Diagnostic and Protective Aspects of Humoral Immune Responses to Epstein-Barr Virus Encoded Proteins in Nasopharyngeal Carcinoma

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Chapter 1

1. General Introduction

Epstein-Barr virus (EBV) was discovered in 1964 (111), initiated by the finding of an unusual lymphoma in African children by Dennis Burkitt during his work in Mulago Hospital in Kampala Uganda around 1950s, which later became known as Burkitt’s lymphoma (BL) (47, 302). In collaboration with Epstein, who already worked on the Rous sarcoma virus, research focused on the search of a virus causing BL. After a long failed investigation, they found a virus that differed from the known human herpesviruses in being unable to replicate in other cultured cells and in being non-reactive with antibodies to other human herpesvirus (205). In the laboratory in Philadelphia, the virus inside BL cells was confirmed serologically as a new human herpesvirus (111).

EBV is classified into the gamma subfamily of potentially oncogenic herpesviruses and is also named human herpesvirus 4 (HHV-4). The gamma herpesvirus subfamily consists of the gamma 1 or Lymphocryptovirus (LCV) and gamma 2 or Rhadinovirus (RDV). EBV is the only human LCV, and is characterized by its capacity to drive B cell growth-transformation (205). Kaposi’s sarcoma-associated herpesvirus (KHSV), also known as HHV-8, is the only human RDV (61).

EBV infects more than 90% of the world population via salivary contact. In developing countries, primary infection occurs in early infancy and is largely asymptomatic. In the West however, primary infection can be delayed until adolescence and in up to 25% cases can present as infectious mononucleosis (IM) (82, 112). Besides being the etiological agent of infectious mononucleosis, EBV is also associated with a variety of lymphoid and epithelial malignancies, including Burkitt’s lymphoma, B-cell non-Hodgkin lymphomas in immunocompromised patients, 40-90% of Hodgkin’s disease cases, T/natural killer (NK)-cell lymphomas, 10% of gastric adenocarcinomas and nasopharyngeal carcinoma. In the tumor cells of these malignancies, different EBV latent genes are expressed (338).

2. The Virus and EBV genome structure

As other herpesviruses, EBV has toroid-shaped protein core which is wrapped with DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and envelope, and an outer envelope with external glycoprotein spikes (106, 107, 111, 183). The most abundant EBV envelope protein is gp350/220 (205). The schematic structure of herpesvirus can be seen in figure 1a.

EBV virions contain a linear double stranded 172 kb genome encoding approximately 85 putative open reading frames (ORF’s) (94, 405). During latent infection EBV DNA is maintained in the nucleus of the host cells as an extra chromosomal circular episome. Twelve viral coding and non-coding genes can be expressed in different combinations during latent viral infection, while the remaining 70 major open reading frames are expressed during the replicative lytic cycle (8). The EBV latent genes on the double stranded viral DNA episome are shown in figure 1b.

The EBV genomic structure is characterized by short and long unique sequence domains (US and UL) that contain almost all the genome coding capacity, as well as internal (IR1-4) and terminal (TR) tandem and reiterated repeats. The EBV contains a linear genome, and upon EBV infection of a cell (typically by a single virion), the linear termini are joined via TR intracellularly to form circular, episomal DNA (338). The architecture of EBV genome can be seen in figure 1c.

The BamHI restriction fragments of B95.8 strain have been sequenced and constitute the first large pathogenic genome of which the full sequence was determined (20). The ORFs are named after BamHI restriction fragment containing the RNA start site and their leftward or rightward transcriptional orientation. BamHI-A is the largest fragment, BamHI-B the second largest, with BALF2 being the second leftward reading frame on the BamHI-A fragment and so on. The ORFs of latent EBV genes can be seen in Figure 1d.

Based on the isolates, EBV can be classified into EBV1 (type A) and EBV2 (type B). Prototypic type A and B viruses differ in the nucleotide sequence for EBNA2, 3A, 3B and 3C by 36%, 10%, 12% and 19% respectively (205). Divergence in the region around EBER genes has also been reported (15), but appears to be less consistence (354). Sequence variation was also found in LMP1 (392), LMP2 (48), EBNA1 (444), and BZLF1 (310). Other genes appear rather conserved, for example vIL-10 (186), EBER (15, 139, 344), and BARF1 [Hutajulu et al., Submitted].

Figure 1a. The schematic structure of herpesvirus
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3. EBV infection & Latency

3.a. B cell infection

EBV mainly infects B lymphocytes through the binding of the major viral envelope glycoprotein gp350 to the CD21 receptor on the surface of B cells. Penetration of virions into B cells requires a complex of three viral glycoproteins, gH, gL, and gp42 (283, 445), with gp42 binding to human leucocyte antigen (HLA) class II molecules as a co-receptor (37, 146, 242, 446, 465). The binding of CD21 is followed by increased mRNA synthesis, blast transformation, homotypic cell adhesion, surface CD23 expression and IL6 production (140, 402, 403).

When the viral genome has been uncoated and delivered to the nucleus, Wp promoter expression initiates a cascade of events that lead to the expression of all of the EBNA proteins and the two membrane proteins. The EBNA-LP and EBNA2 proteins are the first proteins detected (5, 6). EBNA-LP has a role in enhancing EBNA-2 mediated transcriptional activation (205). EBNA2 is the major transcriptional regulator of viral gene expression, including EBV encoded LMP1 and LMP2, and auto-regulates the promoter for a long mRNA transcript that is alternatively spliced to encode EBNA2, LP or the EBNA3 proteins (330, 351). EBNA2 also transcriptionally activates cellular genes by upregulating the expression of certain B cell antigen, CD21 and CD23, as well as viral genes (393, 441). EBNA2 plays an important role in immortalization by activating the Notch signaling pathway. EBNA2 may be a homologue of activated Notch and interacts with the RBP-Jk protein that provides sequence-specific DNA binding and transcriptional activation to the complex (156). EBNA2 also transactivates the viral C promoter (Cp), by inducing the switch from Wp to Cp observed early in B cell infection (205, 393, 441), driving the expression of EBNA3A, 3B and 3C (393, 395, 453). Studies using EBV recombinants have shown that EBNA3A and EBNA3C are essential for B-cell transformation in vitro, whereas EBNA3B is dispensable (341). EBNA3C induces the upregulation of both cellular (CD21) and viral (LMP1) gene expression (7), represses the Cp promoter (331) and might interact with pRb to promote transformation (318). Expression of EBNA3B correlates with the upregulation of vimentin and CD40 and downregulation of CD77 (365). The gene expression pattern, which is characterized by the expression nine viral latent genes under the control of EBNA2, is called growth program latency type III) (411, 414). Together with EBNA-LP, EBNA2 is involved in G0-G1 transition (366), allowing the growth program activated B cells to become proliferating blasts and differentiate into resting memory B cell through the process of germinal centre reaction (248). Expression of EBNA3A and B will downregulate EBNA2 through competition for the binding of RBP-Jc protein (341). EBNA3C will activate G1 progression within the cell cycle. This will activate B cells to become growth-activated B cell blasts not requiring further external signals. EBNA1 is co-transcribed via Cp-transcription and provides essential signals for EBV genome maintenance in dividing cells (410). The only type of EBV-infected cells that express the growth program is the infected naïve B cell in the Waldeyer’s ring in healthy carriers (18, 168).
Freshly EBV transformed B cells will migrate to the follicle, and subsequently downregulate Cp-driven EBNA2 and 3A-C, and switch to Qp-driven EBNA1, and EBNA2 independent expression of LMP1 and LMP2A gene expression, known as default program (latency type II) (Table 1) (19, 410, 411). The default program involves the expression of three latent membrane proteins (LMP), LMP1, LMP2A and LMP2B. These proteins are able to produce signals of the germinal center by mimicking T cell help and antigen-specific B cell receptor triggering to ensure the infected B cell to undergo germinal center reaction, proliferation, and become memory B cells without external signals (51, 207, 410).

The latently infected memory cells shut down the expression of viral protein by promoter methylation, except Qp-driven EBNA1 and the non-coding transcripts EBER1,2 and BARTs. Thus, the viral genome will be maintained in normal memory B cells (168) under the true latency program (latency I, EBNA1 only) (Table 1), and circulate primarily between peripheral blood and Waldeyer’s ring (222).

Finally, latent EBV in memory B cells can be reactivated when B-cells become antibody producing plasma cells via antigen-stimulation and replicates at mucosal sites of the Waldeyer’s ring in the nasopharynx yielding infectious virus that spreads in saliva to new hosts (414).

### Table 1. Five Transcription Program Used by EBV to Establish and Maintain Infection

<table>
<thead>
<tr>
<th>Type of Infected B cells*</th>
<th>Program</th>
<th>Genes Expressed</th>
<th>Function of the Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinal-center cells</td>
<td>Growth</td>
<td>EBNA through Qp, LMP1, LMP2A and LMP2B</td>
<td>Activates B cells</td>
</tr>
<tr>
<td></td>
<td>(latency I)</td>
<td>EBNA1</td>
<td></td>
</tr>
<tr>
<td>Peripher/ blood</td>
<td>Default</td>
<td>EBNA1, LMP1, and LMP2A</td>
<td>Differentiates activated B cells into memory cells</td>
</tr>
<tr>
<td></td>
<td>(latency II)</td>
<td>LMP2A, and</td>
<td></td>
</tr>
<tr>
<td>Memory cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EBNA, only</td>
<td>EBNA1</td>
<td>Allows viral DNA to latently-program cell to divide</td>
</tr>
<tr>
<td></td>
<td>LMP2A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma cell</td>
<td>Lytic</td>
<td>All lytic genes</td>
<td>Replicates virus in plasma cell</td>
</tr>
</tbody>
</table>

*Except where indicated, the types of cell are primarily restricted to the lymphoid tissue of Waldeyer’s ring. Modified from Thorley-Lawson and Gross (414)
3.c. Latent Replication

During latent infection, the host cell is induced to proliferate and the EBV genomes are maintained in the cell nucleus at a stable copy number as double stranded (ds), circular DNA episomes (460). These episomes undergo DNA replication semi-conservatively once per cell cycle only during S phase and are efficiently partitioned to the daughter cell during mitosis, alike to cellular chromosomes (2, 462, 463). Two viral components are required for the replication of the EBV genome, the oriP region on EBV DNA, which acts in cis, and EBNA1 protein, which acts in trans (228, 463). EBNA1 is the only viral protein required for the replication of EBV latently-infected cells and accordingly it is found in all EBV associated malignancies (233). EBNA1 is essential for the maintenance and replication of the episomal EBV genome, through sequence-specific binding to the plasmid origin of viral replication, oriP (254, 463). oriP contains two functional elements, the dyad symmetry element (DS) and the family of repeat element (FR) (20, 334). The DS contains four EBNA1 binding sites and appears to be the initiation site of DNA replication (127, 332, 455). The FR contains 20 EBNA1 binding sites (332) and is involved in three viral processes. It activates DNA replication from the DS, enhances transcription from several promoters, and mediates the segregation of EBV episomes and oriP plasmids in dividing cells (128, 254, 334). EBNA1 has no helicase activity (122), which strongly indicates that the viral episomal replication depends on cellular replication machinery. It has been demonstrated that replication of oriP-containing plasmids is dependent on cellular initiation factors (104). The carboxy-terminal DNA-binding domain (DBD; aa451 to 640) of EBNA1 bind to two clusters of binding sites within oriP (FR and DS) (461). The recruitment of cellular replication system to DS requires the amino terminus of EBNA1 to contain glycine-arginine rich domains A (aa 33 to 89) and B (aa 328 to 378) (261, 356), which have similar function as AT hooks to associate with mitotic chromosomes and ensure stable replication and segregation of the oriP (357).

