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 virus 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.].
Chapter 1

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 of EBV 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). EBNA3A and B will downregulate EBNA2 through competition for the binding of RBP-Jk protein (341). EBNA3C will activate G1 progression within the cell cycle. This will activate B cells to become growth-activated B cell blast not requiring further external signals. EBNA1 is co-expressed 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>Growth (latency I)</td>
<td>EBNA through EBNA3, LMP1, LMP2A and LMP2B</td>
<td>Activates B cells</td>
<td></td>
</tr>
<tr>
<td>Germinal-center cells</td>
<td>Default (latency II)</td>
<td>EBNA1, LMP1 and LMP2A</td>
<td>Differentiates activated B cells into memory cells</td>
</tr>
<tr>
<td>Peripheral/blood</td>
<td>Latency</td>
<td>None</td>
<td>Allows viral DNA in latency-program cell to divide</td>
</tr>
<tr>
<td>memory cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA1 only (latency III)</td>
<td>EBNA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma cell</td>
<td>Lytic</td>
<td>All lytic genes</td>
<td>Replicate virus in plasma cell</td>
</tr>
</tbody>
</table>

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

3.b. Epithelial infection

EBV is frequently found in certain epithelial pathologies, such as nasopharyngeal carcinoma (NPC), gastric adenocarcinomas and oral hairy leukoplakia, indicating that the virus can infect epithelial cells in vivo (323). Most of the EBV+ epithelial cell lines have negative or extremely low CD21 expression, suggesting that the mechanism is CD21 independent (176).

The study in polarized human tongue and oropharyngeal epithelial cells shows that EBV enters these cells through multiple mechanisms, depending on the membrane domain that are distinct from those of lymphocyte infection. The apical membrane of polarized cells is the site of efficiently cell-to-cell transmission from co-cultivated EBV infected lymphoblastoid cells. Cell-to-cell contact of EBV-infected salivary cells with epithelial apical membranes suggest as the primary mode of EBV entry into oropharyngeal epithelium, since the apical site is resistant to cell-free EBV attachment and entry (425). Cell-free virions also affect oropharyngeal cells at the basolateral membrane, mediated in part through an interaction between β1 or α5β1 integrins and the EBV BMRF2 protein, which contain multi-span transmembrane domains and an arginine-glycine-aspartate (RGD) domain. This mechanism may occur when the epithelium is disrupted or injured. After initial infection, virus may spread directly across lateral membrane to adjacent epithelial cells. Virions are released from epithelial cells at both the apical and basolateral membranes (425). BMRF2 is an EBV glycoprotein with highly hydrophobic RGD motif in its major extracellular domain (425, 457). The RGD motif is present in extracellular matrix proteins, which bind to integrins and play a vital role in cell adhesion and cell-to-cell communication (123, 134, 348).

Viral proteins containing the RGD motif may serve as ligands for integrins leading to attachment of virions to host cells (419). BMRF2 is highly expressed in the upper differentiatered layer of oral hairy leukoplakia (OHL) lesions, where the virus actively replicates (221, 311, 457). The promoter of BMRF-2 is up-regulated by mechanisms known to induce differentiation of epithelial cells (221). BMRF2 is associated with the viral envelope and binds to α3, α5, αv and β1 integrin (425, 457). Antibodies to β1 family integrin and to the BMRF2 protein significantly block infection of EBV in polarized oral epithelial cells, which express high levels of integrins on the basolateral membranes. Treatment with the same antibodies does not affect EBV infection in B lymphocytes, which express lower levels of integrins (425). These findings indicate that interactions between EBV BMRF2 and EBV infection in oral epithelial cells but not B lymphocytes (456).

