STRATEGIES FOR MANAGEMENT OF EBV AND ADENOVIRUS INFECTIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

Hamdy Hassan Omar

Stockholm 2010
To my family
ABSTRACT

Viral infections are one of the challenges that can threaten successful allogeneic HSCT especially during the period of immune reconstitution resulting in a high mortality risk. Adenoviruses and EBV are important viruses during this period. Adenovirus infections can cause invasive adenovirus disease and EBV can cause post transplant lymphoproliferative disease (PTLD). Both are associated with significant morbidity and mortality if they are not discovered early and preemptive treatments are not given. IL-7 is a non-redundant cytokine that has important functions in T-cell survival, proliferation and memory formation. It is a growth factor for pre B-cells. It may have a role to improve T cell reconstitution early after HSCT.

The aim of the thesis was to develop strategies to prevent severe viral complications after allogeneic HSCT. In the adenovirus part, we aimed to study the predictive value of adenoviremia for the development of adenovirus disease and to examine a surveillance strategy to control adenovirus disease. In the EBV-PTLD part, we aimed to see the effect of prospective monitoring of EBV load on PTLD control and to study the role of IL-7/IL-7R on PTLD development.

We characterized in paper I adenovirus infections in a Swedish adult cohort after allogeneic HSCT. We found that incidence of adenoviremia was 4.9% in the studied population. CMV and EBV infections may occur to a large extent in patients with adenoviremia. Most patients with positive adenovirus PCR had sustained adenoviremia. Preemptive treatment with cidofovir might be a good treatment option to control adenoviremia. In study II we examined a surveillance strategy to control adenovirus infections. We found that only 5% of the patients had adenoviremia, no one developed adenovirus disease, or required antiviral treatment. This surveillance strategy could be applied to children and high risk adults. Most adult patients had adenovirus specific T-cell immune response in the first three months after allogeneic HSCT.

A monitoring strategy of patients at a high risk of EBV-PTLD was applied. The effect of the strategy was compared with results of a historical control group in whom the strategy was not applied. We showed that 5.6% of high risk patients in the study group developed PTLD and 1.9% died from PTLD with the corresponding numbers in the control group being 9.4 and 5.7% for development of PTLD and death due to PTLD, respectively. Splenectomy was found to be a high risk factor in the study group. This monitoring strategy was able to face the high risk factors and can be applied safely. In study IV we found reduced responsiveness of IL-7 by the STAT5 phosphorylation assay in both CD4+ and CD8+ T-cells in PTLD patients. However, the reduced responsiveness of IL-7 was found only in CD8+ T-cells in the control group. IL-7R was found to be more expressed in PTLD patients than controls and was found to be expressed on other immune cells. This functional dysfunction in IL-7/IL-7R may help to identify and monitor patients at a high risk of EBV-PTLD.

In conclusion, surveillance strategy to monitor high risk patients can help to reduce severe virological complications. Monitoring of IL-7 function might be a predictor of EBV-PTLD.
LIST OF PUBLICATIONS AND MANUSCRIPTS INCLUDED IN THIS THESIS


IV. Hamdy Omar, Raija Ahmed, Andreas Björklund, Åsa Gustafsson-Jernberg, Per Ljungman and Markus J Maeurer. Decreased IL-7 signaling in T-cells from patients with PTLD after allogeneic HSCT (submitted).
CONTENTS

1 Introduction ................................................................................................ 1
  1.1 Stem cell Transplantation ........................................................................ 1
      1.1.1 Procedures .................................................................................. 1
      1.1.2 Immune reconstitution .............................................................. 3
      1.1.3 Complications after HSCT ......................................................... 6
  1.2 Viral infections .......................................................................................... 9
      1.2.1 Monitoring and early diagnosis of viral infections: .................... 9
      1.2.2 CMV ...................................................................................... 11
      1.2.3 Adenoviruses .......................................................................... 12
      1.2.4 EBV ...................................................................................... 16
  1.3 PTLD ...................................................................................................... 18
      1.3.1 Definition .................................................................................. 18
      1.3.2 Classification .......................................................................... 18
      1.3.3 Pathogenesis ........................................................................... 19
      1.3.4 Clinical presentation .................................................................. 19
      1.3.5 Risk factors ............................................................................. 20
      1.3.6 Monitoring and management ................................................... 20
  1.4 IL-7 ........................................................................................................ 22
      1.4.1 Biology .................................................................................... 22
      1.4.2 Functional pathway ................................................................... 23

2 Aims ............................................................................................................. 25

3 Patients and methods .................................................................................. 26
  3.1 Patients .................................................................................................. 26
  3.2 Methods .................................................................................................. 27
  3.3 Statistical methods ................................................................................ 29

4 Results and discussion .............................................................................. 30

5 Conclusion .................................................................................................. 43

6 FUTURE PERSPECTIVES ....................................................................... 44

7 Acknowledgements ..................................................................................... 46

8 References .................................................................................................. 48
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
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<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
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<td>AML</td>
<td>Acute Myeloid Leukemia</td>
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<tr>
<td>ATG</td>
<td>Anti-thymocyte Globulin</td>
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<td>BCR</td>
<td>B-cell Receptor</td>
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<td>BM</td>
<td>Bone Marrow</td>
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<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
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<tr>
<td>CML</td>
<td>Chronic Myeloid Leukemia</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>ELISPOT</td>
<td>Enzyme-linked Immunospot Assay</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
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<td>GVHD</td>
<td>Graft-Versus-Host Disease</td>
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<tr>
<td>HHV-6</td>
<td>Human Herpes Virus-6</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>HSC</td>
<td>Hematopoetic Stem Cells</td>
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<td>HSCT</td>
<td>Hematopoetic Stem Cell Transplantation</td>
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<td>IL7-R</td>
<td>Interleukin-7 Receptor</td>
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<td>iTreg</td>
<td>induced regulatory T cells</td>
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<tr>
<td>Jak</td>
<td>Janus Kinase</td>
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<td>MDS</td>
<td>Myelodisplastic Syndrome</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>n Treg</td>
<td>natural regulatory T cells</td>
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<tr>
<td>NASBA</td>
<td>Nucleic Acid Sequence-based Amplification</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PBSC</td>
<td>Peripheral blood stem cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PD-1</td>
<td>Programmed death-1</td>
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<tr>
<td>PTLD</td>
<td>Post Transplant Lymphoproliferative Disease</td>
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<tr>
<td>RIC</td>
<td>Reduced Intensity Conditioning</td>
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<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>RT-PCR</td>
<td>real-time PCR</td>
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<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
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<td>STAT5</td>
<td>Signal Transducer and Activator of Transcription</td>
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<tr>
<td>TCR</td>
<td>T-cell Receptor</td>
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<td>TGFβ</td>
<td>Transformed Growth Factor β</td>
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<tr>
<td>Th</td>
<td>T helper</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
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<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoecitin</td>
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<tr>
<td>UCB</td>
<td>Umbilical Cord Blood</td>
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**LIST OF ABBREVIATIONS**
1 INTRODUCTION

1.1 STEM CELL TRANSPLANTATION

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective modality of treatment of many hematological diseases. 2009 was the fiftieth anniversary of the first successful HSCT (Thomas, Lochte et al. 1959; Jenq and van den Brink 2010). HSCT was initially used to treat immune deficiencies (e.g. Wiskott-Aldrich syndrome) and was later used to replace the hematopoietic system after infusion of myeloablative doses of radiation and chemotherapy in patients with refractory hematological malignancies (Horowitz, Gale et al. 1990; Jones, Ambinder et al. 1991; Woods, Neudorf et al. 2001). The number of allogeneic HSCT is increasing every year although the indications have changed. During the last few years the number of transplants for CML has decreased substantially while the number of transplants for MDS, AML, and CLL continues to increase (Gratwohl and Baldomero 2009). It has also been recognized that graft-vs-malignancy effect is a very important factor for the outcome of allogeneic HSCT. Allogeneic HSCT is also used in non-hematological diseases such as benign hematological disorders, e.g. bone marrow failure and congenital red cell disorders and some solid tumors (Ljungman, Bregni et al. 2010).

