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Alteration of B cell function by Epstein-Barr virus

Epstein-Barr virus (EBV) is a DNA virus belonging to the γ -herpes virus family. Over 90% of the human population worldwide is infected by the virus. Primary infection usually takes place during childhood and is mostly asymptomatic. However, when primary infection is delayed, frequently a self-limiting disease develops, called infectious mononucleosis (IM). During IM, a massive expansion of virus-harboring cells is observed. These cells are then mostly eliminated by a strong cytotoxic T cell response. The cellular target of the virus and its reservoir during lifelong persistence are B lymphocytes, in which the viral genome is present as a circular episome. In healthy individuals, a stable balance is established between the expansion of virus-infected B cells and their elimination by cytotoxic T cells. However, EBV also plays a role in the pathogenesis of several human lymphomas. In vitro, EBV-infected B cells give rise to stable transformed cell lines, designated lymphoblastoid cell lines (LCL).

Establishment of viral latency in the B cell compartment

Virus carriers produce infectious virus in the lymphatic tissue of the Waldeyer's ring and secrete virus particles into the saliva, so that the virus is predominantly transmitted by the oral route. Studies of D. Thorley-Lawson's group have led to the following scenario of the viral strategy to establish latency in the B cell compartment¹: EBV first infects naïve B cells and in these cells activates a growth program (also termed latency III), which is characterized by expression of nine latent viral genes. A fraction of these cells will be driven into germinal centre (GC) reactions. GC are histological structures in lymphoid organs in which T cell-dependent immune responses take place. In proliferating GC B cells, the process of somatic hypermutation, that modifies the DNA of the variable region of immunoglobulin (Ig) genes, is activated, and GC B cells are selected for improved affini-

ty to the immunizing antigen.² Positively selected GC B cells finally differentiate into memory B cells or plasma cells. It was proposed that also EBV-infected B cells that enter GCs undergo this differentiation process so that the virus gains access to the memory B cell compartment, its main reservoir during persistence, via this route.¹

Strategies of EBV during IM

To study the processes taking place during primary EBV infection in IM, we micro-manipulated single EBV-infected B cells from tonsillar tissue sections of two cases of IM and amplified and sequenced their rearranged Ig genes.³ This analysis revealed that already during acute infection, EBV preferentially resides in B cells with somatically mutated V genes. The virus spreads in the B cell compartment by two main strategies. On the one hand, a large number of B cells is infected by EBV, and on the other hand, virus-harboring cells can expand to large clones. All expanded clones carried mutated Ig genes, but no intraclonal sequence diversity was observed.³ The scenario discussed for persistent infection (see above) does consequently not hold true for primary infection associated with IM. There was no indication for a preferential infection and clonal expansion of naïve B cells. Apparently, GC and/or memory B cells were directly infected and it is these cells that clonally expand preferentially.

In the scenario of the viral strategies to establish persistent infection during asymptomatic infection, the GC passage of EBV-infected B cells plays an important role (see above). As we detected EBV-harboring cells in three GC of two cases of IM, we analysed these cells at the single cell level. In each of the three GC, expanded clones were found among the EBV-carrying B cells.⁴ Interestingly, these expanding clones did not show ongoing hypermutation, while clones of EBV-negative B cells present in the same GC showed, as expected, this feature. Hence, the EBV⁺ GC B cells did not behave like normal GC B cells. Therefore, it

appears unlikely that in IM, EBV-infected B cells undergo a normal GC reaction to finally become EBV-harboring memory B cells. Surprisingly, the EBV⁺ GC B cells were mostly EBNA2 protein-positive, whereas a previous RT-PCR analysis of flow cytometrically isolated GC B cells indicated that EBV⁺ GC B cells lack EBNA2 expression and show a latency II profile.⁵

The role of EBV in lymphomagenesis

EBV is associated with a number of tumours, in particular B cell lymphomas. Nearly 100% of cases of endemic Burkitt lymphoma (BL) in Africa and 30% of BL cases in Europe are EBV-positive.⁶ In BL, only a single EBV-encoded protein is made, namely EBNA1, which is needed for the replication of the viral genome in proliferating cells. BL cells morphologically and phenotypically resemble centroblasts, the proliferating GC B cells, suggesting that the lymphoma cells derive from these cells. This is further supported by the observation that many cases of BL show intraclonal V gene diversity as a result of sustained somatic hypermutation activity in the tumour cells.^{7,8}

