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1. Introduction

Since its discovery, Epstein-Barr virus (EBV) has been associated with a variety of both infectious and malignant human diseases. EBV was first detected by electron microscopy in cultured Burkitt's lymphoma cells in 1964 by Epstein, Achong and Barr. EBV is a DNA virus with a linear genome of 172 kb and belongs to the genus lymphocryptovirus within the family of gamma herpesviruses. These viruses are characterized by (B-cell) lyphotropism, their ability to establish latent infection in host cells and to induce proliferation of these latently infected cells. EBV was distinguished from other, at the time known human Herpes viruses, because of its strong association with Burkitt's lymphoma and its potent growth-transforming ability for B-lymphocytes in vitro. Approximately 90% of humans will become infected with EBV, generally without clinical evidence of disease. Primary infection usually occurs asymptotically in childhood and results in a lifetime carrier state with periodic release of infectious virus into saliva which may cause infection of naive individuals. Sometimes later, e.g. during adolescence, the latter type of infection may be referred to as kissing disease or Pfeiffer's disease. EBV causes various benign syndromes, such as infectious mononucleosis, chronic active EBV infection, X-linked lymphoproliferative disease and oral hairy leukoplakia. EBV has also been associated with malignant diseases including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's lymphoma, and post-transplant lymphoproliferative disease (PTLD). This thesis deals with the development of molecular monitoring of EBV-DNA in plasma of recipients of allogeneic hematopoietic stem cell grafts and the introduction of such monitoring in therapeutic and preventive approaches. In this chapter we shall briefly review the pathogenetic role of EBV in infectious and malignant diseases. Subsequently the clinical presentation, diagnosis, incidence, risk factors and treatment of EBV associated lymphoproliferative disease (LPD) in recipients of hematopoietic stem cell transplants is discussed followed by an outline of this thesis.

2. Infection, immunity, and malignancy

EBV-infection

EBV shedding in the oropharynx occurs intermittently in EBV-seropositive individuals, during such periods of EBV shedding other persons may become infected. When EBV enters the oropharyngeal cavity it penetrates local lymphoid tissue (lingual-, palatine-, and pharyngeal tonsils). Squamous epithelium covering the lymphoid tissue dips into the tonsillar crypts where B-cells are densely situated. Following primary infection these B-cells are the first to be infected. Although earlier studies have discussed elaborately whether EBV replicates in epithelium 9-14, a recent study identified EBV replication in epithelial cells in vitro. Following contact between virus and B-cells, EBV enters the B-cell by binding its major viral envelope glycoprotein 350/220 to the EBV receptor CD21
(receptor for complement C3d) on the surface of the B-cell. Binding of glycoprotein 42 to major-histocompatibility-complex class II (co-receptor) and binding of glycoprotein 350/220 to its receptor facilitate fusion of the viral envelope with the B-cell during endocytosis. Receptor binding results in activation of the B-cell, which may further favor penetration of the virus into the cell. The de-enveloped capsid subsequently travels into the nucleus, degrades and releases viral DNA.

**Immortalization of B-cells**

Linear EBV-DNA is being transported to the cell-nucleus, where transcription and translation of Epstein-Barr virus nuclear antigen (EBNA)-2 and EBNA-leader protein (LP) start within 4 hours following infection. EBNA-2 is essential for B-cell transformation and is an activator of EBV-latent membrane protein (LMP)-1 and LMP-2 and genes involved in growth and transformation of the infected B cell. EBNA-LP augments the ability of EBNA-2 to up-regulate LMP-1. LMP-1 contributes to the growth and transformation of B-cells and is the major transforming gene of EBV. LMP-1 mimics in this respect the function of CD40, a receptor constitutively present on the surface of B-cells. B-cell activation leads to differentiation of the B-cell to a B-blast, that expresses besides EBNA-2, LMP-1 and EBNA-3A, 3B, 3C and EBNA-4. The circularisation of linear EBV-DNA to episomal EBV-DNA (circular form of EBV-DNA) in the B-cell nucleus is completed twenty hours after infection, and with the expression of EBNA-1 the transformation of the B-cell to an immortalized B-cell is completed. LMP-2 is only transcribed once the circular viral episome is formed and prevents B-cell activation stabilising the latent state. The non-translated types of EBV-encoded RNA (EBER) do not encode proteins but may be important for oncogenesis and resistance to programmed cell death, or apoptosis.

**Latency and reactivation**

EBNA-1 plays a pivotal role in the maintenance of EBV in dividing latently infected B-cells. EBV-infected B-cells in vivo can express four different programmes of gene usage depending on the location and differentiation stage of the infected B-cell. One of these programmes is used to produce infectious virus, the other three are all associated with latent infection, in which no virus is produced. These latent growth programmes are known as the growth programme, the default programme and the latency programme (Table 1). During the growth programme EBV infects resting naive B-cells (CD27−, sIgD+) in tonsils and drives these cells out of their resting state to become activated proliferating lymphoblasts. Three viral proteins are expressed in the default programme, of which EBNA-1 ensures replication of the viral genome during cell division. Physiologically, B-cells that encounter antigen become activated and migrate into the follicle of a lymph node where they form germinal centers. Following proliferation and somatic hypermutation, cells that have mutated their immunoglobulin genes become antibody producing plasma cells or memory B-cells, remaining cells that are not selected...
go into apoptosis. Signals through the B-cell receptor and from T helper cells are essential for B-cell survival during this period. EBV mimics these pathways to rescue an activated B-lymphoblast into the memory B-cell pool and create a state of latency. The key proteins to achieve that goal are LMP-1 and LMP-2A.

### Table 1. Latency transcription Programmes (adapted from 27)

<table>
<thead>
<tr>
<th>Programmes</th>
<th>Gene expression</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth programme</td>
<td>EBNA 1-6, LMP-1, LMP-2A, LMP-2B</td>
<td>Activates resting B-cell to become a proliferating lymphoblast</td>
</tr>
<tr>
<td>Default programme</td>
<td>EBNA-1, LMP-1, LMP-2A</td>
<td>Provides necessary survival signals for:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. infected lymphoblasts to differentiate into memory B-cells,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. maintenance of persistently infected memory B-cells</td>
</tr>
<tr>
<td>Latency programme</td>
<td>None (LMP-2A)</td>
<td>Persistence of virus in resting recirculating memory B-cells</td>
</tr>
</tbody>
</table>

EBNA indicates Epstein-Barr virus nuclear antigen; LMP, latent membrane protein.

First, LMP-1 interacts with a set of molecules that act as intermediary in signalling by tumour necrosis-factor-receptor-associated factors (TRAFs). CD40 is a member of TRAF and situated on germinal centre B-cells. Following stimulation by CD154, situated on T helper cells, CD40 can deliver a survival signal for the B-cell, rescuing it from apoptosis and driving proliferation. LMP-1 acts as a functional homologue of CD40 and in addition induces cellular bcl-2 providing apoptosis resistance. Secondly, receptors for LMP-2A and the B-cell receptor contain a common pathway (immunoreceptor tyrosine based activation motifs, ITAM). In the absence of antigen, the B-cell receptor delivers a non-proliferative signal that is essential for survival of B-cells. This is the signal that LMP-2A mimics. During this process the immunodominant EBNA-2, EBNA-3A, 3B, 3C are not expressed. Analogously to normal B-cells encountering an antigen, EBV-infected B-cells enter the follicles and undergo germinal centre differentiation to become memory B-cells. When the EBV-infected B-cell ultimately leaves the tonsil into the peripheral circulation as resting memory B-cell (CD27+, Ig+, CD23−, CD5−) it will switch off all latent gene expression. In this way the virus can persist in a benign state and will not be recognized by the immune system as the main targets for cytotoxic T-cell response lack expression.