Another mechanism was suggested where the epithelial polymeric immunoglobulin receptor (pIgR) can mediate internalization of infectious virus-linked immunoglobulin A (IgA-EBV) complexes (367). Anti-EBV-gp350 IgA were used to prove the ability of EBV to infect epithelial cells (129, 426). The phenomena suggest that antibody-enhanced infection by pIgR-mediated transcytosis of IgA-EBV through epithelium facilitates endogenous spread of EBV in long term virus carriers (129).
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 cells during mitosis, akin 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 act 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 2. Function of EBV latent genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA1</td>
<td>Episome maintenance (binds origin of latent replication) (228)</td>
</tr>
<tr>
<td>EBNA2</td>
<td>GW inhibits processing of EBNA1 through proteasomes, precluding presentation of antigenic peptides with MHC I at the cell surface, which avoiding CD8+ T cells detection (237)</td>
</tr>
<tr>
<td>EBNA3A</td>
<td>Transcription factors, binds RBP-4, activates viral genes (LMP and LMP2A) (1, 443, 468) and cellular genes (CD21, c-fGR, c-MHC, CD23) (79, 447)</td>
</tr>
<tr>
<td>EBNA3B</td>
<td>Like other EBNA3s balances EBNA2 effect on RBP-4, bind C1BP</td>
</tr>
<tr>
<td>EBNA3C</td>
<td>Transcriptional regulator</td>
</tr>
<tr>
<td>EBNA4A</td>
<td>Essential for EBV-mediated transformation of primary B lymphocytes and interacts with RBP-4 (184, 346)</td>
</tr>
<tr>
<td>EBNA4B</td>
<td>Promotes LMP expression in the presence of EBNA2 (162, 467)</td>
</tr>
<tr>
<td>EBNA4C</td>
<td>Key role in upregulating gene expression critical for lymphoblastoid B cell outgrowth (358)</td>
</tr>
<tr>
<td>LMP1</td>
<td>Augment the ability of EBNA2 to transactivate LMP1 (452)</td>
</tr>
<tr>
<td>LMP2A</td>
<td>Activates NF-kB to promote cell growth and survival (208)</td>
</tr>
<tr>
<td>LMP3</td>
<td>MIMics signaling through CD40, a B cell activation and differentiation receptor (301, 427)</td>
</tr>
<tr>
<td>LMP2B</td>
<td>Promotes B cell lymphoma and skin hyperplasia in transgenic mice expressing LMP1 (215)</td>
</tr>
<tr>
<td>LMP2C</td>
<td>Contributes to immune evasion by hIL10 induction and interference with 1-cell activation (109, 110, 121, 218)</td>
</tr>
<tr>
<td>LMP2A</td>
<td>Inhibitor of BCR signalling by sequestering tyrosine kinases through interaction with its immunoreceptor tyrosine-based activation motifs (279)</td>
</tr>
<tr>
<td>LMP2B</td>
<td>Blocking viral lytic cycle by antiviral stimulation; provides B cell survival signals through activation of the same sequestered tyrosine kinases in an antigen- and B cell receptor-independent manner (276)</td>
</tr>
<tr>
<td>LMP3</td>
<td>Function unclear</td>
</tr>
<tr>
<td>EBER RNAs</td>
<td>play a role in oncogenesis (395).</td>
</tr>
<tr>
<td>BARTs</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></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>
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 10^6 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 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 EBER 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 multispliced 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 low 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). 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 post transcriptionally downregulate the expression of mRNAs 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 herpesviruses (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 III Rightward Frame 1) ORF, and one cluster of miRNAs, miR-BART-1 and -2 is located within the BART (Bam III 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 includes 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 coexpressed with miR-BHRF1-1 in latently infected B cell lymphomas, is predicted to target Bc-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 BALF5. BALF5 transcripts decrease in abundance when miR-BART2 is upregulated. miR-BART2 is transcribed antisense to the BALF5 transcript and perfectly complementary to the BALF5 3’ UTR (23). A quantitative PCR was recently described to measure miRNA levels 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,
Chaper 1

immediate early (IE), early (E) and late (L) (Figure 2). Most of the early genes encode functions that are required for host cell survival and viral DNA replication, whereas late gene products are mainly structural proteins, which are expressed after viral DNA synthesis. The IE genes Zta and Rta are transactivators that are activated directly by cellular transcription factors induced by the BCR, independent of the expression of other proteins. BZLF1 appears to be the major IE protein in EBV (33, 280).