The EBNA1 AT-hook domains link the episome to mitotic chromosomes via heterotypic interaction with cellular protein p32/TAP with the A domain (65, 447), and EBP2 to B domain (364). The oriP plasmids can thus “piggyback” on the chromosomes, using the chromosome’s centromere for its own partitioning into daughter cells (389).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA1</td>
<td>• Episome maintenance (binds oriP of latent replication) (228)</td>
</tr>
<tr>
<td></td>
<td>• GW inhibits processing of EBNA1 through proteasomes, precluding presentation of antigenic peptides with MHC-I at the cell surface, which avoiding CDE7 cells detection (337)</td>
</tr>
<tr>
<td></td>
<td>• Transcription factors, binds RBP-Jx, activates viral genes (LMP-1 and LMP2A) (1, 443, 468) and cellular genes (CD21, c-fos, c-Myc, CD31) (79, 447)</td>
</tr>
<tr>
<td>EBNA1A</td>
<td>• Like other EBNA1s balances EBNA2 effect on RBP-Jx, bind C1BP</td>
</tr>
<tr>
<td>EBNA1B</td>
<td>• Transcriptional regulator</td>
</tr>
<tr>
<td></td>
<td>• Essential for EBV-mediated transformation of primary B lymphocytes and interacts with RBP-Jx (184, 540)</td>
</tr>
<tr>
<td></td>
<td>• Promotes LMP-1 expression in the presence of EBNA2 (362, 467)</td>
</tr>
<tr>
<td>EBNA1C</td>
<td>• Key role in upregulating gene expression critical for lymphoblastoid cell growth (359)</td>
</tr>
<tr>
<td></td>
<td>• Augments the ability of EBNA2 to transactivate LMP-1 (159)</td>
</tr>
<tr>
<td></td>
<td>• Activates NF-kB to promote cell growth and survival (208)</td>
</tr>
<tr>
<td></td>
<td>• Mimics signaling through CD40, a B cell activation and differentiation receptor (302, 427)</td>
</tr>
<tr>
<td></td>
<td>• Promotes B cell lymphoma and skin hyperplasia in transgenic mice expressing LMP-1 (245)</td>
</tr>
<tr>
<td></td>
<td>• Contributes to immune evasion by IL10 induction and interference with 1-cell activation (109, 110, 121, 218)</td>
</tr>
<tr>
<td>LMP1</td>
<td>• Inhibitor of BCR signalling by sequestering tyrosine kinases through interaction with its immunoreceptor tyrosine-based activation motifs (279)</td>
</tr>
<tr>
<td></td>
<td>• Blocking viral lytic cycle by antigenic stimulation; provide B cell survival signals through activation of the same sequestered tyrosine kinases in an antigen- and B cell receptor-independent manner (274)</td>
</tr>
<tr>
<td>LMP2A</td>
<td>• Function unclear</td>
</tr>
<tr>
<td>EBER RNAs</td>
<td>• play a role in oncogenesis (168)</td>
</tr>
<tr>
<td></td>
<td>• play a role in countering the antiviral effects of interferon and PKR activation in infected cells (74)</td>
</tr>
<tr>
<td></td>
<td>• Induce IL10 expression, thus contributing to immune escape</td>
</tr>
<tr>
<td>BARTs</td>
<td>• Encoding microRNA that can modulate gene expression by forming imperfect or perfect complementary duplexes with their target mRNAs (22, 84)</td>
</tr>
</tbody>
</table>
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3.d. EBV-encoded non-translated RNA

3.d.1. EBV encoded RNAs (EBERs and 2)

EBER1 and 2 are small noncoding RNAs (167 and 172 nucleotide) that are produced in large numbers in EBV infected cells, at greater than 5 x 10<sup>6</sup> copies per cell (235, 396). EBER RNAs are expressed from their own promoter at 36 hr after infection of primary B lymphocytes in vitro, following other latent gene products (5, 396). The roles of EBERs in viral infection were unknown for long time, but recently several reports demonstrated that EBERs play a role in oncogenesis (398). A speculation about the role of EBERs in the normal EBV life cycle was proposed by Swaminathan (396).

EBERs are similar in size and organization to equally abundant small adenovirus, VAI and VAI, and are also transcribed by polymerase III and bind to cellular La protein. These similarities may indicate analogous function in infected cells. VA RNAs play an important role for adenovirus replication by rescuing cells from the shutdown of protein translation mediated by cellular kinase PKR, which induced by interferon and activated dsRNAs produced during replication of many viruses (133, 171). Mutant adenovirus with EBERs replacing VA RNAs, showed that EBER can functionally substitute the VA RNAs (30, 31). This evidence showed that EBER play a role in countering the antiviral effects of interferon and PKR activation in infected cells. In vitro studies demonstrated that EBER could directly bind PKR and inhibits its activity (74). PKR activation induces apoptosis by multiple mechanism including eIF2-phosphorylation and therefore PKR inhibition by EBERs gives protective effects against apoptosis (292).

Due to its expression in large amount in latently infected cells and all EBV-associated tumors, EBERs had been considered to have an important role in maintenance of transformation. Although in 1990s it was demonstrated that EBERs are not essential for transformation of B lymphocytes in vitro (397), recent findings indicated that EBERs do provide an advantage in viral transforming efficiency (459).

3.d.2. BamHI-A-rightward transcripts (BARTs)

The EBV BARTs were first identified in NPC as multispaced transcripts (370) and were later found in all EBV latency programs, including EBV infected B cells in healthy carriers and a wide range of EBV associated cancers. BART expression is kw in B lymphocytes and high in epithelial tissues (50, 64, 137, 189, 371), suggesting that BARTs may be particularly important in epithelial malignancies. The finding of BARTs encoded miRNAs revealed the function of these transcript in modulating gene expression by forming imperfect or perfect complementary duplexes with their target mRNAs (22, 84). BARTs furthermore contain 3 ORF domains A73, RPMS1 and RK-BARF0 that may encode putative proteins (371). Although interesting functions have been described for these ORFs in artificial expression systems (415), the current consensus is that these ORFs are not translated into proteins in EBV infected cells (94, 415, 432). Therefore the main function of BARTs is considered being the generation of viral miRNAs.

3.d.3. EBV MicroRNA

MicroRNAs (miRNAs) are small noncoding RNAs, generally 21-24 nt in length, that can posttranscriptionally downregulate the expression of miRNAs bearing complementary target sequences (22). miRNAs are related to small interfering RNAs, approximately 22-nucleotide noncoding RNAs that are generated by cleavage of double-stranded RNAs by the RNase III enzyme Dicer (22).

Viral miRNAs were first discovered in EBV (325), and now have been described for Kaposi’s sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (HCMV), mouse gammaherpesvirus 68 (MHV 68), herpes simplex virus I (HSV1) and simian virus 40 (SV40) (49, 145, 324, 350, 391). Five miRNAs were initially found in EBV, clustered into two genomic regions. One cluster of three miRNAs, miR-BHRF1-1, -2, -3 is proximal to the BHRF1 (Bam HI Rightward Frame 1) ORF, and one cluster of miRNAs, miR-BART-1 and -2 is located within the BART (Bam HI A rightward transcript) gene (325). Recent study has identified an additional set of 18 EBV miRNAs, 13 of which map to ~ 12 kb intronic region within the BART gene, which is deleted in the B95.8 strain (50).

There are some possibilities that viral miRNAs could directly act in oncogenesis. For instance, predicted targets of the EBV-encoded miR-BHRF1-1 include the tumor suppressor P53, in which mutations are found in 50% of all cancers (374). On the other hand, miR-BART1, another EBV-encoded miRNA that is compressed with mIR-BHRF1-1 in latently infected B cell lymphomas, is predicted to target Bcl-2, an oncogene with function to block apoptotic cell death (80).

The EBV BARTs produce two clusters of miRNAs, 12 in cluster 1 and 15 in cluster 2. miR-BART2 is an individual miRNA. BART1 Cluster 1 miRNAs target the LMP1 3’ UTR and negatively regulate LMP1 protein expression in NPC at posttranscriptional levels (250). The miR-BART2 serves as inhibitor for viral DNA replication through degradation of the mRNA for the viral DNA polymerase BALK5. BALK5 transcripts decrease in abundance when miR-BART2 is overexpressed, miR-BART2 is transcribed antisense to the BALK5 transcript and perfectly complementary to the BALK5 3’ UTR (23). A quantitative PCR was recently described to measure miRNA level in different EBV linked diseases (81).

4. Lytic replication

Reactivation from latency to lytic replication is essential for transmission of the virus from host to host (280). Lytic replication differs from the latent amplification state and is initiated within oriLyt (150). In contrast to latent replication, the viral lytic replication process is dependent on EBV-encoded proteins (119). EBV latent B cells can be activated to enter lytic replication by surface immunoglobulin cross-linking, cytokines or chemicals activating the transcription and function of two immediate early transactivators, Zta (BZLF1, Z, ZEBRA) and Rta (BRLF1, R) (253).