In a small proportion of latently infected B-cells, EBV eventually may undergo lytic replication. This lytic replication is accompanied by considerable nuclear and cytoplasmic
changes of infected cells, because new virus particles need to be produced. Production of new virus includes, synthesis of viral DNA, assembly of nucleocapsids, and transport of the virus through the nuclear membrane followed by cytoplasmic transport of tegumented capsid and envelopment through budding into Golgi vesicles followed by release of enveloped virions by exocytosis at the plasmamembrane. The switch from latency to lytic replication involves the expression of the immediate early genes BZLF1 and BLRF1 followed by the coordinate expression of a cascade of about 80 early and late genes. Early genes are essential for viral replication. BHRF1, most abundantly expressed during this cycle, shows structural and functional homology to host-encoded bcl-2 having anti-apoptotic activity also. Other important early genes are, BALF1 which regulates the function of BHRF1, BALF2 being important in DNA replication, and BALF5, encoding the DNA polymerase protein. Lastly, viral genome replication, production of structural proteins and subsequent virion assembly takes place. These processes finally lead to donor cell death and release of viral progeny. Important genes in this stage are the BLLF1 gene, which encodes for the glycoprotein 350/220 (capsid antigen) that mediates binding to CD21, and BCRF1, a viral homologue of interleukin-10, which may stimulate B cell proliferation and inhibit the function of cytotoxic T-cells.

Immune response to EBV-infection

Cellular immunity
Initial cell mediated defense against EBV consists of direct cytolysis by non-specific T-cell or natural killer cell responses. These are followed by the development of EBV-specific CD4$^+$ and CD8$^+$ T-cell responses. Cytotoxic T-cells especially recognize epitopes from the EBNA-3 family of latent proteins. Less common, subdominant, reactivities against EBNA-2, EBNA-LP, LMP-1 and -2 have been described. Cytotoxic T-cells may become memory cells after clearance of infection (1/1000 T-lymphocytes versus 1/100,000 EBV infected B-cells), that persist for live and may respond rapidly upon reactivation of the virus. EBNA-1 may escape the immune response by virtue of impaired major histocompatibility complex presentation, as its Glycine/Alanine repeat domain prevents proteasome-dependent processing for presentation by major histocompatibility complex class I. Reactivation of EBV from latently infected B-cells generally results in lytic antigen expression. Several immediate early (BZLF1, BRLF1) and early (BMLF1, BMRF1, BALF2) antigens serve as targets for specific CD8$^+$ cytotoxic T-cells. These lytic-antigen specific T-cells can mount up to 40% of the total CD8$^+$ T-cell population during acute infection, whereas only 2% is targeted to a latent EBV protein sequence.

Humoral immunity
The antibody response to primary EBV infection is characterized by substantial immunoglobulin (Ig) M titres against viral capsid antigen and rising IgG titres to early antigen. Functionally, antibodies to viral membrane antigen (glycoprotein 350) can both neutralize infective free virus and direct antibody-dependent cellular cytotoxicity against
productively infected cells.\textsuperscript{53,54} Such neutralising antibodies develop late after primo-infection and persist lifelong, but they are not able to control the proliferation of latently infected cells, and may only limit infection and prevent superinfection by new incoming strains.\textsuperscript{55} Anti-EBV antibody titres are characterised by a lifelong persistence at a specific level in one patient.\textsuperscript{56} The serologic hallmarks of a non-immuno-compromised EBV seropositive person are: Ig G antibodies to EBNA-1, Ig G antibodies to viral capsid antigen and Ig G antibodies to Zebra (BZLF1, lytic gene).\textsuperscript{16,53,54,56,57}

**EBV-induced infectious syndromes**

*Infectious Mononucleosis*

Most primary EBV infections occur in infancy and are asymptomatic. In adolescence and in adults however up to 50\% of EBV infections may result in symptomatic infectious mononucleosis.\textsuperscript{2} A recent study showed that expansion of T-cells differs depending on whether the infection progresses without clinical symptoms or develops into infectious mononucleosis.\textsuperscript{58} The classical triad consists of fever, lymphadenopathy, and pharyngitis. Most patients have lymphocytosis with cytologically atypical lymphocytes, heterophilic serum antibodies, and elevated serum aminotransferase levels. In general, symptoms subside within a few weeks without sequelae. Complications may include splenic rupture, hepatitis, myocarditis, encephalitis, meningitis, aplastic anemia, and thrombocytopenia. To date no specific therapy has been proven to be effective in infectious mononucleosis.

*Chronic active EBV infection*

Chronic active EBV-infection starts as a primary EBV infection but lasts for more than 6 months and is accompanied by abnormally elevated EBV antibodies titres (anti-viral capsid antigen IgG, anti-early antigen IgG) and low titres to EBNA. In addition, histologic evidence of major organ involvement (pneumonitis, uveitis, hepatitis, splenomegaly, bone marrow hypoplasia) and increased viral load in affected tissues may be present.\textsuperscript{59,60} Although the exact pathogenesis of chronic active EBV-infection is not clear, clonal expansion of EBV-infected T or natural killer (NK) cells may be observed.\textsuperscript{61,62} The T-cell type of disease is characterised by fever, and high titres of EBV antibodies, whereas hypersensitivity to mosquito bites and high IgE may prevail in patients with the NK-cell type of chronic active EBV infection. The cytotoxic T-cell response in chronic active EBV infection is restricted to a few epitopes.\textsuperscript{63} Furthermore, this limited immune response and the low antigenicity (expression of latency genes EBER and EBNA-1 only) of EBV infected T- and NK cells in chronic active EBV-infection may explain the aggressive clinical course. Although allogeneic bone marrow transplantation and cytotoxic T-cell infusion have been reported successful, to date, no specific treatment for chronic active EBV infection has been established.\textsuperscript{64-66}
X-linked lymphoproliferative disease
X-linked lymphoproliferative disease is an inherited disease only affecting males, which results from a mutation in the signalling lymphocyte activation molecule (SLAM) gene located on the X-chromosome. The absence of a functional SLAM gene may impair normal T and B cell interaction, which results in unregulated growth of EBV-infected B-cells. Most patients die of infectious mononucleosis (57%), furthermore, frequent other complications are hypogammaglobulinemia (29%) and malignant lymphomas (24%).

Oral hairy leukoplakia
Oral hairy leukoplakia especially occurs in immunocompromised patients (transplant recipients, human immunodeficiency virus (HIV)-infected patients). It presents as white wartlike epithelial lesions of the oral mucosa, especially the lateral border of the tongue, which contain EBV-DNA and Herpesvirus like particles. Multiple strains are often present in one lesion, showing active viral replication and expression of lytic viral proteins. Aciclovir may be an effective treatment modality, however, frequent recurrences occur after therapy has been stopped.