The product of BZLF1, the ZEBRA protein, plays a role in both viral replication and transcription as an oriLyt binding protein and as transactivator. Therefore it can mediate the switch between latent and lytic forms of EBV infection and alone is sufficient to activate the EBV lytic cascade. Induction of the ZEBRA

Figure 2. Overview of EBV lytic cycle. The lytic cycle can be induced with anti-IgG, which cross-link the BCR to mimic antigen binding. Immediate early, early and late genes are expressed in sequential order. Late gene expression is prevented by inhibitor of lytic DNA replication such as acyclovir (10)."
immunoblasts express LMP1 in the absence of EBNA2 (type II latency) and small lymphocytes express EBNA2 but not LMP1.

Primary EBV infection elicits different cellular immune responses that bring the infection under control. The lymphocytosis in acute IM reflects the hyperexpansion of cytotoxic CD8+ T-cells that are reactive to lytic- and latent-cycle viral antigens. The limited clinical effect of antiviral drugs indicate that not EBV replication, but rather the strong T-cell reactivity during primary infection is causative for the chilcal symptoms of IM. However these drugs do limit the amount of virus in semen, but have no effect on the number of circulating B-cells carrying latent EBV.

The diagnosis of IM relies on the detection of heterophile antibodies, and EBV seroconversion. Sera from IM patients have detectable IgM, IgG and IgA antibodies to one or more EBV marker protein(s). IgM antibody against EAd-p138 and -p47/54 marker to VCA-p18 and p23, without any response against EBNA1. During acute IM IgG and IgA antibodies also can be detected against EAd-p138 and -p47/54 markers, dominant over responses to VCA-p18 and -p23, without responses to EBNA1.

5.b. Lymphoma in immunocompromised patients

5.b.1. 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 poly-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.2. 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.

There are two different types of ARL, which are differ amongst others in their EBV association and EBV gene expression pattern. Most of the cases of diffuse large B-cell lymphoma, particularly those arising in the central nervous system (CNS), are associated with EBV and have an EBV gene expression pattern comparable to that of PTLD. By contrast, only 30-40% of AIDs related Burkitt’s Lymphoma (BL) is associated with EBV. These lymphomas are EBNA2 negative and rarely LMP1 positive. In addition AIDs related BL, like endemic and sporadic BL, carry a translocation involving the c-myc gene.

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 prevailed 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 cell 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 (BZLF1) and early (IR2 and IR4) genes, a putative viral oncogene (BARF1), CST (BART) antisense transcripts and viral bcl-2
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). Interesting EBV+ T-/NK cell lymphomas always have a functionally activated cytotoxic phenotype, expressing granzyme B and perforin than B cells do (118). EBV+ T-cell 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 developed 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 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 is 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
keratinized squamous cell carcinoma (WHO type I) is highly differentiated and characterized by epithelial growth patterns and keratin filaments. The non-keratinizing carcinoma (WHO type II) characterized with retaining epithelial shape and growth pattern. The undifferentiated carcinoma (WHO type III) does not produce keratin and lack of distinctively organized growth (329). Type I is seen in 25% of NPC cases. Approximately 20% of NPCs 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 latero-posterior extension of the tumour to paranasopharyngeal space (tinnitus and deafness); (3) skull-base erosion palsy 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 4768 patients showed the symptoms as neck mass (72%), nasal dysfunction (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 tumors in 75-90% of cases and T3 and T4 tumors 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 compiled 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.

5.2.1.4 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 (35, 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, prognosis, prediction and prevention of the disease. Undifferentiated NPC (WHO type III) is nearly 100% EBV related as demonstrated by EBER in situ hybridization, which remain 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). Immunofluorescence assay (IFA), which allows the separate analysis of antibody responses to VCA, EA and EBNA complexes, is still used as gold standard for NPC diagnosis (130, 157), but this method is time consuming, laborious, and not suitable for mass screening (44, 131, 436). Enzyme-linked immunosorbent assay (ELISA) techniques are increasingly used and show better sensitivity and specificity compared to IFA (87, 113, 130, 190, 294, 295, 316).

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 PTLD (387), the use of anti-EBV immunotherapy strategies has been receiving attention as a possible additional treatment to improve prognosis of advanced NPC (71, 76, 203, 243).

