent infection

Type III latency, the growth program. Human B cells can be infected with EBV *in vitro*. These cultures yield permanent lines referred to as lymphoblastoid cell lines (LCL; refs. 10, 11). The functions and interactions of virally encoded proteins with cellular proteins were characterized in these lines. LCLs can be generated easily by *in vitro* infection of lymphocytes collected from each individual. Because they carry the complete genetic material of the donors, cryopreserved supplies of LCL panels are invaluable for a variety of genetic and functional studies (12).

The EBV-encoded protein complex that induces proliferation is referred to as type III expression or growth program. It comprises six proteins localized in the nucleus (EBNA 1–6 or EBNA-1, -2,

-3A, -3B, -3C, and the leader protein), and three cell membrane–associated proteins (LMP-1, -2A, and -2B.) Importantly, this viral growth program is expressed only in a defined differentiation window of the B cell (13). As detailed in the following sections, this restriction determines the consequence of EBV infection. The two virally encoded proteins EBNA-2 and LMP-1 are pivotal for inducing unassisted proliferation. They are expressed together only in B cells. The B lymphocyte–specific viral growth program is governed by the transcription factors that regulate EBNA-2 expression. The detection of EBNA-2 and LMP-1 is useful as they are markers for the proliferating B lymphocyte. Interestingly, their quantitative expression varies considerably and independently both in tissues and in experimentally infected B-cell populations. The type III B cells represent the immediate danger during primary infection.

Type I latency and Burkitt lymphoma. Infected cells that express only EBNA-1 are designated as type I latency (14). It was detected in the studies of Burkitt lymphoma. It is noteworthy that EBV was discovered in the epidemiologic studies of the African Burkitt lymphoma (15) even though it causes IM, a disease already known and well defined for a long time, although the causative agent was unknown. The discovery was based on a coincident event in the laboratory conducting epidemiologic studies of the African Burkitt lymphoma (16). Discovery of the virus was followed by demonstration of the almost ubiquitous infection (17) and largely symptomless EBV-carrier state in adults. Demonstration that EBV has a direct transforming potential for B cells *in vitro* (10) led to the assumption that the virus is responsible for the genesis of Burkitt lymphoma. However, EBV^- Burkitt lymphoma cases were found outside Africa, and they shared a similar chromosomal translocation with the EBV^+ Burkitt lymphoma cases (18). The translocation involves the Ig and myc genes, indicating that it is the common culprit in the "endemic" and "sporadic" Burkitt lymphoma cases, as they were subsequently designated. The EBV^+ cases predominated in Africa. The geographic localization of these cases coincides with that of holoendemic malaria (14), and was assumed to provide a pathogenic contribution through the activation of the B-lymphocyte compartment (19, 20). However, the differentiation state of the malignant B cells in the EBV^+ and EBV^- Burkitt lymphoma differs. Analysis of somatic mutation rates in 31 cases indicated that the EBV^- BLs may originate from early centroblasts, whereas the EBV^+ BLs originate from late germinal center or memory cells (21). This cell subtype–dependent derivation of the Burkitt lymphoma cases does not correlate with the geography or with the HIV status.

The discovery of EBV^- cases led to the question about the contribution of EBV to the genesis of endemic Burkitt lymphoma. Apoptosis is an important factor in the transient nature of activation/proliferation of lymphocytes during the normal immune response. More recent studies indicate that the EBV-encoded proteins counteract the proneness of infected cells to apoptosis that is a consequence of overexpression of the translocated myc gene (22–24). Therefore, EBV-encoded proteins contribute to the survival of B cells with Ig-myc translocation.

Comparisons of the expression of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) in viral genome–positive and –negative Burkitt lymphoma–derived cell lines also indicated the importance of apoptotic propensity; these lines differ in SAP expression (25). Although the EBV^+ Burkitt lymphoma lines may be either SAP^+ or SAP^-, all of the EBV^- lines are SAP^-. Normal T cells express SAP when activated, but normal B lymphocytes are SAP^-. Transfection experiments showed that SAP is proapoptotic (24). The presence of the antiapoptotic EBNA-1 in EBV^+ Burkitt lymphomas may balance the proapoptotic SAP in EBV^+ Burkitt lymphomas (22, 24).

Type IIa latency and Hodgkin lymphoma. The type IIa expression pattern, designated originally as type II, lacks EBNA-2 but expresses LMP-1. It was first detected in nasopharyngeal carcinoma and thus in epithelial cells (26). Subsequently, EBV-carrying Hodgkin lymphoma cells that originate from B cells, Hodgkin and Reed-Sternberg (HRS) cells, were also found to have this latency type (27). We proposed a designation based on the detection of the two EBV-encoded proteins, EBNA-2 and LMP-1 (13). This designation distinguishes the two reciprocal expressions, EBNA-2–negative LMP-1–positive and EBNA-2–positive LMP-1–negative, as type IIa and type IIb, respectively. Both types can be detected in the lymph nodes of patients with IM (28). The type IIa cells are likely the origin of the subsequent Hodgkin lymphoma that can develop in children and young adults after acquisition of EBV infection with manifested symptoms of IM (29). Type IIa cells can be detected in cord blood mononuclear cell cultures early after experimental EBV infection *in vitro*, before the enrichment of proliferating type III cells in the culture (30).