Malignancies associated with EBV

Hodgkin’s Disease
Hodgkin’s disease is a malignant lymphoma characterised by the presence of mononuclear Hodgkin cells and their multinucleated variant, the Sternberg-Reed cell. The background of these cells consists of lymphocytes, plasma cells, histiocytes and eosinophils. Hodgkin cells and Sternberg-Reed cells are derived from B-cells in most cases. EBV-DNA (BamHI-W region of the EBV genome) can be detected in 40-60% of patients with Hodgkin’s disease, especially in the lymphocyte depleted subtype and mixed cellularity subtype. The EBV genome may be present in the Hodgkin’s and Reed-Sternberg cells, and is monoclonal. Individuals with a history of infectious mononucleosis carry a 2-4 fold increased risk of developing Hodgkin’s disease, the risk being most pronounced during the first 3 years following primary infection. Antibody titres to viral capsid antigen- and early antigen-proteins may be high at diagnosis of Hodgkin’s disease. The latter 3 observations suggest a causative link between EBV and Hodgkin’s disease.

Burkitt’s Lymphoma
Burkitt’s lymphoma is a high-grade lymphoma of small, noncleaved B-cells. The endemic (African) form is EBV DNA positive in 90% of cases. Endemic Burkitt’s lymphoma is characteristically located in the jaw or abdomen and preferentially occurs during childhood. In Africa infection with Plasmodium falciparum is thought to play a role in the pathogenesis of Burkitt’s lymphoma because it stimulates B-cell proliferation and diminishes T-cell control of proliferating EBV-infected B-cells favoring Burkitt’s lymphoma. Children with elevated antibodies to EBV are at risk for developing Burkitt’s lymphoma in endemic areas. Sporadic Burkitt’s lymphoma, occurring in Europe and the
United States, is associated with EBV in only 20% of cases. It most commonly presents with an abdominal mass and is seen at older age. HIV infected individuals are at risk for Burkitt’s lymphoma. The clinical presentation resembles that of the sporadic form. Burkitt’s lymphoma cells often contain chromosomal translocations involving chromosomes 8 (c-myc oncogene), 14 (immunoglobulin heavy chain), or chromosomes 22 and 2 (immunoglobulin light chain). The translocation t(8;14) is apparent in 80% of patients with Burkitt’s lymphoma. The variant translocations, t(2;8) or t(8;22), are evident in 20% of cases. The translocations result in juxtapositioning of the c-myc oncogene to the constant region of the heavy or light chain. Overexpression of c-myc results in increased tumorigenicity of EBV-immortalized B-cells.

T-cell non-Hodgkin’s lymphoma
Although EBV is considered a B-lymphotropic virus, EBV may also infect T-cells and NK-cells. To date, 3 distinct categories of T-cell non-Hodgkin’s lymphomas (NHL) have been associated with EBV. These include: 1. virus-associated hemophagocytosis associated T-cell lymphocytosis/lymphoma, 2. nasal T-NHL, and 3. peripheral T-NHL. It has been postulated that EBV may enter the T-cell during the process of B-cell kill by an activated cytotoxic T-cell. As T-cells may exhibit a very low expression of CD21, EBV may enter the T-cell via CD21, if present and functional, or an alternative receptor or mechanism. Despite intensive chemotherapy outcome of EBV related T-cell NHL is poor. Especially, fulminant T-cell NHL occurring in the setting of a chronic active EBV-infection has a dismal outcome.

Nasopharyngeal Carcinoma
Nasopharyngeal carcinoma is especially prevalent in southern China, northern Africa and Alaskan Eskimos. The incidence approaches 50 per 100,000 persons per year in southern China as compared to <1 per 100,000 in other parts of the world. Almost 100% of anaplastic or poorly differentiated nasopharyngeal carcinoma contain EBV and express EBV proteins, whereas the majority of keratinizing squamous-cell and non-keratinizing nasopharyngeal carcinoma express EBV. The EBV genome is present in transformed epithelial cells but not in the lymphocytes of the tumor. Clonal EBV is found in the early dysplastic lesions or carcinoma in situ, indicating that EBV infection precedes the development of nasopharyngeal carcinoma. The detection of EBV specific IgA antibodies in nasopharyngeal carcinoma patients may be useful in screening individuals for nasopharyngeal carcinoma and monitoring patients during follow-up after treatment of established disease.

Gastric Carcinoma
Approximately 10% of gastric carcinomas are associated with EBV worldwide. The expression pattern is exceptional as only EBNA-1, EBER and LMP-2 are detected, and LMP-1 and EBNA-2 are not expressed. Transcription of the transforming and immortalizing EBV gene BARF1 (BamHI A rightward open reading frame) may be
detected in addition which suggests that it may act as an alternative viral transforming factor instead of LMP-1.94

3. Post-transplant lymphoproliferative disease

Introduction

Most solid organ and bone marrow recipients/donors carry latent EBV at the time of transplantation, as a result of the widespread distribution of EBV. Uncontrolled outgrowth of EBV infected B-cells may occur in patients with a severe immune deficiency, as may occur following allogeneic hematopoietic stem cell transplantation, solid organ transplantation, and congenital/acquired immune deficiencies.16,17,59,68,95-97 Uncontrolled B-cell proliferation may result in B-cell lymphoproliferative disease, which is associated with considerable morbidity and mortality.17 Post-transplantation lymphoproliferative disorders (PTLD) refer to a range of hyperplastic to neoplastic lymphoid proliferations which occur after solid organ- or bone marrow transplantation, they mostly are of B-cell origin, and commonly (90%) contain EBV.98 We shall refer to PTLD as EBV-lymphoproliferative disease (EBV-LPD) only if the presence of EBV was unequivocally demonstrated in malignant lymphocytes, thereby showing the causative role of EBV. Crawford et al were the first to demonstrate the presence of EBNA in a PTLD occurring in a renal transplant recipient.99 The majority of PTLDs is EBV positive (EBV-LPD), as has been concluded from immunohistological and molecular studies. EBV-DNA has been found in PTLD tumor tissue by in situ hybridisation and Southern blotting.17,100-102 Furthermore, EBV-specific proteins have been detected by immuno-histochemistry and Western blotting.102,103 The incidence of PTLD varies among different transplant groups. The incidence is ± 1% among recipients of renal grafts, ± 2% after liver transplantation, 3-4% after heart transplantation, 8% after lung transplantation, 28.5% in recipients of intestinal transplants. The incidence of PTLD after allogeneic hematopoietic stem cell transplantation may vary between 2-25%.104-112 Differences of incidence may reflect the intensity of immunosuppressive measures applied to prevent rejection or graft-versus-host disease after transplantation.