EBV also plays an important role in the development of B cell lymphomas in immunocompromised patients. These are on the one hand AIDS patients, which show a strongly increased risk to develop EBV-positive B cell lymphomas, and on the other hand post transplant patients, which are treated with immunosuppressive drugs.⁹ Presumably, the impaired T cell response in these patients allows EBV-infected B cells to expand unrestricted. This will lead initially to a polyclonal expansion of EBV-transformed B cells, and later some cells that acquired additional growth promoting events may dominate the population of EBV-infected B cells (oligoclonal population). Finally, a fully transformed B cell may give rise to a malignant B cell lymphoma. Such lymphomas in immunocompromised patients usually show the growth program of EBV, with expression of all latent genes.⁹

We analysed five cases of post transplant lymphoproliferative disease (PTLD) for the clonal composition and differentiation stage of the EBV-harboring B cells.¹⁰ In two cases of polymorphic PTLD, oligoclonal expansions of EBV-positive B cells were detected. A monomorphic centroblastic lymphoma was represented by a single clone. In two cases of Hodgkin lymphoma (HL), clonal HRS cell populations were identified. Notably, the centroblastic lymphoma showed ongoing somatic hypermutation, and destructive V gene mutations were detected in this case. Thus, also in PTLD, EBV-infected B cells can acquire features of GC B cells, and in some cases B cell receptor (BCR)-deficient B cells may survive and expand. Similar results were also reported by two other groups.^{11,12}

The role of Epstein-Barr virus in HL pathogenesis

A further lymphoma that shows an association with EBV is classical Hodgkin's lymphoma (HL). About 40% of cases in the Western world are associated with EBV, and among childhood cases of HL in South and Middle America, nearly all cases are EBV-positive.¹³ In these HLs, the virus is found in the tumour cells, the Hodgkin and Reed/Sternberg (HRS) cells.^{14,15} EBV-positive HRS cells express besides EBNA1 also the latent membrane proteins LMP1 and LMP2a (latency program II). HRS cells derive in most cases from B cells, in very rare cases from T cells.¹⁶ B cell-derived HRS cells carry somatically mutated Ig variable (V) region genes, and in a quarter of the cases, obviously destructive somatic mutations were detected in originally functional V region genes.^{16,18} Such mutations happen in normal GC B cells in the course of somatic hypermutation, but normally result very efficiently in the elimination of the respective B cells by apoptosis within the GC. Hence, HRS cells in cases with such mutations most likely derive from "crippled", pre-apoptotic GC B cells, that were rescued from apoptosis by some transforming events. As only a fraction of disadvantageous mutations that normally result in apoptosis of the GC B cell can be easily identified, we speculated that HRS cells in classical HL as a rule derive from pre-apoptotic GC B cells.^{17,19} In EBV-positive cases of HL, EBV-encoded proteins may play an important role in the rescue of the presumptive pre-apoptotic HRS cell precursor from apoptosis: LMP1 and LMP2a apparently replace two important survival signals for GC B cells. LMP1 mimics an activated CD40 receptor, which is one of the main survival signals in the T cell/B cell interaction in the GC (20). LMP2a carries in its cytoplasmic domain an ITAM (immunoreceptor tyrosine-based activation motif), a motif that is also present in the BCR and mediates BCR signaling. LMP2a may thus mimic a crosslinked BCR and thereby replace normal BCR signals. Indeed, in LMP2a transgenic mice expressing this protein in B cell precursors, BCR-deficient B lineage cells are generated that are allowed to leave the bone marrow and survive in the periphery.^{21,22}

While EBV-encoded proteins may well play a decisive role in the early stages of the pathogenesis of EBV-positive HL, it is less clear whether LMP2a can have the same role also in the established HRS cell clone. This is because gene expression profiling studies showed that HRS cells have lost expression of many B cell-specific genes (23). These molecules include the Syk tyrosine kinase and the intracellular adaptor protein SLP-65, two important factors for BCR signalling. Since these molecules appear to be essential for the function of LMP2a as a BCR surrogate,^{24,25} it is questionable whether LMP2a can replace the BCR survival signal in the established HRS cell clone via the conventional

BCR signalling pathway.

