7. General discussion

General discussion

The lack of early and accurate markers of EBV reactivation and disease has long hampered a timely diagnosis of post-transplant EBV lymphoproliferative disease. The introduction of polymerase chain reaction (PCR)-based assays, however, has allowed for sensitive and quantitative monitoring of viral DNA in peripheral blood samples. This thesis has addressed the question whether molecular monitoring of EBV-DNA would accurately predict for EBV-LPD and whether preventive and therapeutic strategies could be developed based on viral load monitoring. High positive and negative predictive values of viral load were retrospectively established in 152 recipients of an allogeneic hematopoietic stem cell transplant. Subsequently a preventive strategy using pre-emptive anti-CD20 monoclonal antibody therapy was developed, which strategy resulted in a reduction of mortality due to EBV-LPD in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplant. Hence, the molecular monitoring of EBV load has great clinical relevance as it offers a convenient predictive assay of EBV reactivation and EBV-LPD in our group of patients. Such monitoring now seems indispensable for prevention of mortality due to EBV-LPD. However, several new questions as regards molecular monitoring emerge which will be discussed in this final chapter.

1. Diagnosis of EBV-LPD

Histology of a pathological lymph node is still considered the gold standard for a diagnosis of EBV-LPD. However, should this standard change with the introduction of PCR-based assays? Are such assays sufficient, necessary or only additive for diagnosing EBV-LPD? In our retrospective study (chapter 3) the positive predictive value reached 100% at a level of 500,000 genome equivalents per ml plasma (geq/ml) in recipients of a T-cell depleted allogeneic hematopoietic stem cell transplant. However, such high viral plasma levels were also observed in recipients of a T-cell replete hematopoietic stem cell transplantation without EBV-LPD, indicating that EBV reactivation as such may be associated with a high viral load without EBV-LPD. Moreover the highest viral load (3×10^6 geq/ml) was observed in a recipient of a T-cell replete allogeneic hematopoietic stem cell transplantation without EBV-LPD. These results compare well to earlier findings by Lucas et al., who measured highly elevated EBV-DNA levels following unmanipulated allogeneic hematopoietic stem cell transplantation in patients not developing EBV-LPD. [1] Therefore, the quantitative result of a PCR test is not sufficient for diagnosing EBV-LPD. Molecular monitoring of viral load, however, does seem necessary, as lymph node histology is not always possible. EBV-LPD following allogeneic hematopoietic stem cell transplantation may present as disseminated disease without overt lymphadenopathy. But lymphoproliferation may already be present as evidenced by the detection of monoclonal B-cells in the peripheral blood or bone marrow. Such patients, who present with aspecific symptoms of malaise and fever without lymphadenopathy, but with high viral load and monoclonal B-cells may be diagnosed as EBV-LPD. Preferably, the detection of EBV within the monoclonal B-cells, for example by anti-LMP antibodies, should then definitely prove a diagnosis of EBV-LPD.

2. Molecular monitoring of EBV-DNA

We defined EBV reactivation as the presence of at least 50 genome equivalents of EBV-BNRF1-DNA per ml plasma. The mere detection of that part of the viral genome does not indicate its origin. It may originate from fully assembled viral particles produced by lytic infection, but it may also come from B-cells latently infected by EBV, but transformed to autonomously proliferating and dying lymphocytes. Earlier studies have suggested that active lytic infection does participate in the development of EBV-LPD. [2-7] Experimentally, Rowe et al. showed that the development of human EBV-LPD lesions in severe combined immune deficiency mice was accompanied by the expression of lytic antigens in all tumors evaluated. [7] Furthermore, expression of lytic genes has also been shown in B-cells of a considerable proportion of patients with established EBV-LPD. [5,8] Expression of lytic genes may be followed by the induction of a specific cellular immune response. [9,10] As described in chapter 6, we observed a strong cytotoxic $CD8^+$ T-cell response to several epitopes from both lytic and latent proteins in patients with EBV-LPD and in patients with

EBV reactivation. These findings suggest that the theoretical sharp distinction between lytic infection and latently infected, autonomously proliferating, B-cells may not apply to the development of EBV-LPD in patients after allogeneic hematopoietic stem cell transplantation. Future studies should address the question, which genes are involved in EBV reactivation and the progression towards EBV-LPD, and to what extent lytic infection may drive the development of lymphoproliferative disease.