Clinical presentation

The clinical presentation of PTLD may vary considerably as B-cell lymphoproliferation may present in nodal or extranodal site(s) and also as a B-cell leukemia. Generally, 3 characteristic clinical presentations may be distinguished.17,98,113-116:

1. A mononucleosis-like syndrome with fever, sore throat, myalgia, tonsillar hypertrophy and cervical lymphadenopathy.
2. A tumorous form with symptoms secondary to the presence of lymphoid tumours including pain, obstruction or perforation.
3. Disseminated disease with monoclonal B-cells in blood and bone marrow, high fever, and/or multiorgan failure.

Most commonly, the first 2 presentations of PTLD are seen following solid organ transplantation, whereas the third form is more frequently encountered following allogeneic hematopoietic stem cell transplantation. The more aggressive clinical presentation of the latter may be explained by an immediate and severe immunopromised condition in the first months following allogeneic hematopoietic stem cell transplantation. PTLD is localised within the allografted organ in 36-100% of recipients of a renal or lung graft. In contrast, patients allografted with a liver or heart rarely experience PTLD at the site of their donor organ. Both origin (donor versus recipient) of the B-cell and virus may differ comparing PTLD following allogeneic hematopoietic stem cell transplantation and solid organ transplantation. PTLD generally develops in donor B-cells in the setting of an allogeneic hematopoietic stem cell transplantation, whereas recipient B-cells usually cause PTLD following solid organ transplantation. PTLD most commonly originates from recipient EBV if the recipient has been EBV-seropositive before transplantation. EBV seronegative recipients of a solid organ transplant may acquire donor EBV and subsequently be at risk of developing PTLD caused by donor EBV.

Diagnosis of PTLD

A diagnosis of PTLD is usually made on the basis of lymph node histology. However, thorough histological examination, including morphology, immunohistochemistry to assess clonality and presence of LMP, and molecular evaluation to detect EBER or EBV-DNA should be performed in order to assess clonality and the causal relation with EBV (EBV-LPD). Investigations required for staging include whole body computer tomography, flow cytometric or molecular detection of monoclonal B-cells in blood, bone marrow, and cerebrospinal fluid (if indicated), or T-cell receptor rearrangement studies in case of a T cell lymphoma. Serology is of little value in the diagnosis of PTLD, and negative serology may even be misleading due to the severe immune dysfunction of patients.

Pathological classification systems of PTLD distinguish 3 subtypes.

1. **Plasmacytic hyperplasia.** The underlying architecture of the lymph node is preserved but is diffusely infiltrated by plasmacytoid lymphocytes with some plasma cells and sporadic immunoblasts. B-cells are preferably polyclonal. It furthermore lacks oncogene and tumor suppressor gene rearrangements, such as N-ras, c-myc, p-53, bcl-2 or bcl-6.

2. **Polymorphic B-cell hyperplasia/lymphoma.** These tumors arise at nodal and/or extranodal sites and mostly show monoclonal lymphocyte infiltration. Histological examination shows a combination of plasmacytic lymphocytes / plasma cells and immunoblasts without atypia and only mild necrosis. No oncogene and tumor
suppressor gene rearrangements are present. When the transition to lymphoma occurs plasmacytoid differentiation is lacking, and atypical immunoblasts prevail in the presence of necrosis.

3. **Immunoblastic lymphoma.** Histologically, large monomorphic atypical immunoblasts are present with abundant necrosis. It may present as a disseminated monoclonal lymphocyte neoplasm, containing only one EBV-strain with alterations in one or more proto-oncogenes or tumor suppressor genes, such as N-ras, c-myc, p-53, bcl-2 or bcl-6.\textsuperscript{124}

From a clinical point of view, the distinction between polymorphic hyperplasia and immunoblastic lymphoma has proven to be rather artificial, because both histological subtypes can be present concomitantly and they do not predict a different clinical course and response to therapy. Lesions may contain monoclonal and/or oligoclonal lymphocyte components, and may or may not express oncogene alterations. The term polymorphic PTLD has been introduced to refer to those PTLDs with characteristics of both immunoblastic and polymorphic B-cell hyperplasia.\textsuperscript{105,128,129}

Immunohistology may include antibody staining with CD19, CD20, LMP-1, EBNA-1, and EBNA-2. In contrast to LMP, both EBNA-1 and –2 are expressed very heterogeneously in PTLD lesions.\textsuperscript{126} In order to differentiate between poly- and monoclonal disease staining with monoclonal antibodies to kappa and lambda can be performed. Alternatively, immunoglobulin gene rearrangement studies can be done. Assessing clonality, however, may be of limited value because both poly- and monoclonal proliferations may coexist in one PTLD lesion.\textsuperscript{130,131} Using molecular diagnostic techniques, such as in situ hybridisation and polymerase chain reaction, EBER and EBV-DNA can be detected.\textsuperscript{132} These assays are important for a diagnosis of EBV associated disease.\textsuperscript{100} Karyotypic analysis of biopsy material from PTLD lesions is usually normal, however, deletions or translocations of chromosome 6, trisomy 11, and t(8;14) can be found.\textsuperscript{116} Untill recently, no systematic analysis has been performed to assess whether the presence of certain oncogenetic mutations in PTLD predicts clinical outcome. In a recent study, the prognostic value of mutations in the proto-oncogene BCL-6 in 33 solid organ recipients with histologically proven PTLD has been reported.\textsuperscript{135} Mutations in BCL-6 appeared to predict failure of response to reduction in immunosuppression.

**EBV load**

EBV serology is the gold standard for diagnosing EBV-infection in immunocompetent individuals, but is of only limited value in immuno-compromised individuals.\textsuperscript{134} Therefore, various authors have explored other ways to monitor EBV-infection in immunodeficient individuals. The diagnosis of EBV infection depends to a large extent on assaying viral nucleic acids (DNA) in conditions of immunodeficiency. Viral nucleic acids can be measured quantitatively in different compartments using various methods. Viral load can be assessed in plasma, mononuclear cells (MNC), or in whole blood samples.\textsuperscript{135-147} Comparative polymerase chain reaction (PCR) assays with end-point dilution, quantitative competitive PCR assays as well as the more recent real-time quantitative PCR assays have
been used.\textsuperscript{144,145,148,149} Real-time PCR monitoring of EBV DNA has emerged as a sensitive and reproducible test.\textsuperscript{144,150,151,152} High levels of EBV DNA in peripheral blood may be associated with a diagnosis of EBV-LPD. However, a high viral load may also be present in patients with infectious mononucleosis and in transplant recipients without definite EBV-LPD.\textsuperscript{142,150,153-155} On the other hand, a gradually increasing EBV load is an important risk factor for impending EBV-LPD.\textsuperscript{135,138,143,149,155-157} In case of established EBV-LPD, EBV load may be used to monitor response to therapy.\textsuperscript{139,151,153,156-159} A recent report suggested that plasma viral load may be more specific for a diagnosis of EBV-LPD following renal transplantation as compared with measurement of EBV-DNA in MNC.\textsuperscript{151} Furthermore, plasma PCR may reflect more closely the response to therapy for EBV-LPD both following solid organ transplantation and allogeneic hematopoietic stem cell transplantation.\textsuperscript{139,151,159}