1.1.1 Procedures

1.1.1.1 Stem cell source:

a- Bone marrow: It was the only stem cell source used during the first decades of allogeneic HSCT. Today, it is still the main primary stem cell source used in children (Eapen, Horowitz et al. 2004) and in patients with aplastic anemia (Viollier, Socie et al. 2008).

b- Peripheral blood: Stem cells from the peripheral blood obtained after G-CSF stimulation has replaced the bone marrow to a large extent in adults with malignant disorders since it is associated with more rapid hematological recovery and lower relapse rates. However, it is also associated with an increased risk for chronic GVHD (Dreger, Haferlach et al. 1994; Schmitz, Bacigalupo et al. 1998; Remberger, Kumlien et al. 2002)
c- Umbilical cord blood: Umbilical cord blood (UCB) graft is a good alternative source of HSC especially in children or in adult patients who do not have suitable related or unrelated donors (Rocha, Labopin et al. 2004; Rocha and Gluckman 2009; Rodrigues, Sanz et al. 2009). An advantage is the immunological immaturity allowing a higher degree of HLA-mismatch than with other stem cell sources. The cell dose is the main limiting factor of using umbilical cord blood especially in adults. However, the use of double UCB and ex vivo expansion of UCB have increased the utilization of UCB (Brunstein and Laughlin 2010).

1.1.1.2 Donor:

An HLA-identical sibling is the best suitable donor. However, in many countries a matched sibling donor can be found for only 25-30% of the patients. Therefore, well HLA-matched unrelated donors are now widely used due to improvement in tissue typing and there are increasing numbers of donors (currently > 13 million) registered in the unrelated volunteer donor registries for example the Tobias registry in Sweden and the Anthony Nolan foundation in the UK. The use of a haplo-identical parent as a donor is a possibility especially in patients who need an immediate HSCT (Aversa, Reisner et al. 2008). An identical twin (syngeneic) is, if available, more suitable in nonmalignant disorders as it does not confer a graft versus leukemia effect (Ljungman, Hagglund et al. 1997; Buckley, Schiff et al. 1999).

1.1.1.3 Conditioning regimen:

a- Myeloablative conditioning: This is the classical conditioning regimen utilizing high dose cytotoxic chemotherapy with or without total body irradiation (Thomas, Storb et al. 1975; Tutschka, Copelan et al. 1991). With this strategy both the dosages of chemo- and radiotherapy and the graft-vs-malignancy effect are used to cure the disease. It is associated with significant toxicity but also with a lower risk for relapse of high risk malignancies compared to reduced intensity regimens (Alyea, Kim et al. 2006; Ringden, Labopin et al. 2009).

b- Reduced intensity conditioning: It is used more in elderly patients and in patients with organ dysfunction who are not able to tolerate the high toxic effects of the myeloablative conditioning but also in some malignancies where intensive therapy is not able to cure the underlying disease (Cahn, Klein et al. 2005; Cho, Lee et al.
2009; Pulsipher, Boucher et al. 2009; Storb 2009; Koreth, Aldridge et al. 2010). The strategy is based on intensive immune suppression especially of existent recipient T-cell function to make donor cell engraftment possible. The direct anti-tumor effect from the conditioning regimen is limited and it utilizes instead mainly the graft-vs-malignancy effect to cure the malignancies.

1.1.2 Immune reconstitution

Reconstitution of the immune system after an allogeneic HSCT takes 12 months or longer (Witherspoon, Matthews et al. 1984; Witherspoon, Goehle et al. 1986; Lum 1987; Roberts, To et al. 1993; Maris, Boeckh et al. 2003; Williams and Gress 2008; Wingard, Hsu et al. 2010). During this period the patient has significant deficiencies in several parts of the immune system considered as the main predisposing factors for opportunistic infections including viral, bacterial, and fungal infections. The kinetics of the immune reconstitution is dependent on the time needed by the immune system cells to expand and mature, the post-transplant immunosuppression, the donor type, the stem cell source, the development of graft versus host disease (GVHD), the age of the patient, and some infections for example cytomegalovirus (CMV) infection (Paulin, Ringden et al. 1987; Heitger, Neu et al. 1997; Matsuda, Hara et al. 1998; Peggs 2004; Komanduri, St John et al. 2007; Cavazzana-Calvo, Andre-Schmutz et al. 2009).

1.1.2.1 The innate immune system:

Recovery of the innate immune response after allogeneic HSCT is more rapid than of the adaptive immune responses. The recovery is not affected of donor/recipient compatibility. The physical barriers as the skin and mucous membranes are the first defense against infection. These are affected by chemotherapy and radiation but usually recover rapidly in patients without GVHD (Chaushu, Itzkovitz-Chaushu et al. 1995). The recovery of donor granulocyte and platelet numbers takes two to three weeks. The natural killer cells return to normal numbers approximately one month after HSCT (Storek, Dawson et al. 2001). These cells have a role in the prevention of herpes virus infections and eradication of residual tumor cells especially in recipients with killer immune globulin like receptor (KIR) mismatch (Ruggeri, Capanni et al. 2002; Barron, Gao et al. 2009). Recipient epithelial dendritic cells may have a role in GVHD development in mice through
priming of alloreactive T cells in the graft but this is not confirmed in human (Merad, Hoffmann et al. 2004; Collin, Hart et al. 2006). However, RIC can affect dendritic cell chimerism and function. A few studies in human showed that numbers of both myeloid and plasmacytoid dendritic cells can reach normal levels between 19-25 days after allogeneic HSCT but these numbers remain low during the first year. Furthermore, these values are significantly lower in case of acute GVHD and can be used as a marker to predict the development of GVHD (Mohty 2007; Horvath, Budinsky et al. 2009). High number of plasmacytoid dendritic cells in the graft is associated with a higher relapse rate and lower overall survival (Rajasekar, Lakshmi et al. 2010)

1.1.2.2 T-cells

The T-cell reconstitution occurs by two main pathways; the expansion of mature donor T-cells which are infused with HSC and de novo generation of thymic-dependent T-cells (Seggewiss and Einsele 2010). The transfer with the graft and expansion of mature donor T-cells are important in protection against infections and rejection of the graft but they are also the main factor for development of GVHD (Leen, Tripic et al. 2010). The generation of thymic-dependent T-cells is affected by several factors; the extent of thymic involution and damage caused by conditioning regimens, the development of GVHD, and degree of engraftment of donor cells (Krenger and Hollander 2010). The thymic dependent pathway is the main pathway of T-cell reconstitution in recipients of T-cell depleted graft and poor T-cell recovery in adults can occur due to a poor thymic function. The thymus can support thymopoiesis, which can be shown by presence of T-cell receptor rearrangement excision circles which are used for measurements of thymic productivity (Krenger and Hollander 2010). Evaluation of thymic function can be done by measurement of the length of the TCRβ CDR3 chains by spectrotyping (Mackall, Bare et al. 1996; Mackall, Hakim et al. 1997; Douek, Vescio et al. 2000; Hakim, Memon et al. 2005). Precursor CD4+ T-cells leave the thymus to migrate and differentiate into four subtypes of effector T-helper (Th) 1,2,17 and antigen induced Treg (iTreg) depending on production of different sets of cytokines to help in clearance of pathogens. Th1 produce IFN-γ, Th2 produce IL-4, IL-5 and IL-13. Th17 produce IL-17 and IL-22 and iTreg function as natural Treg (nTreg) in their suppressive abilities of pathogens (Sallusto and Lanzavecchia 2009). CD8+ T cells differentiate into cytotoxic T-cells with the aim to kill virus infected cells. Cells
surviving after the exposure to a specific antigen are memory cells that have the ability to rapid and effector responses in case of re-exposure to the same antigen. These T-cell subsets are defined by expression of certain surface markers; Precursor T-cells are CD45RA+CCR7+, central memory cells are CD45RA-CCR7+, effector memory cells are CD45RA-CCR7- and terminally differentiated T cells are CD45RA+CCR7- (Sallusto, Lenig et al. 1999)

The recovery of the total number of lymphocytes usually takes one year after HSCT (Storek, Geddes et al. 2008). However, the recovery of CD8+ T-cells occurs earlier than CD4+ T-cells which may require years (Mackall, Hakim et al. 1997). The numbers of CD4+ T-cells are higher in PBSC than BM stem cells. Early after HSCT, most circulating T cells are memory cells while naïve (precursor) T cells take more time to appear. Although the delay in recovery of CD4+ T-cells leads to an inverted CD4/CD8+ ratio for a long time after HSCT, the numbers of activated T-cells (CD4+ and CD8+) are high at first then return to normal after 3 months (Atkinson 1990; Storek, Dawson et al. 2001). Treg cells are of importance in limiting the donor alloreactive immune response. Tregs are divided into two types nTreg CD4+CD25+FOXP3+ produced in the thymus then migrate to the periphery as mature T-cells and iTreg CD4+CD25-FOXP3+ mentioned before. These cells have a role in preventing GVHD by mediating immunosuppression (Karim, Kingsley et al. 2004; Paczesny, Choi et al. 2009).