During the induction, like other herpesviruses, EBV gene expression follows a temporal and sequential order that is divided into three phases,
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To this site. There are seven viral encoded replication proteins, which essential for ZEBRA to activate E and L promoters. ZEBRA and Rta then function synergistically to lytic inducers and mediate ZEBRA transcription. ZEBRA binds to its own promoter and activates Rta promoter. ZEBRA and Rta then function synergistically to activate E and L promoters.

EBV genomic replication is initiated by sequence specific binding of ZEBRA to oriLyt followed by the recruitment of the cellular replication machinery to this site. There are seven viral encoded replication proteins, which essential for oriLyt-dependent DNA replication, the BZLF1 (oriLyt binding protein), BALF5 (DNA polymerase), BALF2 (single-stranded DNA binding protein), BMRF1 (polymerase accessory factor), BBLF2/3 (primase associated factor), BBLF4 (helicase) and BSLF1 (primase). During the induction, these replication proteins are expressed and clustered to newly replicated EBV DNA at discrete sites in the nuclei, termed replication compartment. The EBV core replication proteins are linked to each other through a series of contacts and with the viral oriLyt binding protein ZEBRA, which in turn binds to multiple sites within oriLyt. These proteins together have potential to open up the duplex DNA in the origin region and and synthesise RNA primers. The BALF5 Pol catalytic and BMRF1 Pol accessory subunits form a heterodimer to function as the Pol holoenzyme, which interact with BBLF4/BSLF1/BBLF2/3 complex. This interaction may play an important role in bringing the polymerase into the prepriming complex to initiate DNA synthesis by reducing its affinity for BZLF1, allowing the polymerase-helicase-primase complex to migrate away from oriLyt to the replication forks. The six viral replication proteins appear to work at all the replication fork in forming the replication machinery.

The final step in lytic cycle is initiated by the production of 100 to 1000 copies of viral DNA, which is followed by the transcription and translation of RNAs encoding the late viral protein including structural genes as BFRF1, BFRF2/3, BLRF1/2/3 and RLF1 (major capsid protein), BHRF1 (VCA-p18, small capsid protein), BdRF1 (VCA-p40, scaffold protein) and virion envelopment i.e. BBLF1 (encoding pp 350/220), BLF2 (encoding pp85), BZLF2 (encoding pp42), and BKRF2 (encoding pp25), anti-apoptotic genes as BHRF1 and BLRF1, and also immunomodulation factors such as BCRF1 (vIL-10). This finally results in the formation of progeny virus. The expression of structural proteins (VCA, MA) depend on EA expression, in particular involving BALF5 the virus encoded DNA polymerase, which is a key enzyme during EBV replication. Inhibition of this enzyme by chemical (antiviral) agents such as phosphonoacetic acid (PAA), phosphonoformic acid (PFA), as well as some tannins from Eugenia uniflora will lead to block viral DNA synthesis, production of structural proteins and virus assembly. Another (theurapeutic) way to interfere with virus replication in vivo and in vivo is the use of acyclovir or (val) gancyclovir. Both are nucleoside analogues that, after specific phosphorylation by virus encoded thymidine kinase (TK) enzymes, are incorporated into viral and host DNA genomes, leading to DNA-chain termination, and thus blocking replication.

5. EBV associated diseases

5.1. Infectious mononucleosis (IM)

IM is the clinical manifestation forms of a primary EBV infection. IM is benign disorder with expansion of the paracortex of lymphoid tissues. The proliferating cell populations are EBV infected polyclonal B blast, accompanied by the growth of activated T-cells. IM shows type III EBV latency pattern, even though the expression in a single cell is more heterogeneous. Most cells are EBER positive but do not express EBNA2 or LMP1 (type I latency), whereas some large
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Lymphoma in immunocompromised patients

5.b. Post transplant lymphoproliferative diseases (PTLDs)

The T-cell immunosuppressive therapy given to patients after transplantation is associated with an increased risk for EBV-driven lymphoproliferative diseases, which progress to malignant lymphoma if not treated properly at early stage. These post-transplantation lymphoproliferative diseases (PTLDs) are almost always of B-cell origin and EBV positive. The suppression of T cell responses allows uncontrolled proliferation of EBV-transformed B cells. PTLDs can present as polyclonal expansion or monoclonality. Some EBV-transformed B cell can acquire additional transforming events, which lead to the outgrowth of a malignant B-cell clone from an initially polyclonal or oligoclonal B cell expansion. The EBV-positive B cells in PTLDs overall have a latency III profile, but a more diverse and restricted gene-expression pattern is observed at the single cell level.

The expression of the full spectrum of latent EBV genes in PTLD indicates an important role for EBV in driving proliferation of infected B cells. In many cases of PTLD, the reduction of immunosuppression leads to the elimination of the EBV-positive B cells, at least during early stage of post transplant lymphoma development. Diagnostic monitoring of increased EBV-DNA load is crucial for early and successful intervention and prevention of PTLD. In recent years the use of Rituximab (anti-CD20) has provided the new treatment option for PTLD, but is not without danger.

5.b. AIDS-related lymphomas (ARLs)

A high incidence of malignant lymphoma is seen in highly immunocompromised AIDS patients. AIDS related lymphomas (ARLs) are mostly of B cell origin and occured in EBV positive patients. Like PTLDs, most ARLs contain monoclonal EBV genomes. It is considered that chronic inflammation and antigen triggering is a causative factor in the outgrowth of ARLs.

5.b.3. Oral hairy leukoplakia (OHL)

Oral hairy leukoplakia (OHL) is a benign epithelial disorder with a white epithelial lesion of the tongue, which can be found in immunocompromized individuals. EBV-DNA was first detected in this lesion in 1985, with localization restricted to the superficial epithelial cells (142). Expression of viral lytic cycle antigens has been shown in OHL and abundant production of virus particles has been demonstrated using electronmicroscopy, indicating that epithelial cells can support viral replication (224, 464). The detection of linear EBV genomes in the absence of episomal DNA (136, 299), the exclusive localization of the virus in upper epithelial cells, and the sensitivity of the lesion to acyclovir treatment (335), support this notion. Expression of latent proteins in OHL has also been reported but EBER RNAs are expressed at very low levels in OHL (136). A study by Webster-Cyraque indicates that LMP1 may contribute to the proliferation and apoptosis resistance of OHL epithelial cells (449).

5.c. Lymphomas in immunocompetent patients

5.c.1. Burkitt’s Lymphoma (BL)

Burkitt lymphoma (BL) was first described in children in equatorial Africa and New Guinea. Most of the endemic BL are EBV positive. The tumours also occurs as a rare, sporadic lymphoma outside of the endemic areas, with an EBV association in 20-30% of cases (338). The first indication for a viral etiology was the climate-dependent distribution of the disease. BL had a high incidence in regions where malaria prevalent and EBV infection was present in approximately 95% of the childhood population. However in Europe and in the USA, the EBV association is only 10-20% and this sporadic form of BL mainly occurs in adults. This difference has been ascribed to the early age of infection in Africa compared with industrialized nations (257). Plasmodium falciparum erythrocyte membrane protein 1 (PFEMP1) was recently shown to trigger growth activation of B-cell, and providing immunosuppression, thus setting the stage for unusual EBV B cells activation (66, 85, 108, 342, 412). In the endemic BL the EBV latent gene expression is restricted to the latency type I. EBNA1 are expressed from the Qp promoters (352). In contrast, a study from Malawi showed that BL expressed high proportion of immediate early (BFLF1) and early (IR2 and IR4) genes, a putative viral oncogene (BARF1), CST (BART) antisense transcripts and viral bcl-2
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LMP2, with variable levels of LMP1 (187), expression is restricted to the latency type I/II, with expression of EBNA1 and common in South-East Asia (151). Some of these tumours are CD3 CD56 T-cell lymphomas, but most are CD3 CD56 tumours of NK-cell origin. The viral gene usually present as an erosive lesion (lethal midline granuloma), which is most highly aggressive (151, 185, 208). An example is extranodal lymphoma that (35). These can arise as consequence of chronic active EBV infection and are functionally activated cytotoxic phenotype, expressing granzyme B and perforin than B cells do (118). Interesting EBV+ T-/NK cell lymphomas always have a unclear, since T-cell normally express EBV receptor CD21 at a rate 10-fold lower than B cells do (111). Interesting EBV+ T/NK cell lymphomas always have a functionally activated cytotoxic phenotype, expressing granzyme B and perforin (35). These can arise as consequence of chronic active EBV infection and are highly aggressive (151, 185, 208). An example is extranodal lymphoma that usually present as an erosive lesion (lethal midline granuloma), which is most common in South-East Asia (151). Some of these tumours are CD3 CD56 T-cell lymphomas, but most are CD3 CD56 tumours of NK-cell origin. The viral gene expression is restricted to the latency type I/II, with expression of EBNA1 and LMP2, with variable levels of LMP1 (187).

5.c.2. Hodgkin lymphoma/Hodgkin’s Disease (HD)

Hodgkin’s disease (HD) is characterised by the disruption of normal lymph node architecture and the presence of a minority (1%) of malignant Hodgkin and Reed-Sternberg (HRS) cells in a background of non-neoplastic cell populations comprising T- and B-lymphocytes and other cell types (154, 155). Based on differences in the histology and immunophenotype of HRS cells, the classical nodular sclerosing form of HD, accounting from 95% of cases, is distinguished from a lymphocyte-predominant form (154). HRS cells of lymphocyte-predominant HD are always EBV negative, whereas EBV is found in ~40% of cases of classical HD in the Western world. The association with EBV is higher in children with HD from Latin America, and in HIV-infected individuals, where most of cases are EBV positive (178). In EBV-positive cases, three EBV proteins are expressed, EBNA1, LMP1 and LMP2A (latency type I) (188, 216).

The expression of EBV genes in HRS cells indicates a role for EBV in rescue and transformation of pre-apoptotic germinal centre-B cells. The main survival signals for germinal centre - B cells are mediated through the BCR and through the stimulation of CD40 (249). These signals can be replaced by LMP2A and LMP1 respectively, both of which are expressed in EBV-positive HRS cells (161, 298, 312, 439), allowing cells with disadvantageous mutations to survive as germinal-centre B cells (216). EBV+ HRS cells also secrete multiple cytokines and other factors contributing to autocrine growth and immunomodulation (163, 188, 320, 326).