Detection of EBV specific immunity

By using tetramers of specific HLA-class I molecules loaded with viral peptides, it has become possible to detect peptide-specific T-cells in the setting of cytomegalovirus (CMV)-disease and EBV-lymphoproliferative disease.\textsuperscript{160-163} Marshall et al studied the development of EBV-specific CD8\textsuperscript{+} T-cells using the tetramer technique following unmanipulated- and T-cell depleted allogeneic hematopoietic stem cell transplantation. They showed that EBV-specific T-cells formed a substantial percentage of CD8 T-cells within one month of unmanipulated transplantation. A correlation between T-cell numbers and viral load was demonstrated.\textsuperscript{164} The recovery and function of these EBV-tetramer-binding T-cells following T-cell depleted allogeneic hematopoietic stem cell transplantation is currently unknown, but might greatly add to our understanding of the pathogenesis of EBV-LPD following allogeneic hematopoietic stem cell transplantation. Humoral immunity is of little value in the management of patients with PTLD following allogeneic hematopoietic stem cell transplantation and will therefore not be discussed.\textsuperscript{123}

Risk factors for developing PTLD

The presence of a sufficient number of EBV-specific T-cells with potent anti-EBV activity is pivotal for prevention of PTLD.\textsuperscript{52,98} Therefore, especially factors, which adversely affect the presence and maturation of T-cell-dependent immune responses and generation of cytotoxic T-cells, may become risk factors for EBV-LPD.

Risk factors in recipients of solid organ transplants.

Table 2 shows results observed in large studies performed in at least 100 recipients. Opelz et al assessed the incidence of PTLD among 52,775 solid organ transplant recipients (kidney and heart). They showed that the risk of PTLD correlated with the cumulative intensity of the immunosuppressive regimen applied for prevention of transplant rejection.\textsuperscript{104}
Table 2. Risk factors for developing PTLD following solid organ transplantation *

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of Tx</th>
<th>No of patients</th>
<th>Risk factor</th>
<th>No of patients with risk factor</th>
<th>No of patients at risk developing PTLD</th>
<th>Relative Risk (range)</th>
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<td>Heart</td>
<td>154</td>
<td>OKT3 dose (&gt; 75 mg)</td>
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<tr>
<td>Shpilberg 177</td>
<td>Liver, lung, kidney, heart</td>
<td>303</td>
<td>CD56\textsuperscript{+}DR\textsuperscript{+} count</td>
<td>nr</td>
<td>32</td>
<td>6.9</td>
</tr>
<tr>
<td>Swerdlow 174</td>
<td>Heart, lung, heart-lung</td>
<td>1,563</td>
<td>EBV-serostatus recipient (-)</td>
<td>127</td>
<td>23</td>
<td>10</td>
</tr>
</tbody>
</table>

Tx indicates transplantation; OKT3, anti-T-cell antibody Ortho-Klone; ATG, Anti-Thymocyte globulin; CyA, cyclosporin A; AZT, azathioprin; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; nr, not reported; (-), negative; PTLD, post-transplant lymphoproliferative disease.

*) only risk factors that appeared significant were included in this table.
Patients at highest risk were those, who had received immuno prophylaxis with anti T-cell antibodies (Relative Risk (RR): 1.8) and patients, who received a combination of the immunosuppressive agents cyclosporin A and azathioprine (RR: 1.5).\textsuperscript{104,165} Swinnen et al evaluated the effect of the use of the anti-T-cell antibody Ortho-Klone (OKT3) on the incidence of PTLD in heart transplant recipients.\textsuperscript{165} OKT3 is a murine monoclonal antibody reactive with the human CD3-receptor-T-cell complex and exerts broad anti-T-cell activity.\textsuperscript{165,166} The incidence of PTLD was 1.4\% in patients without OKT3 treatment as compared to 11.4\% in patients who had been treated with OKT3 (RR: 9.5). Successive treatment courses with OKT3 further increased the cumulative incidence of PTLD to 35\%.\textsuperscript{165,167} The immunosuppressive drug mycofenolate mofetil in combination with cyclosporin A did not increase the incidence of PTLD in renal allograft recipients as compared to azathioprine/cyclosporin A or cyclosporin A alone.\textsuperscript{168,169} Both cyclosporin A and tacrolimus primarily inhibit T-helper cell activation through inhibition of interleukin-2 production.\textsuperscript{116} Mycofenolate mofetil exerts reversible inhibition of the enzyme inosine monophosphate dehydrogenase required for purine synthesis during cell division. Mycofenolate mofetil inhibits proliferation of both T and B cells and thereby the production of antibodies and generation of cytotoxic T-cells. Mycofenolate mofetil may therefore have a beneficial effect on prevention of proliferation of EBV infected B-cells.\textsuperscript{170}

Apart from the intensity of the immunosuppressive regimen, the serostatus of the recipient also emerged as an important risk factor. The incidence of PTLD in EBV seronegative recipients may increase more than 20-fold as compared to EBV seropositive patients, which may be explained by the lack of EBV-specific cellular immunity of the recipient.\textsuperscript{17,171-175} In addition, CMV serostatus also proved an adverse risk factor if the recipient had been CMV seronegative prior to transplantation and the donor CMV seropositive.\textsuperscript{167} That association is not entirely clear, but may be explained by reactivation of CMV in the absence of an adequate immune response and subsequent CMV-induced immune suppression.\textsuperscript{167,176} A high absolute count of activated NK-cells (CD56\(^{+}\)DR\(^{+}\)), as assessed prior to transplantation, may also increase the risk for PTLD.\textsuperscript{177} It may be explained by chronic antigenic stimulation, which especially occurs in patients with prior autoimmune diseases and an immune deficiency prior to transplantation.