IL-7 and IL-15 are cytokines important for the peripheral expansion of naïve and memory T-cells. IL-15 was shown to enhance the proliferation of CD8+ memory cells (Li, Zhi et al. 2005). TGFβ has antagonistic action against IL-15 to prevent uncontrolled T cell expansion (Lucas, McNeil et al. 2004).

Il-7 is an essential growth factor for B- and T-lymphocytes. It is secreted from the stromal cells in the thymus leading to development, maturation and survival of T cells by induction of thymopoiesis. IL-7 levels are inversely correlated with T-cell numbers after HSCT. The administration of IL-7 in allogeneic HSCT in mice leads to enhancement of T cell reconstitution through increased thymopoiesis, increased homeostatic proliferation of both T-cell pathways and decreased T-cell apoptosis. However, it can aggravate GVHD but this risk can be decreased by
using T-cell depleted grafts (Mackall, Fry et al. 2001; Sinha, Fry et al. 2002; Broers, Posthumus-van Sluijs et al. 2003; Dean, Fry et al. 2008).

1.1.2.3 B-cells
The reconstitution of B-cells can take 1-2 years after allogeneic HSCT. The numbers of B-cells are very low in the first two months but they increase very rapidly to reach levels more than normal in 1-2 years (Witherspoon, Goehle et al. 1986; Storek, Ferrara et al. 1993). Although B-cell reconstitution is faster than T-cell reconstitution, the response of B-cells to new antigen exposure is impaired due to compromised B-cell functions that can persist for 2 years as a consequence of delayed CD4+ reconstitution and decreased the level of somatic hypermutation in mature B cells (Glas, van Montfort et al. 2000; Omazic, Lundkvist et al. 2003; Marie-Cardine, Divay et al. 2008). Most B cells are naïve (IgD+ high IgM+ high) and mature to IgM more than IgG or IgA (Storek, Ferrara et al. 1993; Storek, Witherspoon et al. 1995; Glas, van Montfort et al. 2000). The B cell reconstitution after allogeneic HSCT follows the same steps of B cell ontogeny as in children but it is slower due to decreased levels of CD4+ and dendritic cells. The responses of B-cells after exposure to antigens are depressed early after allogeneic HSCT. The recovery kinetics of the B-cell response against antigens varies depending on the type of the antigen. Responses to protein antigens recover faster than responses to polysaccharide antigens (Gerritsen, van Tol et al. 1993). CD5+ B-cells that reside in the spleen tend to be low with GVHD than without GVHD (Storek, Ferrara et al. 1993; Avetisyan, Aschan et al.) A delay in the recovery of the B cell repertoire and antibody production diversity is commonly due to the development of post transplant complications e.g. chronic GVHD (Kook, Goldman et al. 1996; Small, Robinson et al. 2009).

1.1.3 Complications after HSCT

1.1.3.1 GVHD
GVHD is one of the most serious complications after allogeneic HSCT. It can occur during the first three months classically defined as acute GVHD or later (after three months) defined as chronic GVHD although these strict definitions have been questioned during the last few years with the increased utilization of donor lymphocyte infusions (Akpek, Zahurak et al. 2001; Mielcarek, Martin et al.
2003; Couriel, Saliba et al. 2004; Cahn, Klein et al. 2005; Filipovich, Weisdorf et al. 2005; Ball and Egeler 2008; Kim, Lee et al. 2009; Vigorito, Campregher et al. 2009). Different factors are involved in the pathogenesis of GVHD. The damage of tissues caused by the myeloablative conditioning regimens can lead to release of inflammatory cytokines such as IL-1 and TNF-α. These cytokines activate donor mature T cells (Th1) by up-regulation of MHC antigens. Th1 cells secrete IL-2 and INF-γ that activate donor derived natural killer cells and enhance maturation of cytotoxic T cells, monocytes and macrophages causing further tissue damage (Akpek, Zahurak et al. 2001; Ferrara, Cooke et al. 2003). The incidence of GVHD varies depending on transplantation related factors: 1) The intensity of GVHD prophylaxis 2) Donor type: An HLA-matched related donor is associated with lower GVHD rates than unrelated donors 3) Stem cell source: Cord blood grafts are associated with less acute GVHD than PBSC and bone marrow while PBSC is associated with more chronic GVHD (Ringden, Labopin et al. 2002; Gluckman and Rocha 2008; Wang, Zhan et al. 2009; Nagafuji, Matsuo et al. 2010). 4) Conditioning regimen: RIC-regimens result in a lower risk for acute GVHD (Le Blanc, Remberger et al. 2004; Pasquini 2008). Acute GVHD can be graded according to the Seattle criteria on a scale from 0 to 4 according to severity of organ involvement involving the skin, the gut, and the liver (Glucksberg, Storb et al. 1974). Grade III-IV acute GVHD is associated with high mortality with high dose corticosteroids, T-cell suppressive drugs such as cyclosporine or tacrolimus, and antibodies (Nagafuji, Matsuo et al. 2010). Severe acute GVHD also results in an increased risk for viral and fungal infections (Miller, Flynn et al. 1986; Wald, Leisenring et al. 1997; Boeckh, Kim et al. 2006; Bow 2009).

Chronic GVHD affects 25 to 65% of long term survivors (Sullivan, Weiden et al. 1989; Mohty, Kuentz et al. 2002; Remberger, Kumlien et al. 2002; Ringden, Labopin et al. 2002; Zecca, Prete et al. 2002). The main target is the connective tissues resulting in dermatitis, keratoconjunctivitis, oral mucositis, and hepatic dysfunction (Lister, Messner et al. 1987). It can be graded as limited or extensive involvement of the affected tissues. Limited chronic GVHD is defined as localized skin involvement or hepatic dysfunction. Extensive chronic GVHD includes either extensive skin involvement or localized skin involvement with or without hepatic dysfunction together with one the followings: eye dryness, salivary gland affection, positive liver biopsy and other organ involvement.
(Zecca, Prete et al. 2002). The National Institute of Health (NIH) in the United States has applied criteria based on a scoring system of organ involvement (0-3) and degree of severity (mild, moderate and severe) (Filipovich, Weisdorf et al. 2005). Chronic GVHD is one of the most important causes of late mortality after allogeneic HSCT (Gratwohl, Brand et al. 2005).

1.1.3.2 Infections

The possibility of infection is high during the period of immune suppression due to many factors that can temporarily disrupt both the innate and cell mediated immune responses. A wide variety of infections can affect the patients resulting in morbidity and mortality. During the first month after allogeneic HSCT, bacterial infections (gram negative and gram positive) are the most common organisms affecting the patients due to severe neutropenia. (Ljungman, Hagglund et al. 1997; Junghanss, Marr et al. 2002; Toro, Morales et al. 2007; Castagnola and Faraci 2009). Fungal infections, especially *candida species*, yet also herpes simplex virus infections are also common in this period (Wade, Day et al. 1984; Slavin, Osborne et al. 1995; Ninin, Milpied et al. 2001; Hwang and Liang 2010) In the period following engraftment (3 weeks to 6 months), other herpes viruses (CMV, EBV, and HHV-6) and adenoviruses are common due to impairment in the T cell function (Meyers, Flournoy et al. 1982; Gratama, Lennette et al. 1992; Wang, Dahl et al. 1996; Ljungman 2002; Ljungman, Perez-Bercoff et al. 2006; Razonable and Eid 2009). Fungal infections caused by molds, especially *aspergillus species*, are also important during this period. Risk factors for these infections include immunosuppressive drugs and acute GVHD (Wald, Leisenring et al. 1997; Williamson, Millar et al. 1999; Hows, Passweg et al. 2006). Later (>6 months) after HSCT, the risk of infection still is present with the most important risk factor being chronic GVHD (Kulkarni, Powles et al. 2000; Gratwohl, Brand et al. 2005; Hows, Passweg et al. 2006; Bjorklund, Aschan et al. 2007; Erard, Guthrie et al. 2007). Important infections during this late period are varicella zoster virus, CMV, community acquired respiratory infections including influenza, Pneumocystis jiroveci, respiratory syncytial virus, and pneumococcal infections (Boeckh, Kim et al. 2006; Bjorklund, Aschan et al. 2007; Avetisyan, Mattsson et al. 2009; Olkinuora, Taskinen et al. 2009).
1.2 VIRAL INFECTIONS