5.c.3. Extranodal T-cell and natural killer (NK) cell lymphomas

EBV is also associated with rare T-cell and natural killer (NK)-cell lymphomas (151, 185). How the virus accesses these cell lineages in vivo is still unclear, since T-cell normally express EBV receptor CD21 at a rate 10-fold lower than B cells do (118). Interestingly EBV+ T/NK cell lymphomas always have a functionally activated cytotoxic phenotype, expressing granzyme B and perforin (35). These can arise as consequence of chronic active EBV infection and are highly aggressive (151, 185, 208). An example is extranodal lymphoma that usually present as an erosive lesion (lethal midline granuloma), which is most common in South-East Asia (151). Some of these tumours are CD3 CD56 T-cell lymphomas, but most are CD3 CD56 tumours of NK-cell origin. The viral gene expression is restricted to the latency type I/II, with expression of EBNA1 and LMP2, with variable levels of LMP1 (187).

5.c.4. Nodal T cell lymphomas

The angioimmunoblastic lymphadenopathy (AILD) is a common human T-cell lymphoma, in which EBV infected cells are often observed (12, 451). EBV can be detected in up to 30% of the nodal AILD-like lymphomas. These AILD-like lymphomas were shown to contain a clonal T-cell population (451), but EBV in these lymphomas is not present in the neoplastic T-cells, but predominantly in B-cells which are usually polyclonal or oligoclonal (369) and rarely become clonal in a later stage (268). This indicates that the proliferation of EBV-infected B-cells may be a secondary event and that EBV is not causally related to the pathogenesis of these lymphomas. The presence of EBV in B-cells in these lymphomas suggests that the expanded meshworks of follicular dendritic cells (FDCs), which are a hallmark of these lymphomas, stimulate proliferation of (EBV-infected) B blasts (276).

5.d. Epithelial and Other malignancies

5.d.1. Gastric Carcinoma (GC)

EBV infection in gastric carcinoma (GC) was first reported by Burke et al in 1990 using PCR (46). More than 90,000 patients worldwide are estimated to develop GC annually in association with EBV (approximately 10% of total gastric cancer) (362, 418, 430). In terms of histological feature, EBV-associated GC occurs in two forms, the first one is lymphoepithelioma-like GC, which has a typical histology of poorly differentiated carcinoma with dense of infiltration of lymphocytes, and the second one is the ordinary type of GC (448). More than 80% of lymphoepithelioma-like GC is associated with EBV (361). The clinical feature of EBV-associated GC are located in the Gardia (upper part of stomach), less lymphnode metastasis, male predominance and relatively younger age compared to EBV-negative GC (60, 433). EBV-associated GC belongs to latency type I, with additional expression of LMP2A and BARF1, but no LMP1 (175, 390, 472). EBV positive GC have a better prognosis than EBV negative ones, possibly due to anti-tumor immune responses triggered by EBV genes in the tumor cells, not having immune escape function imposed by LMP1 (278, 434).

As in NPC, the precise role of EBV in the pathogenesis of gastric carcinoma remains to be determined, but the absence of EBV infection in pre-malignant gastric lesions supports the suggestion that viral infection is a relatively late event in gastric carcinoma (473). EBV-associated GC shows resistance to apoptosis and the production of immunomodulator molecules. Recently, global and non-random CpG island methylation of the promoter region of many cancer-related genes has been demonstrated in GC, correlating with their decreased expression such as p16, INK4A, p73 and E-chaderin. This abnormality is accompanied by methylation of EBV genome, suggesting a process of virus driven hypermethylation in the development of neoplastic cells (428).

5.d.2. Nasopharyngeal carcinoma (NPC)

NPC is a malignant disease that is classified into three types. The
The symptoms in children are generally similar to those in adults (359). Approximately 20% of NPC cases are of the type II, while type III constitutes 55% of the tumours seen in patients with NPC. In endemic areas, undifferentiated type III comprises the vast majority (80%) of NPC, while keratinized squamous cell carcinoma is less common (269). The type III NPC is virtually 100% associated with clonal Epstein-Barr virus infection of all tumor cells that have a characteristic viral gene expression profile, known as Latency-II (default program). WHO type II NPC also is frequently (60-80%) EBV associated. WHO type I shows lower (0-20%) EBV linkage and is more associated with alcohol abuse and betel nut chewing.

NPC is uncommon disease in most countries, with incidence for both sexes is less than one per 100,000 population (319). However, this disease occurs with much greater frequency in southern China, northern Africa, and Alaska (300). The Inuits Alaska and ethnic Chinese people living in Guangdong province are especially prone to the disease. The reported incidence of NPC among men and women in Hongkong is 20-30 per 100,000 and 15-20 per 100,000, respectively (319). The incidence of NPC remains high among Chinese people who have immigrated to Asia or North America, but is lower among Chinese people born in North America than in those born in southern China (43, 105). This finding suggests that genetic, ethnic and environmental factors may have a role in the cause of the disease (450). Recent data suggest that EBV positive NPC is a common tumor also in non-Chinese populations in SE-Asia (103, 372). However poor quality of local cancer registry data collections precludes more thorough analysis.

Patients with NPC can present with one or more symptoms of four categories, which consist of (1) presence of tumour mass in the nasopharynx (epistaxis, nasal obstruction, and discharge); (2) dysfunction of the Eustachian tube, associated with the lateroposterior extension of the tumour to the nasopharyngeal space (tinnitus and deafness); (3) skull-base erosion pathy of the fifth and sixth cranial nerves, associated with the superior extension of the tumour (headache, diplopia, facial pain, and numbness); and (4) neck masses, usually appearing first in the upper neck. Other symptoms such as anorexia and weight loss are uncommon, when such symptoms are present, distant metastasis should be suspected. Unfortunately, due to the non-specific nature of the nasal and aural symptoms and the difficulty of making a clinical examination, most of the NPC patients are diagnosed when the tumour has reached an advance stage (stages III and IV) (450).

A retrospective study to 4760 patients showed the symptoms as neck mass (76%), nasal obstruction (73%), aural dysfunction (62%), headache (35%), diplopia (11%), facial numbness (8%), weight loss (7%), and trismus (3%). The physical sign present at diagnosis were enlarged neck (75%) and cranial nerve palsy (20%) (226, 309). The symptoms in children are generally similar to those reported in adults (359).

Patients with the symptoms should be clinically assessed for the physical signs. An EBV serological test will give further justification and would justify an endoscopic examination and biopsy from the nasopharynx. If the clinical suspicion is high, even though the tumour is not visualized with endoscopic examination, cross-sectional imaging by CT and MRI should be undertaken. A definitive diagnosis of NPC needs a positive biopsy taken from the tumour in the nasopharynx (450).

The standard treatment for NPC is radiotherapy, but it can cause adverse complication because the location of the tumour at the base of skull, closely surrounded by and in close proximity to radiation dose-limiting organs, including the brain stem, spinal cord, pituitary-hypothalamic axis, temporal lobes, eyes, middle and inner ears, and parotid glands. Since NPC tends to infiltrate and spread towards these dose-limiting organs, they are even more difficult to protect. (450).

In radiotherapy a dose of 65-75 Gy is normally given to the primary tumour and 65-70 Gy to the involved neck nodes, whereas the dose for prophylactic treatment for node negative neck is 50-60 Gy. This treatment has successfully controlled T1 and T2 tumours in 75-90%of cases and T3 and T4 tumours in 50-75% of cases (72, 227, 450). Nodal control is achieved in 90% of N0 and N1 cases, but drops to 70% for N2 and N3 cases (72). Treatment schedule should be complied for success of the therapy. Interrupted or prolonged treatment reduces the benefits of radiotherapy (219). In combination with radiotherapy, the use of chemotherapy for the management of locoregional advanced cases have been reported for neoadjuvant, concurrent and adjuvant therapy or for combinations of these approaches (450). A study in 1997 (4) was the first study to show that the use of chemotherapy alongside radiotherapy improved overall survival compared with radiotherapy alone.

s.d.2.a NPC and EBV

NPC is the epithelial tumour most consistently associated with EBV, especially the WHO type III which is reflected by abnormal antibody profiles, increased circulating EBV DNA levels and by distinct EBV gene expression in the tumour cells (39, 40, 58, 114). Early pre-invasive hyperplastic nasopharyngeal lesions already carry clonal EBV, suggesting EBV infection is an early event in carcinogenesis, which contrast to EBV and GC (321, 473). The EBV association was initially suggested by the serological studies characterizing the antibody responses to EBV, which showed that patients with NPC had elevated IgG and IgA antibody titers to the EBV viral capsid antigen (VCA) and early antigen (EA) (157). The detection of EBV DNA and EBNA complex in NPC tumor cells was initially done by zur Hausen (1970) using in situ hybridization and the anticomplement immunofluorescence (ACIF) assay, respectively (474). NPC shows EBV latency type II, by showing several EBV genes consistently expressed in NPC biopsies, including EBERs, EBNA1, LMP1, LMP2A, and BARF1 (48, 98, 115, 137, 167, 193, 276). The early involvement of EBV infection in NPC development was recently further supported by observation that serological abnormalities precede NPC diagnosis by at least 2 years (180)
5.d.2.b. EBV based diagnosis and therapy for NPC

The strong etiological link between NPC and EBV allows laboratory tests for NPC to be based on EBV parameters, which improve the diagnosis, prediction, and prevention of the disease. Undifferentiated NPC (WHO type III) is nearly 100% EBV related as demonstrated by EBER in situ hybridization, which remains the gold standard for identifying latent infection (144).

Sero-epidemiological studies have indicated a close relation between EBV infection and NPC as revealed by elevated IgG and especially IgA to VCA, EA and EBNA complexes (157, 159). NPC patients particularly have elevated titers of IgA anti-EBV-antibodies, reflecting the tumor’s origin in the mucosa of the nasopharynx (100). Abnormal EBV serology is commonly used to support NPC diagnosis and provides an affordable approach for population screening to identify individuals with high NPC risk (294).