**Risk factors in recipients of an allogeneic hematopoietic stem cell graft**

Table 3 shows results observed in large studies performed in at least 100 recipients. As in recipients of solid organ transplants, recipients of allogeneic hematopoietic stem cell grafts may become severely immunocompromised by immunosuppressive agents needed for prevention of rejection and graft-versus-host disease. In contrast to solid organ recipients, recipients of allogeneic hematopoietic stem cell grafts regenerate a new immune system, which originates from the donor. Well known factors impairing immune reconstitution following allogeneic hematopoietic stem cell transplantation include the degree of human leucocyte antigen (HLA)-mismatching between donor and recipient, higher age of the recipient, more intensive radiotherapy applied before transplantation, presence of graft-versus-host disease (GVHD) and T-cell depletion (TCD) of the donor stem cell graft.
<table>
<thead>
<tr>
<th>Reference</th>
<th>No of Patients</th>
<th>Risk factor</th>
<th>No of Patients with risk factor</th>
<th>No of patients at risk developing PTLD</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zutter 122</td>
<td>2,475</td>
<td>OKT3</td>
<td>24</td>
<td>4</td>
<td>nr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCD</td>
<td>64</td>
<td>2</td>
<td>nr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aGVHD II-IV</td>
<td>2,031</td>
<td>6</td>
<td>nr</td>
</tr>
<tr>
<td>Witherspoon 179</td>
<td>2,246</td>
<td>TCD</td>
<td>nr</td>
<td>2</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OKT3</td>
<td>nr</td>
<td>3</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG</td>
<td>nr</td>
<td>7</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA mismatched donor</td>
<td>336</td>
<td>8</td>
<td>3.8</td>
</tr>
<tr>
<td>Gerritsen 115</td>
<td>142</td>
<td>Campath+αCD11a</td>
<td>25</td>
<td>9</td>
<td>nr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA TCD mismatched donor</td>
<td>65</td>
<td>9</td>
<td>nr</td>
</tr>
<tr>
<td>Hale 111</td>
<td>2,582</td>
<td>TCD</td>
<td>20</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Micallef 130</td>
<td>428</td>
<td>TCD</td>
<td>44</td>
<td>5</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG</td>
<td>45</td>
<td>5</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aGVHD II-IV</td>
<td>221</td>
<td>7</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TBI+ATG</td>
<td>38</td>
<td>5</td>
<td>nr</td>
</tr>
<tr>
<td>Curtis 112</td>
<td>18,014</td>
<td>HLA mismatched donor</td>
<td>3,390</td>
<td>30</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCD</td>
<td>2,521</td>
<td>42</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATG</td>
<td>1,101</td>
<td>25</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-CD3</td>
<td>21</td>
<td>3</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aGVHD II-IV</td>
<td>7,063</td>
<td>42</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TBI</td>
<td>13,025</td>
<td>74</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extensive eGVHD</td>
<td>3,872</td>
<td>16</td>
<td>4.0</td>
</tr>
<tr>
<td>Gross 109</td>
<td>2,395</td>
<td>TCD</td>
<td>247</td>
<td>13</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA mismatch</td>
<td>315</td>
<td>11</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*TCD indicates T-cell depletion; aGVHD, acute Graft-versus-Host Disease; HLA, Human Leucocyte Antigen; TBI, total body irradiation; ext eGVHD, extensive chronic GVHD; nr, not reported.

*) only risk factors that appeared significant were included in this table.
All these factors impair reconstitution of a new B-cell and T-cell repertoire following allogeneic hematopoietic stem cell transplantation and thus are also implicated as potential risk factors for PTLD. Several studies have evaluated whether these factors would predict for PTLD and compared them to established risk factors in the setting of solid organ transplantation. The use of anti-thymocyte globulin (ATG) and especially OKT3, as has been observed after solid organ transplantation, were also strongly associated with a considerable risk for PTLD after hematopoietic stem cell transplantation. Furthermore, the degree of T-cell depletion of the donor graft also emerged as an important risk factor. Stringent depletion enhanced the risk of PTLD in several studies. The coexistence of more than 1 risk factor further enhances the risk. The incidence of PTLD rose to 24% in patients, who received a T-cell depleted stem cell graft from a HLA-mismatched donor in a study by Gerritsen et al. The use of unrelated donor stem cell grafts, but especially the use of HLA mismatched donor grafts were also associated with an increased risk of PTLD, although the relative weight was less than the risk associated with the use of OKT3 or T-cell depletion. A greater incidence of PTLD was also observed in patients developing graft-versus-host disease requiring treatment with anti-T-cell immuno-therapy such as anti-thymocyte globulin.

**Therapeutic approaches**

One of the major problems in evaluating treatment of PTLD is the lack of prospective randomised trials. Furthermore, patients often receive multiple therapy modalities concomitantly with a reduction of immunosuppression. Therefore, it has been difficult to assess the merits of each intervention individually. However, various treatments have been evaluated and reported in literature.

**Reduction of immunosuppression**

Reduction of immunosuppression may induce complete remissions of PTLD after solid organ transplantation. Especially patients with localized disease may respond, as patients with disseminated PTLD often show progression despite reduction of immunosuppression. Reduction of immuno-suppression as such has not been studied following allogeneic hematopoietic stem cell transplantation. A retrospective analysis in heart-transplant recipients revealed that the majority of PTLDs occurring in the first year following transplantation respond favorably to reduction in immunosuppression. If PTLD develops more than 1 year after transplantation, responses tended to be rare. Histological subtype of PTLD did not affect the response rate, as responses were readily observed in each subtype and in polyclonal as well as monoclonal PTLDs. A significant disadvantage of reducing immunosuppression is the obvious risk of organ graft rejection or acute graft-versus-host disease in case of allogeneic hematopoietic stem cell transplantation. Therefore, immunosuppression is often interrupted temporarily or the dose reduced only partially. In conclusion, various retrospective studies have shown
that reduction of immune suppression is an important part in the treatment of patients with PTLD.

**Antiviral drugs**

To date no randomised studies have been published addressing the role of antiviral drugs in patients with PTLD. Also no clinical studies have shown that aciclovir and/or ganciclovir may induce durable responses in PTLD. In addition, PTLD has been described consistently in patients receiving both aciclovir and ganciclovir prophylaxis, again indicating the low therapeutic efficacy of these antiviral drugs.\(^{153,180}\) Although EBV shedding from the oropharynx has been reported to be reduced by both aciclovir and ganciclovir, numbers of EBV-infected B-cells were not affected by these drugs.\(^{186,187}\) The lack of efficacy of aciclovir and ganciclovir may be explained by the absence of EBV-induced thymidine kinase in latently infected B-cells, which is needed for both aciclovir and ganciclovir to exert their cytotoxicity.\(^{188}\) Thymidine kinase is expressed during lytic cycle of infection, but not during latency.\(^{188}\) At present it is unknown whether or when B-cells in PTLD lesions express thymidine kinase. It has been suggested to combine ganciclovir with arginine butyrate, as the latter compound may induce the expression of thymidine kinase.\(^{188,189}\) However, no clinical studies have been reported recently. In conclusion aciclovir and ganciclovir cannot be recommended as therapy of PTLD.

**Chemotherapy**

In analogy with the treatment of malignant lymphoma in general, chemotherapy may also induce complete remissions in a high proportion (33-100%) of patients with PTLD. However, long term outcome has been rather disappointing due to high toxicity and sometimes transient duration of responses.\(^{190-193}\) Septic complications in neutropenic patients accounted for the majority of treatment related mortality.\(^{185,190,192,194}\) In order to prevent cardiac- and infectious toxicity, low-dose cyclophosphamide, doxorubicin, oncovin and prednisone (CHOP) like regimes have been studied by Gross and Oertel.\(^{195,196}\) They showed complete remission rates of 75% (6/8) and 100% (3/3), respectively, without severe toxicity in limited numbers of patients. Reduced treatment related mortality was also reported following ProMACE-CytaBOM chemotherapy in cardiac transplant recipients.\(^{194}\) Complete remission rate measured 75% (6 out of 8 patients) and no patient relapsed after a median follow up of 64 months.\(^{194}\) Only case-reports, no larger studies have been published with regard to the use of chemotherapy in PTLD following allogeneic hematopoietic stem cell transplantation. In conclusion, the reduced toxicity profile and high response rates of new chemotherapy schemes may favour the use of chemotherapy in PTLD following solid organ transplantation. Chemotherapy should be applied very cautiously after allogeneic hematopoietic stem cell transplantation as chemotherapy may adversely affect the immune system. Furthermore, the fact that hematopoietic stem cell recipients have often been treated with intensive radio-/chemotherapy prior to transplantation places them at an elevated risk of cumulative toxicity.
**Interferon-α**

Interferon-α is a cytokine with antiviral- and anti-neoplastic activity. Several case reports have described complete remissions in patients with PTLD following solid organ transplantation treated with a combination of interferon-α and intravenous immunoglobulin. Liebowitz studied 18 solid organ transplant recipients with PTLD, who were treated with a reduction of immunosuppression and interferon-α. Fifteen patients obtained complete or partial remissions, but median survival was short and measured approximately 6 months. Three studies evaluated the use of interferon-α for PTLD following allogeneic hematopoietic stem cell transplantation. Durable responses were reported in several patients, however, the specific value of interferon-α could not be assessed in these studies as most patients received additional treatment modalities. Toxic side effects of interferon-α were noted in a considerable proportion of patients. Interferon-α may also negatively contribute to induction of graft rejection or induction of graft-versus-host disease, which may preclude a wider use of interferon-α in PTLD.