The fraction of EBV^+ Hodgkin lymphoma cases varies geographically. The malignancy involves phenotypic changes of the B cell that enters into complex interaction with the microenvironment, resulting in the formation of typical granulomatous tissue. Cellular and humoral factors provided by the microenvironment are required for the proliferation of type IIa cells (27). This is reflected by the lack of proliferative capacity of the Hodgkin lymphoma cells *in vitro*. In spite of considerable efforts, only a few lines have been established from Hodgkin lymphoma, and these lines lack EBV. In Hodgkin lymphoma, the impact of the EBV genome in the B cell is complex. Its dependence on external factors can be exemplified with the *in vitro*–infected EBV^- KMH2 cell line that was derived from an EBV^- Hodgkin lymphoma. The *in vitro* infection resulted in expression of Type I EBV-encoded genes and changed to latency type IIa when treated with lymphokines IL4 or IL13 and CD40 ligand (CD40L; refs. 31, 32).

Type IIb latency. Type IIb latency was identified in studies of chronic lymphocytic leukemia (CLL) cells infected *in vitro*, which express EBNA-2 but not LMP-1 (13, 33). The EBV-carrying CLL cells have a very limited, if any, proliferation capacity (34). Cells with a type IIb expression pattern were first mentioned in studies of IM (28) and posttransplant lymphoproliferative disease (PTLD; ref. 3), but their nature was undefined. The fact that CLL cells express this set of EBV proteins suggests that the differentiation window of the B cells at the event of infection determines the expression of the virally encoded proteins and indicates that CLL cells lack factors required for LMP expression. We assume that, within the normal B-cell population, cells with the corresponding differentiation state can be infected, but do not proliferate. Malignancies with type IIb expression have not been identified to date. Unique cell lines were selected from a Burkitt lymphoma tissue that contained a proportion of type IIb cells (5%–10% in the first *in vitro* passage; ref. 35). The impact of EBV-encoded proteins was assumed to control apoptosis, because proliferation of these cells is probably driven by the Ig-myc translocation (35). B cells with all latency types can be detected in IM (28), PTLD (3),

and in "humanized" (carrying cells of the human immune system) mice infected with EBV (36, 37).

Surveillance mechanisms

The efficient adaptive surveillance mechanism that allows the healthy EBV-carrier state has been studied extensively for decades (1, 2, 9, 38). The virally encoded proteins expressed by viral genome–carrying B cells have been identified. Recognition of the immunoblasts in an MHC-restricted manner by cytotoxic CD8 T cells and, to a lesser extent, by CD4 T cells were shown to play the main role in their elimination (39, 40). The rules of this mechanism have been elucidated in *in vitro* experiments. The results of these *in vitro* studies were in good accord with the emergence of EBV^+ B-cell malignancies in conditions of immunosuppression.

The detailed knowledge and methodologic progress could be "translated" to the bedside and lead to successful immunotherapy. Autologous and, in some situations, allogeneic CD8 T cells that recognize and attack the malignant B blasts based on EBV-encoded protein expression were selected (40–44). These T-cell populations were then expanded *in vitro* and administered to patients. The lymphocyte-mediated elimination of the EBV-carrying cells is thus based on cognitive recognition.

We emphasize an additional mechanism based on the intrinsic regulation of immune responses. We consider that this is particularly important soon after EBV infection, before the development of cognate memory. Given that EBV-infected B cells proliferate efficiently, during the time required for the recognition and effector phase of the immune response, a considerable size of EBV-carrying blast population can develop. We propose that mechanisms inherent in the dynamics of T-cell–B-cell interactions during immune responses can inhibit the B-cell proliferation (45, 46). T cells can modulate EBV latency. EBV-induced B blasts incite a T-cell reaction. Through a mutual feedback loop, the proliferation of both B and T cells becomes limited. Through influencing B-cell differentiation, the T-cell products, IL21 and CD40L, modify the expression of EBV-encoded proteins. They downregulate EBNA-2 that is pivotal for the growth program (45, 46), and thus eliminate the immediate danger of B-cell proliferation (Fig. 1).

Two EBV-harboring cell lines established from CLL cells in different stages of B-cell differentiation

EBV is not involved in the pathogenesis of CLL (47). Presently, it is emphasized that subclonal variation and selection lead to the progression of disease with changes of the biologic behavior, activation state, and proliferation of cells (48, 49).