Quantitative EBV DNA measurement is essential for discriminating the low-level infection of healthy carriers from the high levels characteristic of EBV related disease (379, 381). Patients with active infection or EBV related cancer tend to have high levels of EBV DNA in the circulation. In some types of cancer EBV DNA may reside in the cell-free fraction of blood (plasma or serum), whereas in healthy carriers the virus is restricted to cellular compartment of the blood (144, 379, 381). Circulating free EBV DNA (in serum or plasma) has been reported in patients with NPC (290), but the levels are relatively low (100-2000 copies/ml plasma or blood) in significant subset of patients, therefore making accurate quantification and definite diagnosis difficult (381). Recent studies have shown that plasma EBV DNA is highly fragmented and derives from apoptotic DNA released from dying tumor cells in both NPC and HD (59). This is in agreement with the absence of BARF1 mRNA in the circulation of patients, and excludes presence of circulating intact NPC tumor cells (381).

The increased number of copies of EBV DNA in the blood during the initial phase of radiotherapy suggest that the viral DNA was released into circulation after tumor cell death by apoptosis (59, 251, 381). The quantity of free plasma EBV DNA is related to the stage of the disease in some studies, but not in all. The quantity of EBV DNA copies before and after treatment is significantly related to the rates of overall and disease-free survival (245). A study has reported that the (lower) levels of post treatment EBV DNA compared with pretreatment can be a good predictor of progression-free survival (57).

NPC cells are capable of immunologic processing for cytotoxic T-lymphocyte (CTL) recognition (200, 230), and on the basis of the success of adoptive EBV targeted CTL therapy for EBV-related PTL (387), the use of anti-EBV immunotherapy strategies has been receiving increased attention as a possible additional treatment to improve prognosis of advanced NPC (71, 76, 203, 243).
major role of NK cells in controlling B-cell transformation in vivo, is provided by experiment in T-depleted stem cell transplant recipient. In these patients EBV-driven lymphoproliferative disease (LPD) is most common in the first 3-6-months post transplant, by which time CD8 cell numbers have recovered but the patients remain profoundly T cell-deficient (303). Furthermore, NK cells could also play role in control in lytic infection, since the reduction of HLA I expression on the surface of lytically infected cells, which increases sensitivity to NK cells (315).

6.b. Adaptive immune response

6.b.1. Cellular responses: CD8 T cells

CD8 T cells (CTLs) are known to recognize short peptide epitopes derived from antigenic determinant in association with MHC class I molecules on the surface of appropriate antigen-presenting cells. Although synthetic peptides can directly prime target cells for lysis by CTLs, recognition of a native antigen requires intracellular processing which involves cleavage of the antigen into short peptides and their transport into the endoplasmic reticulum (ER), where they associate with MHC class I (256).

The role of CD3 + CD8 + CTL in controlling primary (53) and latent (337) EBV-infection is well established. CD8 T cell responses to EBV latent antigen are mainly directed to immunodominant epitopes derived from EBNA3A, 3B and 3C, and sometimes to other latent protein, but hardly ever to EBNA1 (36, 166, 197, 289). Endogenously expressed EBNA1 cannot be presented to CD8 T cells because an internal glycine-alanine repeat (GAR) domain protects the protein from proteasomal degradation to peptides suitable for presentation in MHC I (236, 237).

As observed in IM patients, 1-40% of all CD8 T cells responsive to individual epitopes are directed against EBV IE and E antigen. CD4 + and CD8 + T cell responses to latent cycle protein, with individual specificities, are 0.1-5% and mainly directed to immunodominant epitopes derived from EBNA3A, 3B and 3C family (166). The CD8 T cells in IM blood are perforin-positive, and rapidly die by apoptosis without antigen stimulation (54, 376, 377). They express low levels of anti-apoptotic proteins (Bcl-2 and Bcl-x) and high levels of pro-apoptotic proteins (Bax) (52, 400), together with activation marker (CD38) and cell cycle marker (Ki-67) (54, 55, 164, 165).

CD8 T cell memory was studied in asymptomatic EBV carriers with no IM history, showing lackness of activation marker CD38 or CD69 and express higher levels of CD4 + than seen in CD8 T cell during acute infection. From the CD8 T cell memory population, 0.2-2% are specific to lytic epitopes and 0.05-1% to latent epitopes (34). EBV lytic antigen-specific CD8 T cells were identified to be mainly responsive to IE proteins (BZLF1 and BRLF1) or E protein (BMLF1) (355, 376). In EBV infected individuals the CD8 T cell responses particularly to EBNA1 and LMP1 seems to be marginal, which may be an evolutionary achievement beneficial for EBV persistence (271). In NPC patients normal EBV-specific circulating CD4 + and CD8 T cell responses are detected in blood (246), but higher levels of T + cells are also apparent (223). Importantly, specific functional defects are detected in T cells obtained from fresh tumor biopsies indicative of local immune evasion mechanisms operated by EBV tumor cells in vivo (240).

6.b.2. Cellular responses: CD4 T cells

CD4 T cells recognize antigens via MHC II presentation. Extracellular pathogens and viral protein complexes that invade or are taken up into the cell are degraded into peptide in intracellular vesicle, which will fuse with vesicle containing MHC class II. The bound peptide is transported by MHC class II to the cell surface and presented to CD4 T cells (288). CTLs seem to play major role in eradicating virus-infected cells, but CD4 T cell responses to viral infections are critical role for the maintenance of virus-specific memory CTLs (267). Furthermore, CD4 T cells can exhibit a direct effector function against virus-infected or malignant cells that express MHC class II molecules (147, 305).

During the early stage of EBV infection the number of CD4 T cells is low compared to CD8 T cells (258). During primary infection, up to 2.7% of circulating effector/memory CD4 T cells are EBV specific (327). Even though the direct effector role of CD4 T cell in discussion, many lytic antigen-specific CD4 T cell are perforin-positive and cytotoxic, indicating their direct role in control of virus replicative lesions in vivo, especially HLA II positive target cell. CD4 T cells act by reducing EBV transformation efficiency through IFN-γ release, after being triggered by infected B-cells presenting antigen via MHC II upon processing of incoming virus (3).

In IM blood, few CD4 T cells reactive to EBV compared with CD8 T cells (11). Their specificity as detected using individual lytic and latent cycle antigens (166, 327, 454), mainly presenting IE ZEBRA (BZLF1) followed by EA protein (BMLF1) or to latent protein EBNA3A (327). In a recent study, the nuclear antigen (EBNA1) was found to be a main EBV latency antigen for CD4 T cell in healthy carriers (232, 286, 313). It seems that the hierarchy of immunodominance exists among the lytic cycle proteins for the CD4 T cell response, which is different from the CD8 hierarchy (figure 3) (166).

CD4 T cell responses against latent cycle antigens appear to be largely of IFN-γ producing Th1 type, but they are tenfold lower amount than CD8 T cells. The use of HLA class II-recognizing CD4 T cell may be therapeutically useful against endogenously expressing the relevant latent protein (286, 421).

More importantly, potentially, CD4 T helper cells can facilitate the expansion of CTL by direct costimulation through CD70/CD27, 4-1BB/4-1BB interaction and by preventing CTL activation-induced cell death (138, 191). However, additional subsets of CD4 T lymphocytes, known as regulatory T cells (Treg) can inhibit CTL responses, by producing significant amounts of IL-10 (263, 360).
Finally, CD4+ T helper cells are important for induction and maturation of antibody responses, providing CD40 ligand, IL-4 other cytokine to antigen triggered B-cells.

The quantitative changes of CD4+ and CD8+ T cell responses to EBV as from acute phase of IM through convalescence toward long-term asymptomatic virus carriernship can be seen in figure 4. These responses parallel quantitative changes in the level of virus replication in the throat and the numbers of latently infected B cells seen in the blood (166).
6.b.3. Humoral Immune Response

As mentioned previously, host immune responses are important both in limiting the primary infection and controlling the lifelong latency. Combination of EBV specific antibodies and cytotoxic T lymphocytes (CTL) responses against latent and lytic EBV gene product will achieve the control of primary EBV infection. Serological studies have shown that primary and latent infections are associated with different combination of antibody reactivities to lytic and latent antigen (338). Antibodies to major virus envelope glycoprotein gp350 have capacity both to neutralize viral infectivity (413), and to mediate antibody-dependent cellular cytotoxicity against cells in lytic replication (322).

Most infected individuals develop EBV-specific antibodies against viral capsid antigens (VCAs), early (pre DNA replication) antigens (EAs), and EB nuclear antigens (EBNAs). In primary infection such as infectious mononucleosis (IM), serological responses can be classified into heterophile antibody responses and responses against specific EBV antigens (29). Antibody responses during acute IM are directed primarily against lytic cycle antigen, particularly membrane antigen complex, EA and VCA (158, 160, 277, 423, 435), while antibody responses to EBNAs are delayed by several months (Figure 5).

Antibody responses in EBV related cancer patients, is typically directed against EA and IgG VCA with variable anti-EBNA titer, although most have high EBNA titers as well, as long as cellular immunity is not affected (56, 378, 380, 437). However, the results should be interpreted with care since similar patterns are possible in autoimmune disease and other reactive conditions. Moreover, serology is not reliable when the immune system is dysfunction, e.g. acquired immunodeficiency syndrome (AIDS) or allogeneic transplant patients including solid organ and marrow recipients. Unexpectedly, certain abnormal EBV serological patterns are a risk factor for developing EBV negative lymphoma, such as chronic lymphocytic leukemia, suggesting that atypical antibody response to EBV reflects a more general phenomenon and may underly development of other malignancies as well (95, 144).

EBV serology is important in NPC. Patients often have high IgA titers against lytic EBV proteins due to its tumor origin on the mucosal surface of nasopharynx (100, 144). Enhanced EBV lytic replication in nasopharyngeal epithelia is considered to precede or at least parallel NPC development, leading to aberrant infection of epithelial cells, thus predisposing to carcinoma development. The contribution of humoral immunity to protect from tumor growth is poorly studied and will be addressed in this thesis (Chapter 7).

6.c. Immune responses to latent EBV proteins

6.c.1. Immune responses to EBNA1

EBNA1 is the single EBV antigen expressed in all EBV associated malignancies, and has long been thought to go undetected by cell-mediated immune system. EBNA1 is invisible to CD8+ CTL, since its GAr domain prevents proteasome-dependent processing for presentation on MHC I. However, recent studies show that EBNA1 can be presented to both CD4+ and CD8+ T cells, making it a potential new target for immunotherapy of EBV related cancers (285). The EBNA1 protein can be processed and presented normally through the MHC class II processing pathway and elicits consistent CD4+ T cell immune response (32, 125, 199, 286, 301, 375). It was shown that immunization with dendritic cells (DC) loaded with EBNA1-P<br>peptide could elicit antigen-specific immunity, leading to significant inhibition of the growth EBNA1-expressing tumor cells (125).