In conclusion, although initial reports on interferon-α are promising, larger prospective trials are needed.

**Anti B-cell immunotherapy**

Monoclonal antibodies with specificity for the B-cell surface molecules CD21, CD24 and CD20 have been successfully applied in the treatment of PTLD (Table 4, Table 5). These monoclonal antibodies may exert anti B-cell cytotoxicity by opsonization and antibody-dependent cellular cytotoxicity. Anti-CD21 may also block the receptor by which EBV intrudes the B-cell. Recent reports showed that especially complement mediated cytotoxicity plays an important role when CD20 is used. High remission rates (50-80%) were reported in the first two prospective clinical studies using anti-CD21 and anti-CD24 in solid organ- and bone marrow recipients with established PTLD. Relapse rates in those studies were approximately 10% and survival at 12 months from immunotherapy was ± 50%. Comparable response rates were observed in studies using anti-CD20 immunotherapy using the humanized anti-CD20 monoclonal antibody rituximab (± 70%). Both recipients of solid organ transplants and hematopoietic stem cell grafts showed high response rates and overall survival probabilities of 65 to 75%. Mortality from progressive PTLD was mainly observed in patients with multivisceral disease, central nervous system involvement, or late onset PTLD (> 1 year following transplantation). No serious adverse effects have been described following anti B-cell immunotherapy. Mild neutropenia may occur in 40% of patients receiving anti-CD21 and anti-CD24, and hypotensive reactions secondary to cytokine release have been reported in 10% of patients with bulky disease. B-cell lymphopenia of 6-9 months duration may occur following monoclonal anti-CD20 immunotherapy, which as yet has not been associated with a significant increase in the susceptibility to infections nor with decreased serum immunoglobulin levels.
Table 4. Response of PTLD to anti B-cell immunotherapy following allogeneic hematopoietic stem cell transplantation

<table>
<thead>
<tr>
<th>Reference</th>
<th>No of patients</th>
<th>Therapy</th>
<th>Remission</th>
<th>Relapse</th>
<th>Survival (%) at 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fischer</td>
<td>10</td>
<td>αCD21+αCD24</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Benkerrou</td>
<td>28</td>
<td>αCD21+αCD24</td>
<td>16</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Milpied</td>
<td>6</td>
<td>αCD20</td>
<td>5</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Kuehnle</td>
<td>3</td>
<td>αCD20</td>
<td>3</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Faye</td>
<td>12</td>
<td>αCD20</td>
<td>8</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

α CD20/21/24 indicates, monoclonal antibody therapy against CD20/21/24, respectively; nr, not reported.

In conclusion, anti-B-cell immuno therapy has been established in several phase II studies as an effective and non-toxic therapy for PTLD in both solid organ- and allogeneic stem cell transplantation. Since anti-CD20 monoclonal antibody therapy has been licensed (rituximab), that type of immunotherapy will be applied most frequently. The dose and scheme of administration of rituximab may be subject of further study.

Adoptive transfer of T-cell immunity

Infusion of donor lymphocytes from an EBV-seropositive donor has been proven to be effective in the treatment of established PTLD following allogeneic hematopoietic stem cell transplantation (Table 6). A major drawback, however, is graft-versus-host disease ensuing following donor lymphocyte infusion (30-60%), often resulting in considerable morbidity and mortality. To avoid graft-versus-host disease induced by allo-reactive T-cells, Rooney et al infused polyclonal EBV-specific cytotoxic T-cells in patients who had evidence of uncontrolled EBV replication, either as an elevated EBV load (> 20,000 EBV genomes per µg mononuclear cell DNA) or frank EBV-LPD, following allogeneic hematopoietic stem cell transplantation. Two of 3 patients obtained complete remission, the third patient had a complete remission of a histologically proven PTLD. No patient developed graft-versus-host disease. Functional EBV-specific cytotoxic T-cells could be traced by gene-marking until 18 months following initial infusion. Recently, these results were confirmed in a larger study. Anti-EBV cytotoxic T-cell infusion were administered prophylactically at a median of 3 months following allogeneic hematopoietic stem cell transplantation in 39 patients.
Table 5. Response of PTLD to anti B-cell immunotherapy following solid organ transplantation

<table>
<thead>
<tr>
<th>Reference</th>
<th>Tx</th>
<th>No of patients</th>
<th>Therapy</th>
<th>Remission</th>
<th>Relapse</th>
<th>Survival (%) at 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Complete</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>Fischer 208</td>
<td>Heart, heart-lung, liver,</td>
<td>8</td>
<td>αCD21+αCD24</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>kidney, kidney-pancreas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leblond 185</td>
<td>Heart, lung, kidney</td>
<td>10</td>
<td>αCD21+αCD24</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Benkerrou 207</td>
<td>Heart, kidney, lung, heart-</td>
<td>31</td>
<td>αCD21+αCD24</td>
<td>20</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cook 212</td>
<td>Lung</td>
<td>3</td>
<td>αCD20</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Zompi 216</td>
<td>Liver</td>
<td>3</td>
<td>RI+ αCD20</td>
<td>2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Milpied 211</td>
<td>Liver, kidney, heart, lung,</td>
<td>30</td>
<td>αCD20</td>
<td>15</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>kidney-pancreas,</td>
<td>kidney-pancreas, liver-kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RI+ αCD20</td>
<td>7</td>
<td>-</td>
<td>nr</td>
</tr>
<tr>
<td>Oertel 213</td>
<td>Heart, Liver, kidney</td>
<td>6</td>
<td>αCD20</td>
<td>4</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Caillard 226</td>
<td>Kidney</td>
<td>13</td>
<td>RI+ αCD20</td>
<td>7</td>
<td>-</td>
<td>nr</td>
</tr>
<tr>
<td>Verschuuren 221</td>
<td>Lung</td>
<td>3</td>
<td>αCD20</td>
<td>3</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Tx indicates transplantation; αCD20/21/24, monoclonal antibody therapy against CD20/21/24; RI, reduction of immune suppression; nr, not reported.
In these patients no PTLD occurred, whereas 11% of historical controls (7 out of 61) developed PTLD.\textsuperscript{234} PTLD following solid organ has also been treated with EBV-specific cytotoxic T-cells. Complete remissions were observed in several cases without the occurrence of rejection.\textsuperscript{235-237} Although the treatment strategy is highly specific and without major side-effects, preparation of EBV-specific cytotoxic T-cells is time-consuming and technically elaborate. Furthermore, additional drawbacks may include a diminished effectiveness during use of steroids, occurrence of exaggerated inflammatory response in case of bulky disease, and unresponsiveness to EBV-specific cytotoxic T-cells as a result of mutation of EBV epitopes.\textsuperscript{215,234,238} In conclusion, treatment of PTLD following allogeneic hematopoietic stem cell transplantation by donor lymphocyte infusion is effective, but may be complicated by severe graft-versus-host disease. The preparation of EBV-specific cytotoxic T-cells seems promising, but requires rather elaborate technical preparations.