Viral infections are frequent complications after allogeneic HSCT. Many viruses have been recognized to be associated with serious complications and high mortality including herpesviruses, community acquired respiratory viruses for example RSV, adenoviruses, and paroviruses (Ljungman 2002). Viral infections can cause direct effects such as pneumonia, encephalitis, gastroenteritis, and hepatitis but also indirect effects such as immunosuppression. During the history of HSCT, antiviral drugs have been developed against many of the viruses important after transplantation. However, early diagnosis has been shown to be important to the effective usage of these drugs. Therefore, the application of different strategies for control of two important viruses (adenovirus and Epstein Barr virus) is the focus of this thesis. The strategy to control these two viruses by early diagnosis and preemptive treatment has previously been successfully applied against cytomegalovirus (CMV) infection after allogeneic HSCT (Goodrich, Mori et al. 1991; Einsele, Ehninger et al. 1995; Ljungman 1995; Boeckh, Gooley et al. 1996; Ljungman, Lore et al. 1996; Boeckh, Bowden et al. 1999; Ljungman, Perez-Bercoff et al. 2006; de la Cruz-Vicente, Cerezuela Martinez et al. 2008)

1.2.1 Monitoring and early diagnosis of viral infections:

Historically, viral infections were diagnosed by serology, viral isolation, or direct histopathological analysis of biopsy material. These methods were either too insensitive (serology), too slow (virus isolation), or required invasive diagnostic techniques (Meyers 1988; Meyers, Ljungman et al. 1990). The need of rapid and accurate diagnosis of infections has been shown to be important for the prevention of viral complications (van der Bij, Schirm et al. 1988; Ljungman, Gleaves et al. 1989; Meyers, Ljungman et al. 1990; Einsele, Steidle et al. 1991; Einsele, Ehninger et al. 1995; Whelen and Persing 1996; Boeckh, Leisenring et al. 2003; Ince and McNally 2009). During the last decades rapid and sensitive methods that can be used to diagnose viral infections have been developed. Most of these techniques in use today are based on the detection of nucleic acids by molecular biology tools.

PCR is a powerful tool for quantification of nucleic acid that was first developed by Kary Mullis in 1987. It can amplify defined nucleic acid sequences to large
numbers to be analyzed (Mullis and Faloona 1987; Kubista, Andrade et al. 2006). The reaction is based on presence of DNA template (the sample DNA), DNA polymerase to synthesize a new strand of complementary DNA, primers that are short single stranded DNA complementary to the target sequence leading to accumulation of millions of copies of the desired sequence. The amplification of viral DNA is a very important tool for diagnosis of a viral infection. However, for the detection of RNA sequence in RNA viruses, a reverse transcriptase (RT) step is done to convert it to DNA. NASBA (nucleic acid sequence based amplification) technique was used to amplify the nucleic acid sequence by the simultaneous activity of enzymes without thermal cycling. The process involves three enzymes and two primers. It was used mainly in diagnosis of HIV (Kievits, van Gemen et al. 1991; Romano, Williams et al. 1997) but has also been used for diagnosis of CMV (Gerna, Baldanti et al. 2000).

RT-PCR is a rapid, accurate and sensitive method for detection of pathogen developed in 1992 (Higuchi, Dollinger et al. 1992). It has the advantage that it can be used to study viral load kinetics (Emery, Sabin et al. 2000) (Emery, Sabin et al. 2000; Jebbink, Bai et al. 2003). RT-PCR was used to measure the viral load of EBV for the first time in 1999 (Kimura, Morita et al. 1999) and it was recommended to be used to measure the adenoviral load by Lion et al., 2003 (Lion, Baumgartinger et al. 2003).

![Figure (1): Taq-Man RT-PCR steps (adapted from the website of Division of Molecular Medicine, Louisiana state University)](image-url)
The extraction of DNA can be made using a ready nucleic acid isolation kit as the AmpliPrep or the MagNA Pure LC kit (Roche Diagnostics Scandinavia AB) or workstations like the biorobots M48 or 9604 (QIAGEN, Germany). Detection and amplification of the targeted gene can be made using intercalating dye, e.g., ethidium bromide or SYBR Green I (Higuchi, Dollinger et al. 1992; Zipper, Brunner et al. 2004) in which the dye binds to DNA and intercalate into a double stranded DNA molecule. However, the detection of DNA/RNA in viruses is frequently done by using probes as hydrolysis probes (TaqMan) figure (1) (Applied Biosystems AB, USA) which are oligonucleotide probes that are labeled with a fluorescent reporter dye on 5’ end and a quencher dye on 3’ end. The DNA polymerase extends the primers. The quencher and the reporter molecules are separated by the action of 5’exonuclease and fluorescence emission from the reporter can be measured at the end of each extension step. Detection can be done by hybridization probes, which based on the use of two oligonucleotide probes; the donor dye 3’ end and acceptor dye 5’ end that hybridize during the annealing step. The fluorescence emission is proportional to the number of PCR copies produced. Molecular beacon is another detection method based on oligonucleotide probes that have stem loop structure that labelled on their ends by fluorescent and non-fluorescent quenching dye. A new generation of probes and primers are used, e.g., peptide nucleic acid probes, Minor Groove Binding probe and nucleic acid primers and probes (Watzinger, Ebner et al. 2006). The interpretation of RT-PCR depends on the type of materials studied as in liquid materials viral load is indicated per volume unit, in samples with cells the count is indicated per number of cells and in solid specimen the load is indicated per mass unit.

1.2.2 CMV

Cytomegalovirus (CMV) is a double stranded DNA virus. The infection is usually asymptomatic in normal individuals but it is one of the important complications as it can cause both early and late diseases after allogeneic HSCT. CMV can cause pneumonia, gastroenteritis, hepatitis, retinitis and encephalitis (Ljungman 2002). The strategies to control CMV infections after allogeneic HSCT are the models that can be applied to other viruses. Monitoring with the aim to early detect a CMV infection is today most commonly performed by the weekly measurement of the CMV load using a real-time polymerase chain reaction (RT-PCR) during the first
100 days after HSCT. The use of quantitative RT-PCR allows analysis of viral load kinetics including response to antiviral therapy (Emery, Sabin et al. 2000; Gerna, Lilleri et al. 2005; Hows, Passweg et al. 2006; Lengerke, Ljubicic et al. 2006; Schonberger, Meisel et al. 2010). At Karolinska University Hospital, a whole blood RT-PCR is used with duration of monitoring depending on patient risk factors. Preemptive antiviral treatment is initiated when the CMV DNA level reaches 1000 copies/ml and with this strategy the risk for CMV is low (Avetisyan, Aschan et al. 2007; Boeckh and Ljungman 2009).

1.2.3 Adenoviruses

1.2.3.1 Biology

Adenovirus infections have emerged as important viral infections after allogeneic HSCT with high mortality in disseminated disease (Shields, Hackman et al. 1985; Flomenberg, Babbitt et al. 1994; Lion, Baumgartinger et al. 2003; van Tol, Kroes et al. 2005) Adenoviruses are non-enveloped lytic DNA viruses. There are 52 serotypes distributed in 7 subspecies (A-G) (Xu, McDonough et al. 2000; Haque, Wilkie et al. 2007). The serotypes are divided on the basis of morphological, hemagglutinating, oncogenic potentials, and DNA homologies. Adenoviruses are ubiquitous human pathogens generally causing self-limited febrile illnesses in early childhood (Hierholzer 1992; Leen, Bollard et al. 2006) but severe infections can occur also in immune competent individuals. The different serotypes have some predilection for different organs with some types being more commonly found in respiratory tract infections, others in gastrointestinal infections, and some serotypes commonly associated with hemorrhagic cystitis. Adenoviruses can be transferred from person to person by shedding from body secretions as feces, tears, and respiratory secretions. The virus attaches to the endocytosis receptor on the cell membrane where the virus proceeds to the nucleus. Adenoviral proteins lead to viral assembly, cause host cell lysis and escape from the host cell immune response (Leen, Bollard et al. 2006). Adenoviruses can remain in a persistent state especially in the tonsils and adenoids in immune competent healthy people, especially in children (Garnett, Talekar et al. 2009). The gp19 protein is responsible for the persistence state preventing the transport of MHC class I molecules to the surface of infected cells and reducing recognition by antigen-specific cytotoxic T-cells (Lichtenstein, Toth et al. 2004; Echavarria 2008). Therefore, detection of
adenovirus infection in HSCT recipients can be due both to activation of persisting virus and outside sources.