Several mechanisms of CD4+ T cell mediated immunity have been proposed. Studies of EBNA1 specific CD4+ T cell lines established from healthy human donors have shown that some CD4+ Th1 cells can directly kill Burkitt's lymphoma (BL) cells in an 18-hour "Cr release assay (313), which suggest that CD4+ T cells might inhibit EBV-infected cells through cytotoxicity mediated by perforin or Fas ligand expressed by CD4+ effector cells (125, 429). However, the experiment using EBNA1-P<br>peptide activated T cell did not show any cytotoxic activity. Therefore, CD4+ T cells might indirectly kill target cells through the production of cytokines, such as IFN-γ, which have inhibitory activity in EBV-induced cell growth (13).

A new hypothesis in EBNA1 specific CD8+ T cell recognition suggest that...
EBNA1-derived CD8+ T cell epitopes are not derived from full-length EBNA1 but from proteins that are prematurely truncated during translation or mal-folded after translation (defective ribosomal products or DRiPs), and these DRiPs are degraded by proteasomes for MHC class I presentation (285, 353).

EBNA proteins including EBNA1 are the first EBV-induced proteins expressed in an infected cell. However, anti-EBNA1 antibodies are not present during primary infection, but appear few months after infection and persist for life (9, 408). This early absence of anti-EBNA1 antibodies is thought to be due to late emergence or defective T-cell function (28, 56, 265, 438). Most of EBV healthy carriers show high IgG reactivity to EBNA1 (114).

6.c.2. Immune Response to LMP1

LMP1 can be a target for CTL recognition and LMP1-specific MHC class I-restricted epitopes have been reported, but, in fact, LMP1 is a subdominant target for CTL responses compared to other EBV latent and lytic antigen (166, 194, 198, 271). A previous study found that first transmembrane domain of LMP1 contains the sequence LALLFWL, which is highly homologous to immunosuppressive domain of retrovirus encoded transmembrane protein p15E (LULULFL) (73, 396). This short sequence proved to have strong inhibitory effects on T cell proliferation and NK cytotoxicity in vitro (110), and modulates expression of immunosuppressive cytokine (IL-10 induction and IL-2 & IL-12 inhibition) (153, 263). In addition LMP1 is secreted in association with exosomes. These small MHC-II rich vesicles are directly and strongly immunosuppressive when incorporating LMP1 (121). This effect is directly mediated by full length LMP1 transfered to the T-cell, but not by LMP1 deleted LALLFWL (192). In the case of latency type II malignancies, when LMP1 is expressed, such as in EBV positive Hodgkin’s Diseases tumor cells, LMP1 has direct role in local immunosuppression. It was demonstrate that EBV positive HRS cells expressing LMP1 abundantly produce IL-10, which can suppress CD8+ T cell responses (109).

Several studies reported that LMP1 derived peptides were recognized by human CD4+ T cell (101, 213, 232). Kobayashi show that LMP1 antigen is processed and presented to CD4+ T cells in the context of HLA class II molecules through the endogenous (direct presentation by transformed cells) or the exogenous (antigen captured and processed by conventional APC) pathways (213). Antigen presenting cells (APC) presenting LMP1 protein or synthetic peptides from LMP1 stimulated CD4+ T cells from EBV-seropositive donors to produce significant amounts of IL-10 and to inhibit a variety of T cell responses (263). However, Kobayashi showed that one of LMP1 peptide (LMP1_1-20)-specific CD4+ T cells, although not tested for IL-10 production, show to secrete abundant of IFN-γ in response to antigen stimulation, indicating that these cells are not typical TΦ lymphocytes (213). These findings show that much research is still needed to understand the role of LMP1 in escape of host immune responses.

Little is known about the humoral immune responses to LMP1, but it has been detected in subgroups of patients with EBV associated diseases, such as NPC, HD, IM, BL patients using several approaches and different antigen protein preparations. The result of those studies indicated that LMP1 is a protein with a low immunogenicity for the humoral immune response in humans (63, 272, 273, 262, 345).

6.c.3. Immune response to LMP2

LMP2 is one of the few EBV-latent cycle proteins expressed in malignancies such as HD and NPC. LMP2 can serve as a CD8 T cell target (166, 225, 231). One of major EBV specific CTL response in healthy virus carriers is directed towards LMP2A (196). It has been demonstrated that LMP2A can be processed independently of TAP, furthermore tumor cells with down regulated TAP expression can be efficiently recognized by virus-specific T cell following infection with recombinant vaccinia virus encoding this protein. This indicates that LMP2A is a potential target in the immune control of EBV-associated malignancies such as HD and NPC (48, 97), and forms an obvious target for vaccination and immunotherapy.

CD4+ T cells reactivities to LMP2 have been considered to have additional therapeutic benefits, either through their helper or direct effector function (148, 232, 386). A recent study demonstrated the CD4+ T cell clones reactive to LMP2 display Th1-like reactivity secreting IFN-γ, TNF-α and IL-2 in absence of typical Th2-like cytokines such as IL-10 and IL-4 (148).

Few studies addressed in detail the role of LMP2 protein as targets for humoral immune responses. Antibodies to LMP2A/2B were detected at low titer in about 40-60% of NPC sera from different ethnicity (234, 272).

6.c.4. Immune response to BARF1

BARF1 protein is frequently expressed in undifferentiated NPC (98, 358). It may be also secreted after signal sequence (residue 1-20) cleavage (349, 388). Moreover, BARF1 protein may function as soluble receptor (scavenger) for human colony-stimulating factor-1 (388), and regulate immune response by inhibiting or interferon secretion by mononuclear cells (96). Recent study showed strong CD4+ T and CD8+ T cell responses to BARF1 in healthy donors and NPC patients, by using autologous monocyte pulsed with recombinant BARF1p29 protein, in healthy donors and NPC patients (264). In contrast to the low CD8+ T cell responses observed for latent EBV protein LMP1 and LMP2, CD8+ T cell responses to whole BARF1 protein and derived peptides was significantly higher in NPC patients than in healthy donors. This discrepancy may relate to the biological property of the proteins. BARF1 protein can be actively secreted by undifferentiated NPC cells, and in the tumor microenvironment BARF1 protein may be captured and processed by local dendritic cells or other APC, which may provide a sustained presentation of antigenic BARF1 peptides. This may lead to the increased numbers of BARF1 specific T cells circulating in the blood of NPC patients, although it remains to be elucidated whether these effectors are detectable and functional within NPC lesions (264).

BARF1 is also able to induce humoral immune responses in EBV-seropositive individuals and may serve as a target for antibody-dependent cellular
cytotoxicity in NPC patients (404). Like LMP1 and LMP2, BARF1 appear to be a protein of low immunogenicity for humoral immune responses (431). However, this remains to be confirmed in further studies.

6.d. Immune responses to lytic EBV proteins

EBV can establish both latent and productive (lytic) infection. The CD8 cytotoxic T lymphocytes (CTL) response to latently infected cells is well characterized, but very little is known about T cell control over lytic infection. Studies by Scotet (1996) and Steven (1997) shows that EBV lytic antigen specific CD8 T cells are present in the inflamed joint of rheumatoid arthritis patients (355) and IM blood (376). As mentioned previously, the EBV lytic antigen-specific CD8 T cells were identified mainly to IE proteins (BZLF1 (ZEBRA) and BRLF1 (Rta)) or E protein (BMLF1, BRLF1 (EAd-p47/54), BALF2 (EAd-p138) and BALF5) (355, 376). (see Adaptive immune response-Cellular responses-CD8 T cells section). The CD4 T cell responses have been detected not only to ZEBRA and BMLF1, but also to late proteins such as BCRF1 and the gp350 and gp110 envelope components (3, 440). We here limit discussion to immune responses against IE and E protein.

6.d.1. Immune response to IE proteins

Primary CD8 T cell response to EBV in IM patients as well as rheumatoid arthritis contains multiple lytic antigen-specific reactivities. Chlamyta analysis revealed individual responses to two immediate early proteins BZLF1 and BRLF1 (355, 376). In the case of autimmunity, the finding of reactivity to IE antigen provide new understanding into the fine specificity of the anti-EBV T cell response and the immune control of EBV reactivation, because they provide the first clear-cut evidence that the ZEBRA EBV-transactivating proteins can be targets for CD8 T cells (355). T cell recognition to ZEBRA has been suspected in several EBV-associated diseases (e.g. acute IM, NPC, EBV reactivation in immunocompromised patients) during which antibody titers against ZEBRA and other early antigens were significantly increased (338). The physiological significant of ZEBRA recognition by T cells can be understood from its function during virus replication. ZEBRA is the first gene expressed during the immediate early stage of the lytic cycle and is thought to turn on expression of other early genes (205). It is therefore likely that T cell responses against this protein play a central role in controlling virus reactivation and spread under physiological and pathological situations (220).

Antibody responses against ZEBRA are marker of EBV reactivation and regularly found in undifferentiated nasopharyngeal carcinoma (NPC) patients (65-87%), but rarely among healthy people (181, 182, 266, 406, 407). Furthermore, studies done by Bardari (87, 89) found that IgG-ZEBRA in combination with IgA-EA-p47/54+138 improve the sensitivity of NPC diagnosis, and the IgG-ZEBRA can be used as diagnostic and post-therapeutic prognostic marker in juvenile NPC with low titers of IgA-VCA and EA.