Several PCR based techniques have been used to determine viral load, including semiquantitative PCR, quantitative competitive PCR and quantitative real-time PCR (reviewed by Stevens et al. [11]). A disadvantage of semiquantitative PCR assays is the inability for adequate standardisation. [12] These problems were circumvented with the introduction of quantitative competitive PCR assays, which are based on competitive co-amplification of EBV-DNA with a fixed amount of an internal calibration standard added to the reaction. [13] Competitive PCR proved to be reproducible and accurate. But competitive PCR also proved very time-consuming and it requires intensive sample handling and calculation. Recently, real-time PCR has been introduced, based on direct detection of fluorescent PCR products in a closed-tube system. It is associated with a low risk of contamination due to few handling procedures, thereby allowing high-throughput screening. [14] Real-time PCR also appeared reproducible, sensitive, and standardisation among different laboratories can effectively be accomplished (chapter 2).

Several specimens have been used to determine viral load, including peripheral blood mononuclear cells (MNC), serum, plasma, and whole blood. So far, all studies have shown that an elevated EBV load, irrespective of the source of the specimen, after allogeneic hematopoietic stem cell transplantation and solid organ transplantation increases the probability of developing EBV-LPD. [11] Although several studies have shown a correlation between EBV-DNA assessed in MNCs and plasma or serum, differences in sensitivity and specificity have been reported. [15-18] To date, only few comparative studies have addressed this issue in detail. Two studies compared plasma and MNC as the source of EBV-DNA assessed by real-time PCR in recipients of solid organ transplantation. [15,17] Both studies revealed a higher sensitivity of real-time PCR for EBV-DNA in MNC as compared to plasma, but they also showed a higher specificity if plasma was used as the source of EBV-DNA. As a result, the positive predictive value was greater using plasma samples.

In contrast, Stevens et al. compared whole blood samples versus plasma or serum samples in 4 patients with EBV-LPD following solid organ transplantation using a quantitative competitive PCR. [19] They found no correlation between viral load measured in whole blood as compared to plasma or serum. Furthermore, the EBV burden seemed restricted to the cellular blood compartment in most patients as several serum or plasma samples yielded negative results, despite a high viral load in corresponding whole blood samples. The authors concluded that whole blood samples are to be preferred as they may better reflect the total virus load by combining different blood compartments. However, as long

as the latter findings have not been validated in a larger longitudinal study in both patients with definite EBV-LPD and patients at risk for EBV-LPD, it remains uncertain whether whole blood is to be preferred.

We have longitudinally assessed positive and negative predictive values in a group of 152 recipients of an allogeneic hematopoietic stem cell transplant (chapter 3) using a quantitative real-time PCR. Viral reactivation as defined by $\geq$ 50 geq/ml preceded the development of EBV-LPD in all patients by a median number of 22 days (range, 13-120 days). The positive predictive value of a viral load $\geq$ 1,000 geq/ml was 39% at 2 months and 50% at 4 months, while the corresponding negative predictive values were 100%. These results indicate that the plasma viral load in recipients of an allogeneic hematopoietic stem cell transplant timely and reliably predicts for EBV-LPD (chapter 3). The specificity of the assay was further demonstrated in patients with established EBV-LPD who did or did not respond to therapy (chapter 4). While all responding patients showed rapid clearance of plasma viral load, all non-responders showed a progressive increase of EBV-DNA. These results are in contrast with several studies evaluating cellular viral load during and after therapy for EBV-LPD. High copy numbers were found to persist in a substantial number of responding patients, which did not differ from those in non-responding patients. [20]

Thus, while the cellular viral load may more sensitively reflect an early increase of EBV-DNA, the plasma viral load may be associated with a higher specificity and a higher positive predictive value. From a clinical point of view, decisions as regards pre-emptive treatment or adaptation of therapeutic regimens for established EBV-LPD will especially need to rely on assays with a high specificity and high positive predictive value. Quantifying the viral load in plasma by real-time PCR currently seems to meet these requirements best, in recipients of a solid organ or allogeneic hematopoietic stem cell transplantation. Further improvement of the positive predictive value can be achieved by combining real-time PCR with techniques to assess the EBV-specific cellular immune response. As shown in chapter 6, absence of EBV-specific $CD8^+$ T-cells, as quantified by the tetramer technique in patients with a high viral load ($\geq$ 1,000 geq/ml), was strongly associated with progression to EBV-LPD. Thus, the combination of these assays may permit a further improvement in accurately identifying patients at high risk for EBV-LPD.

3. Prevention of EBV-LPD

Outcome of clinically established EBV-LPD is still not optimal, although new promising treatment modalities have been introduced, such as monoclonal anti B-cell antibody therapy (rituximab) and adoptive T-cell immunotherapy. [21-24] Therefore, preventive strategies are to be preferred. Prevention may be applied as prophylaxis in patients at risk before the onset of EBV reactivation or, prevention may be performed by pre-emptive treatment in patients with established reactivation at high risk of progressing to EBV-LPD.