Further details of EBV based therapy for latency type II malignancy, especially NPC, is mentioned in part 8 of introduction (strategy for immunotherapy).

5.d.2.c. NPC in Indonesia

In Indonesia, NPC is the most prevalent head and neck cancer and the 4th and 6th the most prevalent cancer overall in male and female respectively, with estimated incidence of 6.2/100,000/year in males and 4.6 in females (218, 372). The incidence peaks at the age of 45-54, but juvenile NPC is also quite common (372). Histopathologically about 70-90% of the cases are WHO type III (Kurniawan, unpublished) (372). Most of the patients come to the hospital at stage III and IV (>80%) when they already have metastasis in the cervical lymph node. Whereas, late stage disease has poor prognosis and requires combined chemoradiotherapy, early stage NPC may reach complete remission by radiotherapy only (244). Therefore, screening for early stage NPC among the population is important and clinically relevant.

6. EBV Immunology

Primary EBV infection is followed by establishment of viral latency in memory B lymphocytes. Infection in humans triggers both humoral and cellular immune responses to virus-encoded structural and non-structural proteins (antigens). In healthy individuals, EBV is effectively controlled by the immune system especially by antigen specific T lymphocytes (166, 339). Although the detection of antibodies against viral structural proteins and the EBV nuclear antigen is important for diagnosis, the cellular immune response, especially involving CD8+ cytotoxic T lymphocytes (CTL) is more important in controlling primary (53) and life-long latent infection (337). The major targets for controlling EBV are reactivating infected memory B cells. By killing these cells and preventing the spread of infectious virus by neutralizing antibody, the immune system may reduce the infection. However, the immune system is unable to eliminate the virus completely, and as consequence, viral shedding and virus infected cells persist at a low level, approximately 1 in 10,000 to 100,000 memory B cells (222, 414). New evidence is emerging for a critical role played by natural killer (NK) cells and CD4 effector T cells to control virus infected cells that is complementary to the CTL (314, 452).

6.a. Innate immune response

Natural killer cells are a key component of early innate immune response to many microbial infections. An important role of NK cells in controlling EBV infection is revealed by the increasing numbers of activated NK cells in the peripheral lymphocyte inversely correlating with viral load (452). This finding suggests an active role for NK in controlling acute virus replication. In vitro study showed that NK cells can inhibit the EBV-induced transformation of resting B cells through the release of interferon (IFN)-γ (303). However, the role of NK cells in controlling B-cell transformation in vivo is still in debate. Direct evidence against a
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 NK 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 lyrically 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 IFN-γ 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). 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 or CD69 and expressing higher levels of Bcl-2 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 Treg 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 cells is in discussion, many lytic antigen-specific CD4+ T cell are perforin-positive and cytoxic, indicating their direct role in control of virus replicative lesions in vivo, especially MHC II positive target cells. 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 progressing 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 composing EBZEBRA (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 relevance of studying latent antigen-specific CD4+ T cell responses is that most EBV-associated tumors are HLA class II-positive and express latent antigen (199). The use of HLA class II-recognizing CD4+ T cells 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, 4-1BBL/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 carrier state 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 condition. 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 underlie 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-Lys6 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 1Cr release assay (313), which suggest that CD4+ T cells might inhibit EBV-infected cells through cytokotoxicity mediated by perforin or Fas ligand expressed by CD4+ effector cells (125, 429). However, the experiment using EBNA1-Lys6 peptide activated T cell did not show any cytokotoxic 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 malfolded after translation (defective ribosomal products or DRPs), and these DRPs 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 (20, 56, 265, 438). Most of EBV healthy carriers show high IgG reactivity to EBNA1 (114).

### 6.c.1. 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 antigens (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 (LDLLFL) (73, 306). 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 abundantly produced 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) 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 T_h 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, 282, 345).

### 6.c.2. 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 cells 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 lower in about 40-60% of NPC sera from different ethnicity (234, 272).

### 6.c.3. 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 reponses 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 appears 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 Scocot (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, BMF1 (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. 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. Cholosty analysis revealed individual responses to two immediate early proteins BZLF1 and BRLF1 (355, 376). In the case of autoimmunity, 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 immunodepressed patients) during which antibody titers against ZEBRA and other early antigens were significantly increased (339). 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.
Chapter 1 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).