The influence of B-cell differentiation on its interaction with EBV can be illustrated with experimentally infected *ex vivo* CLL cells (34), and by the two lines, MEC1 and MEC2, derived from a patient with CLL (50). The MEC lines emerged one year apart from explanted blood-derived malignant cells when the disease had gradually acquired characteristics of prolymphocytic leukemia (PLL). These lines carry the same EBV strain harbored by the patient, indicating that the cells of origin became infected probably *in vivo*. The patient was EBV-seropositive, but according to the original report, no gene expression that encoded viral protein was detected by PCR in these leukemic cells at the time of explant.

In addition to the confirmation that EBV-infected PLL cells can proliferate *in vitro*, comparisons of the lines demonstrate that even though both lines express type III latency and proliferate in culture, their phenotypes differ. The MEC1 cell line was established from the explant when the clinical picture was characterized as CLL, albeit the disease was already beginning to transition to PLL. Analysis of the cell population at this time showed already the expression of some PLL markers. Although the MEC1 cells do not correspond to the vestige of *in vivo* CLL cells, they were used in several studies as a "CLL cell line" (51–53). The MEC1 line is unique because it lacks the typical phenotype of type III cells.

One year after the establishment of the MEC1 line, the condition of the patient deteriorated. The leukemia disseminated with a marked increase of the white blood cell count and other characteristics of PLL. The MEC2 line established at this time originated from the same CLL clone but with a changed differentiation state, as revealed through an analysis of the EBV terminal repeat and promoter usage for EBNA-2 expression (54). This line had several similarities with LCLs, but differed conspicuously from the earlier established MEC-1 line. Since MEC1 and MEC2 are progenies of one cell from the malignant populations, the phenotypic difference between these two MEC lines reflects the different phases of the disease (54).

The rare EBV genome–carrying CLL cells were probably under the influence of T-cell–derived factors *in vivo*, and lymphokines could have suppressed EBNA-2 expression. Both our group and others have shown earlier that EBNA-2 expression can be suppressed by IL21 in type III cells (54–56). Therefore, it is likely that when released from the effect of lymphokines *in vivo*, the viral program in infected cells changed to type III and the cells proliferated. The experiment in which the MEC lines were treated with IL21 or CD40L is a corollary of this scenario because they show the modulation of the program under the influence of T-cell–produced factors (46, 54, 55, 57). Our recent results showed that the T-cell product IL21 induced plasmacytoid differentiation in type III LCLs and concomitantly changed the viral program; in these B cells, EBNA-2 was downregulated and LMP-1 was upregulated (55).

Conclusions

EBV can infect human cells with variable efficiency, and B lymphocytes are exceptionally sensitive. Almost all adults carry B cells positive for EBV genome. Normally, these cells either lack the expression of virally encoded proteins or express EBNA-1. The EBV genome is expressed differently in various phases of B-cell differentiation. Importantly, in a defined differentiation window, the set of EBV proteins imposes proliferation. Unless controlled, these proliferating cells can manifest as malignancy.

The pathogen-carrier state of humans is accompanied by the development of efficient immune surveillance mechanisms. Pathogenic complications occur when these mechanisms are impaired. The study of EBV immune surveillance mechanism has a long history, and it was focused initially on the cytotoxic T cells, which eliminate B lymphocytes induced to proliferate by the EBV genome they carry. Evidence is emerging, however, that an additional mechanism contributes to the elimination of risk for proliferation of these infected B cells. This mechanism is particularly important in the early phase of EBV infection

and is based on the restricted expression of the EBV growth program to a defined window of B-cell differentiation. The experiments of Leo Sachs emphasized that certain leukemias could be suppressed by inducing the differentiation of tumor cells (reviewed in 58). The evidence that modification of the differentiation window of the EBV genome–containing B cells can downregulate the growth-inducing program suggests that a "differentiation therapy" may also be designed for EBV-associated B-cell malignancies.

Dedication

We dedicate this article to the memory of Leo Sachs, one of the giants in cell and tumor biology. His outstanding experimental and conceptual contributions to several fields are examples of thoroughness and clarity. His early work, the cytologic basis of prenatal diagnostics, provided a great practical importance. His discoveries have pioneered new approaches to basic and medically applied aspects of stem cell biology, development, hematology, and oncology, and led to new therapies.

Importantly, and with particular relevance to this article, Leo Sachs showed that murine leukemia cells can be induced by natural products (cytokines and lymphokines) and artificial compounds (TPA and DMSO) to differentiate terminally and to cease malignant behavior. He extended this work to human leukemia cells. Results from these studies underscore the proposal that modification of differentiation state can be exploited for therapy.

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