1.2.3.2 Immune response against adenoviruses
The innate and adaptive immune responses start to react early after the virus infection. The innate immune response recognizes the virus by extracellular and intracellular receptors including Toll-like receptors. Release of inflammatory mediators (INF \(\gamma\), TNF \(\alpha\), IL-1 and IL-2) occurs as a consequence of viral recognition. These mediators prevent virus aggregation and maturation by direct antiviral response and activation of other innate immune cells. The response of T-cells is directed to the capsid protein (Molinier-Frenkel, Gahery-Segard et al. 2000). However, the hexon protein consisting of two parts (hyper variable and conserved regions) is the main stimulus for the cell mediated immune response (Rux, Kuser et al. 2003). Both MHC class I &II molecules have been reported presenting adenoviral antigens; MHC class I as well as MHC-II restricted epitopes residing within the hexon protein have been described (Gaudin, Rosado et al. 2004; Tang, Olive et al. 2004). Both specific CD4 and CD8 T-cells can recognize different hexon protein epitopes; they can also recognize different adenovirus subspecies (Fujita, Leen et al. 2008). CD4+ adenovirus specific cytotoxic T-cells are the main immune response against adenovirus infections as they have the ability to lyse adenoviral infected cells by a perforin dependent mechanism.

1.2.3.3 Clinical presentation
The symptoms of adenoviral disease after allogeneic HSCT vary from mild fever, mild diarrhea, respiratory symptoms and hematuria to severe involvement of the affected organs, e.g., severe hemorrhagic cystitis, pneumonia and hemorrhagic enteritis. Disseminated adenovirus disease is associated with high mortality after allogeneic HSCT and can be associated with encephalitis, myocarditis, nephritis, elevated liver enzymes, or multi-organ failure (Shields, Hackman et al. 1985; Howard, Phillips et al. 1999; La Rosa, Champlin et al. 2001; Lion, Baumgartinger et al. 2003; Feuchtinger, Lang et al. 2007; Kalpoe, van der Heiden et al. 2007; Anderson, Guzman-Cottrill et al. 2008; Gustafson, Lindblom et al. 2008).
Diagnosis of adenovirus infection and disease:
The diagnosis of adenovirus infection is commonly done today by detection of adenovirus DNA by RT-PCR in plasma or in whole blood, stool, respiratory secretions, and urine. The diagnosis of adenovirus infection can also be made by rapid antigen detection of adenovirus in stool, urine, bronchoalveolar lavage (BAL), biopsies or autopsy material by viral isolation and immunocytochemistry. Adenovirus disease has been defined differently by different investigators. Proven adenovirus disease is usually defined as detection of adenovirus in material from the involved site by tissue culture or histologic findings together with clinical symptoms commonly associated with adenovirus infections. Disseminated disease is defined as detection of adenovirus at multiple sites together with clinical symptoms (Flomenberg, Babbitt et al. 1994; La Rosa, Champlin et al. 2001; Ljungman, Ribaud et al. 2003). These criteria of diagnosis of adenovirus disease have been modified by including RT-PCR in the diagnostic criteria as follows: diagnosis of adenovirus disease is based on positive PCR in peripheral blood together with isolation of adenovirus from other sites other than respiratory or gastrointestinal (Chakrabarti, Collingham et al. 2000), Probable adenovirus disease is defined as PCR positivity in stool or respiratory secretions together with clinical symptoms and probable adenovirus disseminated disease when PCR is positive in peripheral blood with or without PCR positive at other sites (Chakrabarti, Collingham et al. 2000; Suparno, Milligan et al. 2004).

1.2.3.4 Risk factors
The incidence of adenovirus infection in allogeneic HSCT ranges between 12 and 27% (Chakrabarti, Mautner et al. 2002; Lion, Baumgartinger et al. 2003; Leruez-Ville, Minard et al. 2004; Hakim, Memon et al. 2005). However, the incidence can reach 40-47% in other centers (Hoffman, Shah et al. 2001; Kampmann, Cubitt et al. 2005). This incidence is influenced by the presence of recognized risk factors. Adenovirus disease is most frequently found in high risk children especially in those younger than 5 years old (Hale, Heslop et al. 1999; Lion, Baumgartinger et al. 2003; van Tol, Kroes et al. 2005) Delayed immune reconstitution as well as the use of haplo-identical, mis-matched cord blood, and T-cell depleted grafts are recognized risk factors for both adenovirus infections and disseminated adenovirus disease (Flomenberg, Babbitt et al. 1994; van Tol, Kroes et al. 2005; Feuchtinger,
Lang et al. 2007; Robin, Marque-Juillet et al. 2007). It has been shown by several investigators the effect of using in vivo and ex vivo T-cell depletion on the incidence of adenovirus disease (Runde, Ross et al. 2001; Kampmann, Cubitt et al. 2005; Sivaprakasam, Carr et al. 2007). The use of an unrelated donor graft is one of the risk factors especially together with in vivo or in vitro T-cell depletion. CMV infection and acute GVHD are also considered as risk factors due to the intensive immunosuppressive treatment used in GVHD treatment (Watcharananan, Kiertiburanakul et al. 2010; Flomenberg, Babbitt et al. 1994; Avivi, Chakrabarti et al. 2004; Myers, Krance et al. 2005; Robin, Marque-Juillet et al. 2007; Symeonidis, Jakubowski et al. 2007).

1.2.3.5 Monitoring and management

Detection of adenovirus DNA by RT-PCR in peripheral blood is helpful in monitoring of patients at high risk as it can predict disseminated adenovirus disease (Lion, Baumgartinger et al. 2003; Kalpoe, van der Heiden et al. 2007). In addition, Lion and coworkers showed recently that monitoring of the AdV DNA load in stool by RT-PCR can help to prevent disseminated adenovirus disease by preemptive treatment with antiviral drugs (Lion, Kosulin et al. 2010).

Antiviral therapy: There is no ideal antiviral drug in the treatment of adenovirus infection or disease. Three drugs have been used in with data reported from retrospective studies and small case series.

A. Cidofovir (cytosine analogue): It is the most effective drug against adenovirus in vitro. An advantage is that cidofovir is also effective against the herpesviruses including CMV and acyclovir resistant HSV. It inhibits the DNA polymerase and has been shown to give lower adenoviral loads during treatment and some efficacy in treatment of adenovirus disease. However, no controlled study has been performed but it is associated with nephrotoxicity, uveitis and cytopenias (Legrand, Berrebi et al. 2001; Ljungman, Ribaud et al. 2003; Morfin, Dupuis-Girod et al. 2005; Yusuf, Hale et al. 2006; Neofytos, Ojha et al. 2007).

B. Ribavirin (a purine nucleoside analogue): The drug has been used against many different viruses (RSV, parainfluenza, measles, influenza, HCV) and has also been studied against adenovirus infections. The results from different case series have
been variable but ribavirin is today regarded as having lower efficacy than cidofovir possibly except for subgenus C (Bordigoni, Carret et al. 2001; La Rosa, Champlin et al. 2001; Gavin and Katz 2002; Lankester, Heemskerk et al. 2004; Morfin, Dupuis-Girod et al. 2005)

C. Ganciclovir: Its efficacy against adenovirus disease is not proven due to limited data, however it might have a prophylactic effect when used against CMV (Chen, Liang et al. 1997; Bruno, Gooley et al. 2003).