6.d.2. Immune response to EA proteins

The primary CD8 T cell response to EA proteins was evaluated in IM patients and rheumatoid arthritis as well as against ZEBRA and Rta (355, 376). The analysis revealed dominant responses to three early proteins BMLF1, BMRF1 (EAd-p47/54) and BALF2 (EAd-p138) (376). Clones reactive to other E and L proteins are detected but only in small number of patients and are only a minor component of total CD8 response (164, 328, 377). Antibody responses to EA proteins were detected as well to VCA and EBNA (29). In acute IM, IgG reactivity to EAd-p47/54 and -p138 was detected. IgM and IgG reactivity in IM patients to EAd proteins was also detected but in a lesser extend compare to the IgG reactivity (277, 317). Antibodies to EAd especially IgA are important diagnostic and prognostic indicators in NPC as well (157, 158). Previous studies using recombinant proteins as well as synthetic peptide showed that IgA EA has high sensitivity and acceptable specificity for the diagnosis of NPC, either being used as single marker or in combination with other EBV antigen such as VCA or EBNA1 (69, 173, 252, 401, 422). Antibody responses to other EBV proteins are detected against DNAase and TK. EBV DNAase neutralizing antibodies have been found in 83%-94% of NPC patients (68). Stolzenberg et al. (384) detected IgA antibody against recombinant DNAase in NPC patients but rarely in patients with other EBV-related malignancies. The use of thymidine kinase (TK) as antigen for diagnosis and screening of NPC was also suggested (78). However, present data indicate that a single EA maker protein as antigen might not be adequate to comprise the diverse anti-EBV antibody responses for NPC diagnosis (114). Therefore multiple EA protein species may be required (67, 88, 114, 247). This thesis proposes the use of native EA proteins extracted from natural EBV infected cell lines for NPC diagnosis.

7. EBV Immune Escape

7.a. Immune escape in Latent infection

Successful viral persistence in immunocompetent individual is facilitated by immune evasion strategies. In purpose of doing this, EBV has developed several mechanisms to escape from immune recognition. Latently infected cells express a restricted set of viral genes, which are usually non-immunogenic, making them invisible to the immune system. EBV is found in circulating memory B-cells that express low levels of MHC class I and co-stimulatory molecules (17, 281), which negatively affects the presentation of antigenic peptides (124). The true latency programme where EBV gene expression is completely shutdown or limited to the expression of LMP2A, may not be recognized by immune system (16, 17, 62, 281).

Another escape mechanism in latent infection is inhibition of antigen processing by the G5-A2a repeat (G5-A2a) present in EBNAs (29). G5 generates a cis-acting inhibitory signal that interferes with antigen presentation, possibly by altering the kinetics of interaction with the proteasome, or sequesters the
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is considered to be strong targets for virus-specific CTLs. Immunological characterization by expression of EBNA1, BARF0, LMP1 and LMP2. LMP1 and LMP2 presentation of other potential tumor-associated epitopes (204). Immunogenic antigens (EBNA3A, 3B and 3C), but also blocking the endogenous resistance control by not only downregulating the expression of highly immunogenic genes (EBNA3A, 3B and 3C), but also blocking the endogenous presentation of MHC-I epitopes (236). Furthermore, BL cells express viral antigen, EBNA1. EBNA1 can evade from immune recognition through its GAr, a mechanism. In latency type I tumor, such as BL, the tumor cells express only one latency phenotypes (latency type I, II and III), which showing different escape mechanisms ubiquitously in all latently EBV infected cells.

LMP1 effects to ensure persistent IL10 production since EBERs are expressed (102). IL-10 induction, which was also shown for Hodgkin Lymphoma by Dukers et al (109). IL-10 allows it to reveal the ingenious strategy of EBV to survive in immune hosts. This study demonstrated that EBERs were responsible for IL-10 induction. IL-10 allows it to escape the immune response by suppressing Th-helper1 (Th1) cytotoxic T lymphocytes (117). Several studies have indicated that IL-10 is induced by EBV infection, in particular via LMP1 (109). The majority of EBV-transformed lymphoblastoid cell lines and EBV carrying cell lines derived from BL and AIDS related lymphoma produce detectable amount of IL-10 (45). Most of these cells express LMP1, and Nakagomi (291) demonstrated that LMP1 is responsible for IL-10 induction, which was also shown for Hodgkin Lymphoma by Dukers et al (109). This may be a universal immune evasion effect in EBV cells, combining EBER and LMP1 effects to ensure persistent IL10 production since EBERs are expressed ubiquitously in all latent EBV infected cells.

NK cells are considered to have an important role in controlling EBV infection, but are made inactive by the action of EBV induced gene 3 (EBI3). EBV transformed B-lymphocytes express high levels of EBV induced gene 3 (EBI3), and in a matter of immunosuppressive activity, a peptide derived from EBI3 bind to HLA-G, which is the ligand of inhibitory receptors expressed on NK cells and CTls (102).

Another escape mechanism was demonstrated by Kitigawa (209) revealing the ingenious strategy of EBV to survive in immune hosts. This study demonstrated that EBERs were responsible for IL-10 induction. IL-10 allows it to escape the immune response by suppressing Th-helper1 (Th1) cytotoxic T lymphocytes (117). Several studies have indicated that IL-10 is induced by EBV infection, in particular via LMP1 (109). The majority of EBV-transformed lymphoblastoid cell lines and EBV carrying cell lines derived from BL and AIDS related lymphoma produce detectable amount of IL-10 (45). Most of these cells express LMP1, and Nakagomi (291) demonstrated that LMP1 is responsible for IL-10 induction, which was also shown for Hodgkin Lymphoma by Dukers et al (109). This may be a universal immune evasion effect in EBV cells, combining EBER and LMP1 effects to ensure persistent IL10 production since EBERs are expressed ubiquitously in all latent EBV infected cells.

EBV associated malignancies are characterized by three different types of latency phenotypes (latency type I, II and III), which showing different escape mechanism. In latency type I tumor, such as BL, the tumor cells express only one viral antigen, EBNA1. EBNA1 can evade from immune recognition through its GAr, which acts as a cis-inhibitory signal for proteasomal degradation and blocks endogenous presentation of MHC-I epitopes (236). Furthermore, BL cells express very low levels of antigen processing genes (TAP-1 and TAP-2), which play a crucial role in the endogenous processing of CTL epitopes (195, 347). Therefore, BL cells resist CTL control by not only downregulating the expression of highly immunogenic antigens (EBNA3A, 3B and 3C), but also blocking the endogenous presentation of other potential tumor-associated epitopes (204).

Hodgkin disease and NPC represent the type II malignancies, which characterized by expression of EBNA1, BARF0, LMP1 and LMP2. LMP1 and LMP2 is considered to be strong targets for virus-specific CTLs. Immunological and biochemical analysis of LMP1 and LMP2 sequences associated with HD and NPC have shown that these proteins are poorly immunogenic in murine models when compared with LMP1 sequences derived from normal EBV-infected B cells (270, 420). Moreover, Reed-Sternberg cell in HD secretes anti-inflammatory cytokines (IL-10 and TGF-b), which can block tumor-infiltrating virus specific T cell responses (110, 162, 172).

Several mechanisms are proposed for immune escape in NPC. LMP1 and LMP2 specific CD8+ T cells are abundant in tumor infiltrating lymphocyte, but their function is impaired (240), due to the present of CD4 CD25 FoxP3+ natural regulatory T cell in the tumor tissue, which could suppress EBV specific immune responses against NPC even after correct homing of effector T cells (223, 240). The majority of NPC down regulate MHC-I antigen processing machinery (304, 308, 467). High levels of granzyme B-positive tumor-infiltrating lymphocytes (TILs) in NPC associated with poor treatment outcome (308). It is assumed that under the pressure of a strong CTL-mediated immune response, selection for apoptosis-resistant tumour cells occurs, resulting in resistance to both CTL and radio therapy-induced apoptosis. This is substantiated by decreased activated caspase-3 levels in these tumors (307). Another strategy of immune escape by LMP1 in NPC is directly mediated by immunosuppressive sequence of LMP1 LALLFWL (see part 6.c.2 immune response to LMP1) (278). It is suggested that NPC tumor cells impair EBV specific immune control locally, while systemic immune response against EBV remain intact (287). TILs, freshly obtained from NPC biopsies showed non-responsiveness and failed to produce IFN on specific stimulation (240). The general failure of CTL therapy in NPC also reflects the presence of local immune evasion by this tumor microenvironment (386).

7.b. Immune escape in Lytic infection

In EBV lytic replication, more than 80 viral genes are expressed (182, 405). The escape strategies of the virus in this phase are different from those affecting the recognition of latently infected cells (336). EBV infection inhibits the development of DCs from monocytes precursors (241), may temporarily halt the onset of immune responses during primary infection, creating time window for efficient viral replication. This could permit the accumulation of a sufficiently large pool of virus infected B-lymphocytes and allow their access to the memory B-cell compartment. Lytic EBV infection is associated with production of other factors that may affect the function of monocytes in trans. BARF1 gene that is transcribed early after EBV infection of B cell, encodes the soluble colony-stimulating factor-1 (CSF-1) receptor, which neutralizes the proliferative effect of CSF-1 on monocytes and inhibit the poly induced secretion of IFN-b by this cells (75). Therefore BARF1 may interfere with the IFN-b dependent antiviral effects of innate immunity and with delayed responses that depend on monocyte proliferation and differentiation (238).

During the lytic phase EBV has several strategies to escape antigen presentation and immune activation. Several immediate early and early product that can interfere directly or indirectly with T cell mediated immunity (238). EBV...
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encodes BCRF1 protein, which close structural and functional similarity to human IL-10 (172), which affect the development of effector functions by suppressing IL-2 and IFN-γ production by T-cells and enhancing the proliferation of EBV-infected B-cells (26). IL-10 also suppresses the migration of DCs to regional lymphnodes by inhibiting the expression of chemokine receptor CCR7 (399).

The early protein encoded by the EBV BHRF1 and BALF1 genes are homologues of the cellular hsc-2 gene and confer resistance to various apoptosis inducing signals including granzyme-B mediated cell killing by CTLs (27, 70, 91, 260).

One of the well described features of herpesvirus is host shut off, and results in down regulation of surface expression of MHC class I and II molecules, which normally presenting peptide fragment of viral antigen for activation of specific T cells (214, 368). A recent study by Rowe (346) revealed a mechanism affecting MHC class I and II restricted antigen presentation by lytic cycle EBV cells (productively EBV-infected B cells). Expression of early-lytic gene product BLF5 results in the mRNA degradation and causes the impairment of protein synthesis.