Table 6. Response of PTLD to donor lymphocyte infusion or EBV specific CTLs following allogeneic hematopoietic stem cell transplantation

<table>
<thead>
<tr>
<th>Reference</th>
<th>No of patients</th>
<th>Therapy</th>
<th>Complete Remission</th>
<th>Relapse</th>
<th>Survival (%) at 1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papadopoulos\textsuperscript{228}</td>
<td>5</td>
<td>DLI</td>
<td>5</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Rooney\textsuperscript{234}</td>
<td>3</td>
<td>CTL</td>
<td>3</td>
<td>0</td>
<td>nr</td>
</tr>
<tr>
<td>Lucas\textsuperscript{148}</td>
<td>5</td>
<td>DLI</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CTL</td>
<td>1</td>
<td>0</td>
<td>nr</td>
</tr>
<tr>
<td>Rooney\textsuperscript{234}</td>
<td>6</td>
<td>CTL</td>
<td>6</td>
<td>0</td>
<td>nr</td>
</tr>
<tr>
<td>Gross\textsuperscript{109}</td>
<td>3</td>
<td>DLI</td>
<td>0</td>
<td>0</td>
<td>nr</td>
</tr>
</tbody>
</table>

\textit{DLI}, indicates donor lymphocyte infusion; \textit{CTL}, cytotoxic T-lymphocyte infusion; \textit{nr}, not reported.

Prevention of PTLD following solid organ transplantation and allogeneic hematopoietic stem cell transplantation

As morbidity and mortality of established PTLD following solid organ transplantation and allogeneic hematopoietic stem cell transplantation is high, the emphasis should be on prevention of PTLD. The therapeutic approaches described above may also be applied for prevention. To date, no trials have been reported specifically addressing prophylaxis with antiviral agents such as aciclovir or ganciclovir. A reduced incidence of PTLD following solid organ transplantation was suggested in patients treated prophylactically with aciclovir or ganciclovir. However, these studies mostly had a non-randomized design without a
control group or PTLD was a secondary endpoint.\textsuperscript{339-244} In the setting of allogeneic hematopoietic stem cell transplantation, no benefit has been reported of aciclovir or ganciclovir prophylaxis with respect to PTLD.\textsuperscript{122,178} B-cell depletion of the hematopoietic stem cell graft using monoclonal anti B-cell antibodies was reported very effective in the prevention of PTLD following allogeneic hematopoietic stem cell transplantation as compared to historical controls.\textsuperscript{245,246} Furthermore, B-cell depletion did not delay engraftment, or increase incidence of infection. Rooney et al. evaluated the prophylactic infusion of EBV-specific cytotoxic T-cells in patients with high risk features such as HLA mismatch and T-cell depletion. A significant reduction in the incidence of PTLD was observed as compared to historical controls (0/39 versus 7/100).\textsuperscript{234} Gustaffson et al. prophylactically administered EBV-specific cytotoxic T-cells following allogeneic hematopoietic stem cell transplantation guided by viral load and observed 1 PTLD out of 4 patients treated.\textsuperscript{246} In contrast to the experience with CMV, prophylaxis guided by viral load as yet has gained little interest in the prevention of PTLD sofar.

4. Outline of this thesis

Until recently, EBV-LPD could only be diagnosed in recipients of an allogeneic hematopoietic stem cell transplantation with overt established EBV-LPD, usually presenting as a critical illness in patients with generalized lymphadenopathy. The diagnosis was made on the basis of lymph node histology, since early and sensitive markers of EBV infection and reactivation were lacking. Recently the development of PCR-based assays has created the possibility to sensitively and quantitatively monitor EBV-DNA in peripheral blood samples and to evaluate their diagnostic value in immunocompetent patients with EBV infection and in immuocompressed patients with (impending) EBV-LPD.

The development of a real-time quantitative PCR assay for detection of EBV-DNA is described in chapter 2. The assay was evaluated in-vitro using an EBV standard determined by electron microscopy and in-vivo using plasma samples of patients with EBV-infection or EBV-LPD. Using that assay, we then retrospectively assessed the predictive value of the assay using plasma samples from a large cohort of recipients of an allogeneic hematopoietic stem cell transplantation. One hundred and fifty-two patients of whom 2-weekly plasma samples were available, were examined for predictive parameters as regards the incidence of EBV reactivation and EBV-disease. In addition, risk factors for reactivation and disease were assessed and correlated to transplant outcome measures (chapter 3).

In a cohort of 14 patients presenting with EBV-LPD, we next asked the question whether quantitative follow-up levels of EBV-DNA would predict response to therapy and survival. Quantification of EBV-DNA appeared as a very accurate and sensitive marker of response (chapter 4). Moreover, quantification of EBV-DNA before the onset of clinical overt EBV-
LPD appeared to accurately predict impending EBV-LPD. High positive and negative predictive values of EBV-DNA were established. Based on these results a prospective clinical study was designed aiming at the prevention of EBV-LPD and EBV-LPD-mortality. This prospective phase II study included 49 recipients of an allogeneic hematopoietic stem cell transplantation, and 15 patients received pre-emptive treatment when EBV load in plasma exceeded a threshold level of 1,000 EBV genome equivalents per ml. Comparison of prospectively followed patients to a recent historical cohort showed effective prevention of EBV-LPD and EBV-LPD-mortality (chapter 5).

Although the positive predictive value of quantified EBV-DNA appeared relatively high, most patients with viral reactivation were able to mount an immune response and clear their viral reactivation. So far, little was known with respect to the recovery of EBV specific cellular immunity following allogeneic hematopoietic stem cell transplantation. The recent introduction of HLA-class I tetramers presenting viral peptides has enabled the monitoring of peptide specific cytotoxic CD8\(^+\) T-cells in peripheral blood samples following allogeneic hematopoietic stem cell transplantation. We were able to use several EBV peptide specific tetramers and monitor the recovery of EBV specific CD8\(^+\) T-cells. The question whether impaired recovery of cellular T-cell immunity would identify patients at serious risk of EBV reactivation and progression to EBV-LPD was addressed in 61 recipients of a T-cell depleted allogeneic hematopoietic stem cell transplantation. Results described in chapter 6 suggest a pivotal protective role of T-cell immunity against EBV in recipients of an allogeneic hematopoietic stem cell graft. Finally, the overall results of our studies and future issues with respect to diagnosis, prevention and treatment of EBV-LPD are discussed in the general discussion (chapter 7).
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