Adoptive transfer of adenovirus specific T cells:

Feuchtinger et al., have studied T-cell immunotherapy in 6 patients with adenovirus disease; 5 of them had a decrease in the adenoviral load and no associated toxicities. The method is still experimental but it can be a feasible treatment option against adenovirus disease (Feuchtinger, Richard et al. 2008). The use of multivirus specific T-lymphocyte therapy can be used especially in high risk patients to prevent and treat adenovirus, EBV and CMV (Fujita, Leen et al. 2008; Leen, Christin et al. 2009).

1.2.4 EBV

1.2.4.1 Biology

The epstein-barr virus (EBV) was first described in 1964 by Epstein, Achong, and Barr in lymphoblastoid cell lines derived from a Burkitt lymphoma tumor biopsy. EBV is a gamma herpes virus (type 4). It is a DNA virus which infects > 90% of individuals. The primary exposure to EBV usually occurs in childhood. EBV infects B cells by attachment of the envelope glycoprotein to CD21; a component of the complement receptor. Endocytosis of the virion leads to release of the nucleocapsid into the cytoplasm, then the capsid dissolves, and the EBV genome is transported into the nucleus where the viral genome fuses to form a closed circle (Sato, Takimoto et al. 1990; Martin, Marlowe et al. 1994).

EBV and B-lymphocytes:

In healthy EBV carriers, the virus remains dormant in B-lymphocytes which express a number of latency genes that encodes for 6 nuclear proteins ((EBNA)-1,
-2, -3 (or 3A), -4 (or 3B), -5 (or LP) and -6 (or 3C)) and 3 membrane proteins (LMP)-1, LMP-2A and LMP-2B (Dolcetti and Masucci 2003; Pattle and Farrell 2006). One of four EBV latency programs is expressed on B-lymphocytes. The latency 0 program (complete silencing of the viral genome) and Latency I (LMP-2A is expressed alone or together with EBNA-1)(Miyashita, Yang et al. 1997) are present in healthy EBV carriers. The latency I program is usually expressed in patients with Burkitt lymphomas. In immunosuppressed patients such as patients with AIDS, the latency III program (growth program) is expressed in which all 9 latency proteins are present. It is associated with autonomous B-cell proliferation. EBV infected B-lymphocytes in the germinal centers of lymphoid follicles express the latency II program (rescue program) in which EBNA-1 and the three LMPs are expressed (Babcock and Thorley-Lawson 2000).

Latent proteins:
EBNA1 is a viral nuclear DNA-binding protein, which is important for maintenance of the viral episome (Yates, Warren et al. 1985; Grossman, Johannsen et al. 1994; Middleton and Sugden 1994). EBNA2 is a transcriptional nuclear protein that activates the expression of a variety of cellular and viral genes (Grossman, Johannsen et al. 1994; Henkel, Ling et al. 1994; Hsieh and Hayward 1995). LMP1 is an intrinsic membrane protein that leads to loss of contact inhibition in immortalized murine cell lines and tumor formation in nude mice (Wang, Liebowitz et al. 1985). Two more latency proteins other than membrane and nuclear proteins are also present. These are EBV encoded RNAs (EBER1 and 2), their functions are unknown but they can be important in the detection of latent EBV infections (Ambinder and Mann 1994).

Lytic proteins
BZLF1 (Zta) is an early DNA binding protein and transcriptional activator which leads to expression of the viral DNA polymerase. BHRF1 is another lytic protein which is homologous to Bcl-2 and inhibits apoptosis (Henderson, Huen et al. 1993). BCRF1 is also a lytic protein which exhibits high homology to interleukin-10 (IL-10) (Moore, Vieira et al. 1990), which has a role in the modulation of antigen presentation, helper T-cell function, and B-cell growth (Moore, O'Garra et al. 1993).
1.2.4.2 Clinical presentation:

A primary EBV infection in immune competent individuals is usually asymptomatic but producing a lifelong persistent infection. However, EBV causes infectious mononucleosis in 25% of primary infections occurring after puberty. In immunocompromised patients activation of a persistent infection is frequent (Gratama, Oosterveer et al. 1992; Wang, Dahl et al. 1996; Brunstein, Weisdorf et al. 2006). EBV is an oncogenic virus which is associated with a number of malignant diseases such as lymphoproliferative disorders in immunocompromised patients, Burkitt’s lymphoma/NHL, nasopharyngeal carcinoma, NK-cell leukemia/lymphoma, Hodgkin disease, hemophagocytic lymphohistiocytosis and angioimmunoblastic T-cell lymphoma (Cohen 2000).

1.3 PTLD

1.3.1 Definition

PTLD is the common denomination for a wide range of lymphoid and plasmacytoid proliferations that can occur after allogeneic HSCT or solid organ transplantation. 85% of the cases are of B-cell lineage in which more than 80% of the cases are EBV positive. 10-15% of the cases are of T-cell lineage 30% of these are EBV positive (Taylor, Marcus et al. 2005).

1.3.2 Classification

The classification of PTLD is based on the histopathological classification of the World Health Organization (WHO) in 2008 (Swerdlow SH 2008)

The WHO classification of PTLD

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<th>Early lesions</th>
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<td>plasmacytic hyperplasia</td>
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<td>Infectious mononucleosis- like disease</td>
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Polymorphic PTLD

Monomorphic PTLD (B- and T/NK- cell types)

Classical Hodgkin lymphoma type PTLD
1.3.3 Pathogenesis

In the healthy individual, the EBV infection is controlled by humoral and T-cell mediated immune responses. The B-cells are the primary target of EBV staying persistent in healthy individuals. Early after allogeneic HSCT, T-cells are markedly suppressed which increase the chance of uncontrolled proliferation of B cells (Dolcetti 2007).

In the early phases of PTLD, the latency III program is expressed and all EBV proteins are detected. The majority of B-lymphocytes in PTLD cases have germinal center and postgerminal center origin (Brauninger, Spiker et al. 2003), EBV-positive germinal center B-cells are the site of somatic hyper mutation and half of the PTLD cases do not express a functional B-cell receptor due to presence of mutations of Ig genes during the somatic hyper mutation process (Brauninger, Spiker et al. 2003; Timms, Bell et al. 2003). A clonal outgrowth can be promoted by mutations in viral genes that may alter either the functions or the antigenicity of their protein products. Several other factors beside EBV have roles in the development of PTLD including exogenous antigens (Gottschalk, Ng et al. 2001). Furthermore, CD4+ T-lymphocytes and other infiltrating cells may promote tumor growth or modulate cytotoxic T-cell responses with the help of other growth factors as IL-2 and IL-6 (Veronese, Veronesi et al. 1992; Johannessen, Asghar et al. 2000).

IL-7 was found to be expressed in EBV positive B cell lines in a greater amount as compared to EBV-negative B cell lines (Benjamin, Sharma et al. 1994) suggesting a role of IL-7 in the context of B-cell survival and/or B-cell proliferation. The progression of EBV infected B cells to a post transplant lymphoma needs genetic and epigenetic changes within cellular DNA. Somatic hypermutations, aberrant promoter hypermethylation, p53, c-MYC and BCL-6 are frequent mutations affecting PTLD patients (Gottschalk, Rooney et al. 2005).

1.3.4 Clinical presentation

EBV associated PTLD can be either localized to lymph nodes, tonsils, and other single organs or be disseminated to several organs. It can present with general symptoms like fever and other constitutional symptoms, severe GI, and respiratory
symptoms and can also be associated with signs such as tonsillar enlargement, lymphadenopathy and hepatosplenomegaly (Ocheni, Kroeger et al. 2008).

1.3.5 Risk factors
PTLD is associated with high rates of morbidity and mortality and usually develop during the first year after SCT (82%), with its highest incidence during the third month (Curtis, Travis et al. 1999). The incidence of EBV-associated PTLD varies (1–29%) between centres, presumably because of the varying presence of high risk factors in each centre’s SCT population. Different risk factors have been identified by various investigators (Hale and Waldmann 1998; Curtis, Travis et al. 1999; Brunstein, Weisdorf et al. 2006; Sundin, Le Blanc et al. 2006; Landgren, Gilbert et al. 2009) and include HLA mismatch, T cell depletion, the use of antithymocyte globulin (ATG), splenectomy, and pre-transplant EBV serological mismatches between donor and recipient. An increased risk has also been reported in patients receiving cord blood (CB) grafts, possibly since several of the recognized risk factors are present in such patients, including HLA mismatch and use of ATG (Aversa, Tabilio et al. 1998; Brunstein, Weisdorf et al. 2006). Early onset of PTLD is common in patients with myeloablative conditioning, in vitro manipulation of the donor graft, and the use of immunosuppressive drugs for prophylaxis or treatment of GVHD (Curtis, Travis et al. 1999). Patients with age >50 years have been reported having a higher risk than younger patients. The risk of late PTLD development is higher in patients have chronic GVHD and in patients with selective T-cell depletion (Landgren, Gilbert et al. 2009).