Role in the endogenous processing of CTL epitopes (195, 347). Therefore, BL cells very low levels of antigen processing genes (179). Endogenous presentation of MHC-I epitopes (236). Furthermore, BL cells express viral antigen, EBNA1. EBNA1 can evade from immune recognition through its GAr, 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 LMP1 effects to ensure persistent IL10 production since EBERs are expressed (230). IL10 induction, which was also shown for Hodgkin Lymphoma by Dukers et al (109).

Related lymphoma produce detectable amount of IL-10 (45). Most of these cells in infection, in particular via LMP1 (109). The majority of EBV-transformed 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 latently 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-β), which can block tumour-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 (183, 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-γ by this cells (75). Therefore BARF1 may interfere with the IFN-γ 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
Chapter 1

were shown for EBNA1-specific CD4+ CTLs (285, 287, 301). More recently similar results were efficiently recognized by EBNA2 reactive T cells. On the other hand, a study by Herbst et al (162) showed that BL cell lines pathways result in the limitation of using antigen-specific immunotherapeutic strategies. In the type I malignancies, low expression of immunogenic antigens (eg. EBNA3A, 3B and 3C) important basis for the development of immunotherapeutic strategies. In the type II malignancies such as BL, HD, and NPC limits the efficacy of this kind of immunotherapeutic strategies (204).

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. Adoptive 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 (eg. 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).
Chapter 1

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 (203), 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 Cha 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). Bhanna 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 CTLs 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 leukaocyte antigen identical siblings, showing an increasing of endogenous tumor-infiltrating CD8 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 Strathoff 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) (146).

Totally different approach by vaccination inducing antibody against EBV were also developed. The first prophylactic vaccination with vaccinia gp350 result 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.

9. Aims and outline of this thesis

Nasopharyngeal carcinoma (NPC) is one of the EBV associated malignant diseases, which shows latency type II by expression of several EBV gene products including EBER-1, EBER-2 and BART transcripts encoding miRNAs, as well as EBNA-1, LMP-1, LMP-2A, -2B and BARF1. This etiological link between EBV and NPC is also indicated by the elevated IgG and especially IgA antibodies against EBV antigen complexes prior to clinical manifestation. Aberrant EBV serology is commonly used to support NPC diagnosis and provides an affordable approach for population screening to identify individuals with high NPC risk and to monitor treatment outcome. For the development of affordable diagnostic tools we developed in this study a protocol to provide a reliable and reproducible EA antigen and analysed each component in the EA complex for its diagnostic value.

The LMP1 and LMP2 “non-self” proteins can be an effective target for the immune response, despite the fact that tumor cells expressing LMP1 and LMP2 are not eradicated by natural immune responses. Many studies developed T cell therapy specific to these proteins, but their effectiveness is still under debate. Importantly, LMP1 and LMP2A/B are suggested to stick out from the cell surface via several conserved small loop domains connecting the transmembrane helices. These loop-domains have not been studied as target for humoral immune response. Such anti-kop antibodies may have potentially important function in targeting complement and/or FcR-bearing killer cells to LMP1, 2 expressing tumor cells. In this study we evaluate the humoral immune responses to the protein expressed on the tumor i.e. LMP1, LMP2 and BARF1 and analysed the possibility to use specific antibody to extracellular loop domains of these proteins for therapeutic approach for 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 identified IgG and IgA responses to individual EBV antigens and defined their diagnostic value.
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, DNAase, 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 consist 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 S9 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 cytolyis 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.
Chapter 1

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Chapter 1


Chapter 1


Chapter 1

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Chapter 1


Chapter 1


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Chapter 1


Introduction

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Chapter 1


1. Introduction

2. The Epstein-Barr Virus and Its Infection of B Cells

3. Biology of Epstein-Barr Virus Infection

4. Clinical Manifestations of Epstein-Barr Virus Infections

5. Immunology of Epstein-Barr Virus Infections

6. Diagnosis of Epstein-Barr Virus Infections

7. Treatment of Epstein-Barr Virus Infections

8. Future Directions in Epstein-Barr Virus Research

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