The latter approach critically depends on a timely and accurate identification of such patients. As described in this thesis, abrogation of EBV-LPD-mortality can effectively be achieved by molecular monitoring and pre-emptive rituximab in accurately identified high-risk patients. However, patients at risk may also be identified, albeit less accurately, by pre-transplant risk factors, such as the application of T-cell depletion, the use of anti-thymocyte-globulin, and alternative donor stem cell transplantation (reviewed in chapter 1, table 3). Instead of monitoring viral load in these patients, an alternative approach would be the administration of prophylaxis in all patients in order to prevent reactivation and the progression towards EBV-LPD. Such prophylaxis would result in significant over-treatment, because EBV-LPD is still a rare complication of allogeneic hematopoietic stem cell transplantation. On the other hand prophylaxis would be attractive if an effective agent is available with few side effects and at low cost. Are such modalities available? To date, prophylaxis with antiviral drugs such as aciclovir and ganciclovir have not been shown to prevent EBV reactivation and EBV-LPD. [25-27] In our retrospective study (chapter 3), 75 out of 152 patients were treated prophylactically with aciclovir, but the incidence of EBV reactivation did not differ between patients with or without prophylaxis.

Another way of prophylaxis is the depletion of B-cells from the donor stem cell graft, which has already been shown a highly effective approach. [28,29] Several groups using the monoclonal antibody alemtuzumab for both T-cell and B-cell depletion have reported a low incidence of EBV-LPD. [28,29] More recently, Liu et al. reported favourable but preliminary results of the in-vivo application of rituximab for B-cell depletion shortly after stem cell transplantation. [30] Although B-cell depletion of the donor graft (either performed in-vivo or in-vitro) may be very effective, the approach may be associated with a delayed immune recovery in general and a delay in EBV-specific T-cell immunity. [30] Furthermore, the approach would imply significant over-treatment. Therefore, weighing the pros and cons of prophylaxis versus pre-emptive treatment, the balance may turn in favour of pre-emptive treatment if one prefers to avoid unnecessary (expensive) treatment of patients with a low probability. Lastly, 67 recipients of unmanipulated hematopoietic stem cell grafts described in chapter 3 did not develop EBV-LPD, although they experienced no less frequent reactivations than did recipients of T-cell depleted grafts. Clearly, the T-cells infused with the donor graft were able to mount an immune response to prevent recurrent reactivation and the progression to EBV-LPD. Therefore, these patients do not require prophylactic treatment or intensive molecular monitoring and pre-emptive treatment for prevention of EBV-LPD. The question then arises whether the benefits of T-cell depletion still outweigh the disadvantages such as the risk of EBV-LPD.

Retrospectively, treatment related mortality did not differ between recipients of T-cell depleted stem cell grafts versus recipients of unmanipulated grafts (chapter 3). Furthermore, the incidence of acute graft-versus-host disease did not differ either. However, the probability of developing chronic limited and extensive graft-versus-host disease was significantly less following T-cell depletion (38 % ± 6% versus 83% ± 5%). These results compare well to a number of earlier studies evaluating the incidence of acute

and chronic graft-versus-host disease following partial T-cell depletion. [31,32] An even better prevention of acute and chronic graft-versus-host disease may be achieved by a more stringent, near complete depletion of T-cells. However, such reduction is achieved at the expense of an even slower immune recovery and a higher relapse rate of the primary malignancy (reviewed by Ho,[33]).
Therefore, partial T-cell depletion was developed in order to prevent acute and chronic graft-versus-host disease, while retaining some graft-versus-leukemia activity. [34] Although survival differences have not been demonstrated between recipients of T-cell depleted versus unmanipulated stem cell grafts, the reduction of acute and especially chronic graft-versus-host disease may be of significant benefit in terms of prevention of long lasting treatment related morbidity, necessitating prolonged use of immunosuppressive drugs. Disadvantages of T-cell depleted allogeneic hematopoietic stem cell transplantation such as viral reactivation and the need for cautious monitoring should therefore be weighed against the treatment related morbidity associated with a higher incidence of chronic graft-versus-host disease following unmanipulated allogeneic hematopoietic stem cell transplantation. So far, the approach pursued by hematopoietic stem cell transplant centers in the Netherlands has focussed on the prevention of acute and chronic graft-versus-host disease and the concurrent prevention of opportunistic infections and secondary malignancies such as EBV-LPD.