One of the consequences of host shut off is a block in the synthesis of HLA class I and II molecules, indicated by reduced levels of these antigens presenting complexes at the surface of lytic-cycle EBV infected cells (471). More recently, BILF1 was shown to down regulate MHC class by increasing the degradation (Rowe et al., 2008). Finally, like other herpesviruses EBV encodes an inhibitor of TAP function in the BNLF2a gene (170). These effects combined can lead to escape from T cell recognition and elimination of EBV-producing cells, and allowing generation of viral progeny in the face of memory T cell responses. Mechanism of immune escape in EBV latency & replication can be seen in figure 6.

8. Strategy for Immunotherapy

Understanding of the role of cytotoxic T lymphocytes (CTLs) in controlling EBV-associated malignancies and overall biology of these diseases lead to the development of novel therapeutic strategies designed to specifically target viral antigens expressed in these malignancies. The success of these strategies is limited by the latency phenotypes in different diseases. Adaptive transfer of polyclonal virus specific CTLs has been used successfully to reverse the outgrowth of post-transplant lymphoproliferative disease (PTLD), which express full spectrum of EBV antigens (latency type III). However, limited viral gene expression in other EBV-associated malignancies such as IL, HD, and NPC limit the efficacy of this kind of immunotherapeutic strategies (204).

Phenotypic differences in EBV-associated malignancies provide an important basis for the development of immunotherapeutic strategies. In the type I malignancies, low expression of immunogenic antigens (e.g. EBNA3A, 3B and 3C) and a consistent loss of antigen-processing function through the MHC cabs I pathways result in the limitation of using antigen-specific immunotherapeutic strategies. On the other hand a study by Herbst et al (162) showed that BL cell lines are efficiently recognized by EBNA2 reactive T cells. More recently similar results were shown for EBNA1-specific CD4+ CTLs (285, 287, 301).
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Another strategy for treatment of the BL cells is using soluble CD40 ligand (CD40L). This treatment can upregulate the TAP-1 and TAP-2 gene expression involved in MHC class I-restricted presentation (201), therefore CD40L-treated BL cells regain susceptibility to EBV-specific CTL-mediated lysis.

EBV associated malignancies expressing latency type II genes provide an excellent opportunity to adopt a CTL-based immunotherapy for the treatment of HD and NPC. LMP1 and LMP2 proteins are expressed in these malignancies, and have been proposed to be used as targets for immunotherapy. The first challenge to treat NPC using adoptive T cell therapy was reported by Chu et al (71). LCL-reactivated T cell lines were generated in vitro by stimulating the peripheral blood lymphocytes with EBV-transformed lymphoblastoid cell lines (LCLs) and used to treat advance cases of NPC. However, there was no evidence of tumour regression. The major limitation of this study is that LCL reactivation of PBMCs preferentially stimulate T cells that are specific for EBNA3 antigens rather than to viral protein expressed in NPC (196, 230, 289). Another approach involves stimulation of T cells with autologous dendritic cells transduced with a viral vector that expresses individual LMP antigen. This approach can be used to selectively expand LMP2-specific CTLs with minimal reactivity to other EBV antigens (343). Khan et al (204) developed two therapeutic strategies for HD and NPC, based on the T cell activation with viral or DNA expression vectors encoding multiple LMP1 and LMP2 CTL epitopes known as polyepitope. The first approach is adoptive transfer of in vitro activated LMP-specific CTLs. These CTL were stimulated by autologous DCs loaded with recombinant vaccinia virus containing full length LMP2A or LMP polyepitope. The second approach is based on the active immunization with DNA vaccine or a recombinant viral delivery system containing LMP polyepitope construct (202, 255).

A later study by Comoli proposed a therapeutic approach for NPC using adoptive transfer of EBV-specific CTLs reactivated ex vivo from human leukocyte antigen identical siblings, showing an increasing of endogenous tumor infiltrating DC/T lymphocytes and a long-term increase of latent membrane protein 2-specific immunity (76). Another study by Comoli used the same approach on ten patients with EBV related NPC stage IV with progression after radio and chemotherapy (77). Additional study by Straathoff et al also supported this idea (385). Because of CTL resistance and immunosuppressive phenotype of NPC, CTL therapy may not be efficient and required improved targeting (LMP2A) and resistance to local immunosuppressive effect(s) (116). Totally different approach by vaccination inducing antibody against EBV were also developed. The first prophylactic vaccination with vaccinia gp350 resulted in increasing EBV MA-neutralizing antibodies in juvenile (143). Another phase I/II studies using a recombinant gp350 in healthy adults were also done shows that the gp350 vaccine induce gp350 specific antibody responses, including neutralizing antibodies. The adjuvanted gp350 vaccine display significant higher than non-adjuvanted gp350 vaccine (284, 373). In the same study by Sokal (373), which suggest that 3 doses of vaccine are necessary before full protection showed that no cases of infectious mononucleosis was declared in the vaccinated healthy individual group after completion the vaccination schedule, whereas cases still occurred regularly in the placebo group. This is the first demonstration of considerable efficacy of vaccine to prevent infectious mononucleosis. A recent phase-I trial failed to induce protective immunity in young transplant recipients by using a gp350 based vaccine (333). The use of potent adjuvant seems crucial. Surprisingly, in contrast to T cell based and gp-350 based vaccination or immunotherapy, very little attention has been done for Ab based for immunotherapy directed to tumor. A combination of early stage detection of NPC and specific vaccination or immunotherapy targeting tumor-associated antigens may provide an effective approach in prophylactic treatment of NPC.

Chapter 2

What is the predominant EBV marker for NPC diagnosis?

In this chapter we analysed the IgG and IgA responses against individual EBV antigens in NPC patients and healthy EBV carriers using serum panels of Indonesian, Caucasian, and Chinese ethnical background. By employing immunoblot-strips containing EBNA1 and a large spectrum of EBV lytic antigens separated by molecular weight we further identify IgG and IgA responses to individual EBV antigens and defined their diagnostic value.
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Chapter 3. Can we use individual EBV markers alone or in combination for NPC diagnosis?
A number of Epstein-Barr virus (EBV) proteins were defined as being immunodominant markers for diagnostic serology. Specific reactivity patterns to these proteins have been described for infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC), various types of lymphoma, and healthy EBV carriers.

In this study we compare the NPC-related diagnostic value of EBV RecombLine test containing EBNA1, VCA-p18, VCA-p23, EAd-p47/54 and EAd-p138 with a standardized immunoblot assay (chapter 2). Furthermore, we define the diagnostic value of individual EBV marker proteins in a population with high incidence of NPC.

Chapter 4. What is the diagnostic value of individual EA components versus the complex “native” EA proteins for NPC?
The EA protein complex contains multiple proteins, which are relevant markers for diagnosis of acute, chronic and malignant EBV related diseases, including nasopharyngeal carcinoma (NPC). Multiple studies have addressed the value of individual EA marker proteins for NPC diagnosis, but no consensus is obtained thus far. Considering the diagnostic relevance of EA-specific antibody responses in NPC patients, we evaluate in chapter 4, the antibody responses of patients with NPC and healthy EBV carriers against individual recombinant EA proteins expressed in SF9 insect cells (TK, DNAse, ZEBRA) and E. coli (EAd-p47/54 and EAd-p138), by using immunofluorescence assay (IFA) and immunoblotting (IB). Evaluation of the responses was also done to the synthetic peptides of immunodominant epitopes EAd-p47/54 and -p138 using enzyme-linked immunosorbent assay (ELISA).

For comparison with individual recombinant EA proteins and synthetic peptides, an EA-specific extract was prepared by differential salt extraction from induced HH514.c16 cells, and used as antigen for ELISA to evaluate the antibody responses of patients with NPC in compare with healthy EBV carriers and other EBV associated diseases. Furthermore we defined the diagnostic value of native EA extract for NPC.

Chapter 5. Can we use IgA-EA ELISA as confirmation test in the screening of NPC.
Therapy failure in NPC cases is high, since most patients come to the hospital at advanced stage of disease. Therefore screening for early-stage NPC is needed. In this chapter we propose to use two-step EBV ELISA for screening and confirmation of NPC. The first step consists of a cheap peptide based test for IgA [EBNA1+VCA-p18] with high sensitivity and second step consist of IgA EA ELISA with high specificity described in chapter 4 as confirmation test.

Chapter 6. Can we use the Epstein-Barr Virus (EBV) DNA Load in Blood of NPC Patients as confirmation test for screening?
NPC patients generally show strong IgG and especially IgA responses to EA, VCA, and EBNA1. Serodiagnostic assays based on defined EBV-derived epitopes may facilitate population-based screening to identify NPC at early stage. However, an independent marker might be useful as confirmation for serology testing. In this chapter we use a real time PCR method targeting a conserved region in the single copy EBNA1 gene of EBV-DNA to quantify EBV-DNA levels in whole blood of NPC patients and controls.

Chapter 7. Is there any antibody response in patients with NPC against EBV protein expressed on the tumor i.e. LMP1, LMP2, EBNA1 and BARF1 and what is the potential of this response?
NPC is one of the latency type II tumors and is characterized by expression of EBNA1, LMP1, LMP-2A/-2B and BARF1. It is surprising that LMP1, LMP2 expressing tumors occur in immunocompetent individuals, who are considered to have the capacity of mounting an effective immune response to these “non-self” proteins.

Many studies addressing CD8 T cell responses against EBV latent antigens, identified immunodominant epitopes derived from the EBNA3A, 3B, and 3C protein family, and subdominant epitopes from the same EBNA3 family or from LMP2, and much less often to epitopes from EBNA2, EBNA-LP, or LMP1. Only limited data are available for T cell responses to BARF1.

Using IFA and IB methods, in this chapter we evaluate the humoral immune responses (IgG and IgA) of NPC to the individual recombinant EBV tumor associated protein (EBNA1, LMP1, LMP2A and BARF1) expressed on SF9 cells. PEPSCAN analysis of LMP1 and LMP2 was used to define immunogenic parts of the proteins, followed by the generation of synthetic peptides of each immunogenic epitopes. IgG and IgA responses to epitope-specific synthetic peptides were evaluated using ELISA. Immunization of rabbits with synthetic peptides representing extracellular domain yielded specific antibodies serving as positive control. Furthermore, we show that antibodies against putative LMP1 and LMP2 extracellular domain can mediate complement-driven cytolysis of LMP1 and LMP2 expressing cell lines. This opens possibilities for inducing therapeutic antibodies in NPC patients targeting tumor associated antigen expressed on the NPC tumor cells, using either using active or passive immunization.
Reference:


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