Another issue regarding the role of EBV in the pathogenesis of HL relates to the question at which step in the pathogenesis of HL HRS cell precursors become infected by EBV. According to the scenario discussed above, in healthy virus carriers, EBV predominantly infects naive B cells and (some of) these cells undergo GC reactions to gain access to the memory B cell compartment. Thus, EBV would be present already in a B cell that is driven into a GC reaction and that after acquisition of additional transforming events gives rise to a malignant HRS cell clone. While it is normally not possible to identify the differentiation stage at which an HRS cell precursor was infected by EBV, we recently encountered a classical HL in which only a fraction of HRS cells was EBV-infected.²⁶ Interestingly, the EBV-positive and -negative HRS cells carried not only shared somatic mutations in the rearranged Ig V genes, but also a few mutations that were found only in the EBV+ subclone. This suggests that the EBV infection happened in a member of a mutating GC B cell clone and that this cell as well as a non-infected other member of the clone later gave rise to the HRS tumour clone (presumably, the common precursor of the two HRS subclones already carried a shared transforming event). The alternative scenario, namely that EBV was lost from an originally homogeneously EBV-infected HRS cell clone is unlikely due to the fact that the EBV- HRS cell subclone carried less somatic mutations than the EBV+ subclone. Thus, this case is the first example for a HL case with indication that EBV infection happened in a GC B cell, suggesting that at least in a fraction of cases of EBV-positive HL the viral infection may happen in GC and not in naive B cells.

Survival and expansion of Ig-deficient EBV-infected B cells in T cell lymphoma of AILD-type

Angioimmunoblastic lymphadenopathy with dysproteinaemia (AILD) is one of the most frequent T cell lymphomas. Besides the T cell tumour clone, occasionally expanded B cell clones have been reported in such cases, and EBV-infected B cells are often found at increased frequency in AILD tissues. EBV-positive B cells were microdissected from six cases of AILD with a high frequency of EBV-positive cells and their rearranged Ig genes were sequenced. In all cases expanded clones were found among the EBV-infected B cells.²⁷ Most clones carried mutated Ig genes and showed intraclonal V gene diversity, indicating active somatic hypermutation during clonal expansion. Surprisingly, many members of expanded clones carried destructive somatic mutations in originally functional Ig gene rearrangements, and in several clones, it was evident that hypermutation was ongoing after acquisition of destructive mutations.²⁷ Such a sustained

hypermutation in the complete absence of selection for functionality of the BCR has not been observed before for B cells in vivo, but appears to be characteristic for EBV-positive B cells in AILD. Moreover, the survival and clonal expansion of BCR-deficient EBV-infected B cells represents a novel type of viral persistence in B cells.

Establishment and characterization of EBV-immortalized GC B cell lines

On the background of the indications that most EBV-associated B cell lymphomas appear to derive from GC B cells, it is of particular interest to study EBV-infected GC B cells. One way to address this issue that we are pursuing is to generate EBV-immortalised LCL from flow cytometrically isolated tonsillar GC B cells. More than 50 monoclonal cell lines were established so far. We are now characterizing these lines to find out whether they retain typical features of normal GC B cells, and whether they acquire some features of the EBV-associated B cell lymphomas. In the later case, such lines may represent valuable models to study early stages in the pathogenesis of such lymphomas.

Since we had speculated that in EBV-positive cases of HL the virus might be involved in early stages of the pathogenesis by rescuing crippled GC B cells from apoptosis, we were specifically interested to find out whether EBV could rescue and immortalize crippled GC B cells upon in vitro infection. Therefore, the monoclonal GC B cell-derived LCL were screened for surface Ig-negative lines, using a highly sensitive surface staining for κ and λ light chains. Indeed, a considerable fraction of lines appeared surface Ig-negative. To unequivocally identify lines with crippled BCR, we sequenced the rearranged Ig heavy and light chain genes of these lines. For two of the lines, destructive Ig VH gene mutations were indeed identified in originally productive V gene rearrangements. Expression of a heavy chain from the second IgH allele was ruled out for both lines by showing that these alleles carried DHJH joints, and no other in-frame VHDHJH rearrangements. Thus, EBV is indeed able to rescue GC B cells with a crippled BCR from apoptosis. This finding supports a role for EBV in early stages of HL pathogenesis and also in the subset of PTL with crippled BCR. Further characterization of these lines is currently underway.

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