1.3.6 Monitoring and management
An early diagnosis of PTLD is of importance since delay in treatment is associated with a mortality rate of more than 90%. Real time PCR is a sensitive and specific tool for monitoring patients for EBV replication and it could be used in patients at a high risk of development of PTLD (Gartner, Schafer et al. 2002; van Esser, Niesters et al. 2002; Wagner, Cheng et al. 2004; Gaeta, Nazzari et al. 2006; Kinch, Oberg et al. 2007). There is a correlation between the number of EBV DNA copies in blood and the likelihood of developing PTLD (Gartner, Schafer et al. 2002; Merlino, Cavallo et al. 2003; Wagner, Cheng et al. 2004). However, the sensitivity, specificity, and positive predictive value vary greatly between different studies (Weinstock, Ambrossi et al. 2006). Weekly monitoring of EBV DNA load by real time PCR has been
recommended for patients at a high risk of PTLD, allowing early detection of a high EBV load, the close follow-up of viral load kinetics and early intervention by way of preemptive treatment (van Esser, Niesters et al. 2002; Sundin, Le Blanc et al. 2006; Kinch, Oberg et al. 2007; Styczynski, Einsele et al. 2009).

The diagnosis is based on clinical, laboratory and radiological findings. However, the pathological diagnosis is the gold standard method for confirmation of the diagnosis. There is a clinical need to provide a standardized reference system of the disease and its prognosis. PET (positron emission tomography) scanning is one of the imaging methods important in the diagnosis as it can detect extranodal disease, it can also differentiate between viable tumor and necrotic or fibrotic tissue (Noraini, Gay et al. 2009). It might also be used for monitoring the response to therapy and predicting prognosis. The histopathological diagnosis is based on the recent WHO classification mentioned above.

Three approaches are used for early management. If possible, the immunosuppression should be reduced to increase the likelihood of mounting a specific immune response against EBV, preemptive treatment of an elevated EBV load before the appearance of symptoms, or prompt (early) treatment in which treatment is given only when elevated EBV loads and symptoms indicate progression to EBV-PTLD. The preemptive therapy can be rituximab or infusion of EBV specific CTL (Rooney, Roskrow et al. 1998; Gustafsson, Levitsky et al. 2000). The treatment of EBV-PTLD consists of reduction of immunosuppression (if possible) and infusion of rituximab (anti CD20 monoclonal antibody) (375mg/m²) or, alternatively, an infusion of EBV specific CTL (Rooney, Aguilar et al. 2001; Haque, Wilkie et al. 2007; Meijer and Cornelissen 2008; Styczynski, Einsele et al. 2009). Antiviral drugs have been used but there is little data to support its efficacy in prevention or management of PTLD (Styczynski, Einsele et al. 2009).

There is no accurate test assessing the response of PTLD to therapy. The assessment of the EBV viral load has been used as a follow-up after treatment but it is not a reliable predictor of the treatment response. Aqui et al. showed that the amount of M protein increased with disease progression and decreased with symptoms improvements. They concluded that M protein can be used as a predictor of PTLD progression and response to treatment (Aqui, Tomaszewski et al. 2003).
1.4 IL-7

1.4.1 Biology

IL-7, a growth factor for B- and T-cells, is a non-redundant cytokine composed of 177 amino acids located on chromosome 8q12-13 in humans (Sutherland, Baker et al. 1989). IL-7 is produced in different tissues including thymus, bone marrow and intestinal epithelium (Maeurer 2003). IL-7 represents a growth factor for B lineage progenitors and it has a main role in B-cell survival, proliferation and maturation. IL-7 provides the survival signals for B-lymphoid precursors and leads to proliferation of pro and large pre-B-cells (Brown, Hulitt et al. 2007). IL-7 is not only able to bind to its nominal receptor (IL-7Rα chain, CD127), but also to the TSLP-receptor that forms a heterodimer with CD127. The nominal ligand for the TSLP receptor is TSLP produced in thymic tissue (Ziegler and Liu 2006).

The IL-7R is located on chromosome 5p13 and composed of 439 amino acids (Venkitaraman and Cowling 1992). It consists of two subunits; the IL-7R alpha chain and the gamma chain which is also called the common cytokine receptor as it shares the gamma chain with the specific alpha chains of IL-2, IL-4, IL-9, IL-15 and IL-21 receptors. (Asao, Okuyama et al. 2001). IL-7Rα is expressed on different hematopoietic cells in peripheral blood (Vudattu, Kuhlmann-Berenzon et al. 2009). IL-7Rα has an important role in pre B cell expansion, it starts to be expressed after occurrence of the pre- B cell receptor (BCR) after proliferative expansion in the large pre B cell stage (Erlandsson, Licence et al. 2005). The density of percentage of CD127 expression is associated with T-cell differentiation/maturation defined by CD45RA and CCR7 expression. IL-7 rescues T-cells from activation-induced cell death (Kinter, Godbout et al. 2008), it represents a crucial cytokine for the induction of long-term memory T-cell responses (Boyman, Letourneau et al. 2009), particularly in intracellular infections (Maeurer, Trinder et al. 2000). IL-7 has also been described to overcome tumor-induced immune-suppression. This was more recently shown in a murine pancreatic cancer model (Pellegrini, Calzascia et al. 2009) and previously in a vaccine setting in patients with melanoma (Moller, Sun et al. 1998).
IL-7 has a major role in different hematological malignancies, e.g. T-ALL (Scupoli, Perbellini et al. 2007), B-ALL (Brown, Hulitt et al. 2007) or cutaneous T-cell lymphomas (Yamanaka, Clark et al. 2006). IL-7 induces lymphoma development with dependence on IL-7R expression along with an enhanced mortality risk (Abraham, Ma et al. 2005). IL-7 has been shown to play a role in solid tumor formation, it promotes cell growth in breast cancer, esophageal, renal and head and neck squamous cell carcinoma (Al-Rawi, Rmali et al. 2004). IL-7 isoforms, generated by alternative splicing have been described in hematological malignancies (Korte, Moricke et al. 1999) as well as in solid tumors (Trinder, Seitzer et al. 1999).

The IL-7R has several isoforms, some of these are expressed in hematological malignancies (Korte, Moricke et al. 1999). More recent data show that at least three alternate forms of the IL-7R exist (Rane, Vudattu et al. 2010) associated with alternative mRNA splicing leading to a soluble IL-7R: a situation which may increase the risk to develop MS (Gregory, Schmidt et al. 2007), some studies also suggested a role in GVHD (Azarpira, Dehghani et al. 2010).

1.4.2 Functional pathway

IL-7 induces signaling by promoting heterodimerization of the IL-7Rα chain and the common γ chain (Olosz and Malek 2000). IL-7Rα is attached to the janus kinase-1 (Jak-1), IL-7Rγ chain is attached to Jak-3, which leads to trans-phosphorylation of the IL-7Rα associated Jak-1 proteins. Jak-1 phosphorylates the tyrosine residue (Y449) present in the cytoplasmic portion of IL-7Rα (Fry and Mackall 2002). Transcription factors are then activated, i.e., the STAT family, the central transscription factor in the IL-7 signaling pathway is STAT5A/B. STAT5 phosphorylation activates target genes that have γ-interferon binding sites (GAS) (TTCNNGAA) (Foxwell, Beadling et al. 1995; Al-Rawi, Mansel et al. 2003). IL-7 signals also via alternate pathways, other than the JAK-STAT pathway, i.e. the PI3K and the Shc-Ras-ERK kinase pathway. Figure (2) shows a simplified model of the IL-7 signaling pathway (Hofmeister, Khaled et al. 1999).