4. Treatment of EBV-LPD

Before the introduction of molecular monitoring of viral load, a diagnosis of EBV-LPD was often made relatively late following the onset of LPD. Patients usually presented with a critical illness and outcome was very poor despite the application of multiple treatment modalities. Surrogate markers for response were lacking, precluding a careful evaluation of different treatment modalities. The picture has significantly changed during the last 5 years following the introduction of molecular monitoring. The merits of molecular monitoring are several fold. First, EBV-LPD presents itself at diagnosis no longer as a medical emergency. Impending EBV-LPD alerts the clinician to institute pre-emptive treatment or to begin therapy at a relatively early time-point in the course of EBV-LPD. Secondly, different treatment modalities can now be evaluated by a highly specific surrogate marker of response. As described in chapter 4, the molecular quantification of viral load in patients with established EBV-LPD allows for a very early (< 72 hours) and accurate prediction of response, which now enables us to carefully select and adjust successive treatment modalities.

Which therapeutic approach should be pursued in patients with established EBV-LPD following allogeneic hematopoietic stem cell transplantation? Improving host defence and eliminating EBV-infected autonomously proliferating B-cells remain the current cornerstones of therapeutic management of EBV-LPD. But molecular monitoring now allows for a stepwise approach. Malignant B-cells can effectively be eliminated by

rituximab infusion guided by viral load. High response rates were observed in recipients of stem cell grafts and in recipients of solid organ grafts following multiple infusions of rituximab (reviewed in chapter 1). While complete peripheral blood B-cell depletion may already be achieved by a single infusion of rituximab, some patients may require multiple infusions. We observed incomplete peripheral blood B-cell depletion in 5 out of 17 patients (chapter 5) after a single rituximab infusion. Molecular monitoring in 2 out of these 5 patients revealed a progressive increase of viral load following the first infusion concurrent with the development of overt EBV-LPD. Subsequently, a decline of EBV-DNA accompanied with complete peripheral blood B-cell depletion was observed in both patients following a second infusion of rituximab. Future studies should address the question whether and how the scheme of rituximab can be optimized.

Host defence may be improved by interruption of immune suppressive drugs and/or the adoptive transfer of donor T-cells. As described in chapter 6, patients with established EBV-LPD may rapidly recover EBV-specific cytotoxic T-cells up to a protective level already within the first weeks following interruption of immunosuppressive agents. In order to allow for sufficient endogenous T-cell recovery, the adoptive transfer of donor T-cells may be postponed in patients with EBV-LPD for at least 1-2 weeks. T-cell immunotherapy may then very selectively be applied in patients, who do not recover EBV-specific immunity and who show a progressive increase of viral load despite cessation of immunosupression and rituximab infusion. Adoptive immunotherapy can be performed with unselected donor leucocytes or with donor-derived EBV-specific cytotoxic T-cell lines as developed and pioneered by Rooney and Heslop. [23,24] While unselected donor T-cell infusion may be complicated by graft-versus-host disease, they do provide immunity not only towards EBV but also to a number of other potential lethal opportunistic infections, including CMV, adenovirus, Aspergillus, etc. As recently reported by Einsele et al., patients lacking CMV-specific immunity may be treated with CMV-specific cytotoxic T-cells, but the lack of immunity towards other pathogens may still be associated with the development of lethal infections. [35] In addition, although effective, the laborious technical procedures needed to prepare cytotoxic T-cells may preclude their application on a wider scale.

Is there still a role for chemotherapy? Elimination of malignant B-cells may effectively be performed by rituximab, but relapse of EBV-LPD has been reported in 20-30% of patients treated for EBV-LPD following solid organ grafting (chapter 1). Failure of treatment may be due to development of resistance, viral immune evasion, rapidly progressive disease, and loss of CD-20 antigen expression. [22,36-38] Earlier studies evaluating response following chemotherapy showed high response rates in recipients of solid organ grafts with acceptable toxicity, if intensified dosages were avoided. [39-42] In contrast, the side effects of chemotherapy applied for EBV-LPD in recipients of allogeneic hematopoietic stem cell grafts appeared excessive, which may be explained by cumulative toxicity added to the preceding high dose chemo-radiotherapy. Therefore, cytotoxic chemotherapy may selectively be applied in the treatment of EBV-LPD following solid organ transplantation.

Preferably, a combination of rituximab and chemotherapy, such as has been studied by Coiffer et al in patients with Non-Hodgkin's lymphoma [43], would need to be studied in recipients of solid organ grafts to address the question whether the response rate can be improved and relapse can be prevented by combining rituximab with chemotherapy.

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