During the period of immune reconstitution and severe T-cell depletion, IL-7 is needed for survival, homeostasis and proliferation of mature T-cells (Capitini, Chisti et al. 2009) However, there could also be adverse effects, as IL-7 may be able to induce a proliferation of reactive T-cells against the host which can
exaggerate GVHD (Azarpira, Dehghani et al. 2010). Efforts to increase immune recovery by administration of IL-7 without precipitation of GVHD are still clinically tested. IL-7 has also a role to induce T-cell reactivity in autoimmune diseases, e.g., multiple sclerosis and autoimmune arthritis (Snyder, Mackall et al. 2006). It has recently been shown that infusion of IL-7 increases the level of antigen-specific IL-17-producing T-cells instrumental against intracellular pathogens (Pellegrini, Calzascia et al. 2009).

Figure (2) IL-7 signaling pathway
2 AIMS

General:
To develop strategies to control severe viral complications after allogeneic hematopoietic stem cell transplantation.

Specific:
1- To characterize AdV infection after allogeneic HSCT in a Swedish cohort.
2- To apply a monitoring strategy to control AdV infection after allogeneic HSCT.
3- To evaluate a strategy to monitor patients at high risk of EBV associated PTLD.
4- To study the role of IL-7 in development of EBV associated PTLD.
3 PATIENTS AND METHODS

3.1 PATIENTS

Patients included in all studies in the thesis underwent allogeneic HSCT at the Karolinska University Hospital, Huddinge. However, in study II, 31 of 101 study patients were transplanted at Sahlgrenska University Hospital, Gothenburg. The regional ethical review board in Stockholm approved the studies.

Study I: Seventeen patients with AdV viremias were retrospectively analyzed. The patients were selected from a cohort of 344 allogeneic HSCT patients transplanted between the years of 2002 and 2006.

Study II: 101 patients who underwent allogeneic HSCT during the period of March 2006 to September 2007 in Karolinska University Hospital (50 adults; 20 children) and 31 adult patients transplanted in Sahlgrenska University Hospital were prospectively included. Four patients were excluded due to few collected samples. Thus, 97 patients were analyzed.

Study III: 131 patients, who were monitored for EBV DNA according to a predefined strategy during the period of July 2005 to June 2007, were retrospectively analyzed. A control group of 150 patients who transplanted between January 2003 and June 2005 was selected to study the impact of the monitoring strategy.

Study IV: PBMCs from 7 patients with confirmed diagnosis of PTLD have been studied. Samples from 10 EBV DNA positive patients who did not have PTLD were used as controls.

Patients’ characteristics, transplantation procedures and complications are described in detail in the papers.
3.2 METHODS

Quantitative Real Time-PCR (RT-PCR): This technique was used in study I-III. Plasma samples were used for adenovirus diagnosis, serum samples were used for EBV diagnosis and whole blood samples were used for CMV diagnosis.

A monitoring strategy to control viral infections is now routinely used to monitor CMV by weekly measurement of CMV DNA by RT-PCR and preemptive therapy is given when the viral load reaches 1000 copies/ml (Yun, Lewensohn-Fuchs et al. 2003; Hows, Passweg et al. 2006; Boeckh and Ljungman 2009). This strategy was applied in the first three papers.

TaqMan based RT-PCR techniques were used for the detection of viral DNA. The following genes were used for diagnosis of CMV, adenovirus and EBV respectively: a conserved region in the CMV polymerase (pol) gene, conserved region of hexon gene of adenovirus, the EBV BNRF gene of EBV (Niesters, van Esser et al. 2000; Heim, Ebnet et al. 2003; Yun, Lewensohn-Fuchs et al. 2003; Gustafson, Lindblom et al. 2008). Details of the whole RT-PCR procedures are described in their respective papers.

Study II: Patients were monitored for adenovirus DNA by RT-PCR on a weekly basis during the first 9 weeks and after that at three, six and twelve months. In addition, an ELISPOT technique was used to measure adenovirus specific T-cell responses. ELISPOT was first described in 1983 on the immunoenzematic principles of ELISA but it is 200 times sensitive than ELISA (Czerkinsky, Nilsson et al. 1983). It can detect cytokines detection from a low number of cells (10-100 cells /well). It can for example detect IFNγ, TNF-α, IL-2 and IL-4 secretion from the cells by the direct visualization of cytokines secreting cell as a spot (Letsch and Scheibenbogen 2003). The technique depends for its results on the quality of the four main steps in the assay; the choice of the capture and detection antibodies, the enzyme conjugate, the substrates, and the coated plates. The capture and detection antibodies can recognize epitopes on the target antigens, The antibodies can be either monoclonal or polyclonal. We used alkaline phosphatase as the conjugate, Enzyme substrates as BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) are used to produce stable color, then developing of the spots (Kalyuzhny 2005). PBMCs were isolated from heparinized peripheral blood samples by LymphoPrep gradient. The cells were incubated overnight with adenolysate
(kindly provided by Dr. Tobias Feuchtinger, Tübingen, Germany), negative and positive controls. Biotinylated mAb (7-B6-1-biotin) and then streptavidine-alkaline phosphatase were added for detection of the spots that were measured after drying of the plate by an ELISPOT reader (Feuchtinger, Lang et al. 2004).

Study III: Serum samples were analysed for detection of EBV DNA by RT-PCR once weekly during the first three months after HSCT. Preemptive treatment with rituximab was given when the EBV DNA reached 10,000 copies/ml. In the control group, samples for EBV DNA detection were analyzed on clinical suspicion and no routine intervention strategy was defined.

Study IV: PBMCs from 7 PTLD patients were used in the experiments. We had access to PBMCs at time points before, at and after PTLD development. However, in two patients we had only PBMCs at and after the PTLD diagnosis. For comparison, a control group of 10 patients was selected who were EBV positive at the time of sampling (6 patients and before sampling time (4 patients) Samples were chosen from a time point approximately equivalent to the time of PTLD diagnosis in the study patients.

The following methods were used:

Flow cytometry was used to assess the expression of IL-7R (CD127) on different immune cell subsets (B- and T-cells), EBV specific CD8+ T cells were enumerated by tetramer-guided analysis (Jager, Benninger-Doring et al. 1998). Tetramer-reactive T-cells were examined for CD107a expression, a marker indicative of T-cell degranulation (Magalhaes, Vudattu et al. 2008). The CD127 antibody binding capacity values per cell (enumeration of IL-7 receptor molecules / cell) were determined using the median value of CD127 as compared to APC-coupled beads as described (Vudattu, Kuhlmann-Berenzon et al. 2009). STAT5 phosphorylation (p- STAT5) as a result of IL-7 or IL-2 stimulation of CD4+ or CD8+ T cells was performed using a STAT5 phosphorylation assay (Magalhaes, Vudattu et al. 2008; Vudattu, Kuhlmann-Berenzon et al. 2009). A summary of the flow cytometry panels is listed below in the table (1). Data analysis was performed using FlowJo software 8.8.6.
Table (1) Flow cytometry panels used in the study.

<table>
<thead>
<tr>
<th>Panel 1</th>
<th>Panel 2</th>
<th>Panel 3</th>
<th>Panel 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3- ECD</td>
<td>CD19 Amcyan</td>
<td>CD19 Amcyan</td>
<td>pSTAT5 - Alexa 488</td>
</tr>
<tr>
<td>CD8a- APC Cy7</td>
<td>CD16/56 – PE</td>
<td>CD20 Pacific Blue</td>
<td>STAT5-CF</td>
</tr>
<tr>
<td>CD4- Pacific blue</td>
<td>TCRαβ-PerCP</td>
<td>CD 138 PerCP Cy 5.5</td>
<td>CD3 - ECD</td>
</tr>
<tr>
<td>CD127- APC</td>
<td>TCRγδ -PE Cy5.5</td>
<td>CD127- APC</td>
<td>CD4 - PE Cy5</td>
</tr>
<tr>
<td>CD45RA PerCP</td>
<td>CD127- APC</td>
<td>CD 77 FITC</td>
<td>CD8a – APC Cy7</td>
</tr>
<tr>
<td>CCR7- PE Cy7</td>
<td>CD8a- APC Cy7</td>
<td>CD 27 APC-H7</td>
<td>CD19 – PE</td>
</tr>
<tr>
<td>CD107a- APC Alexa700</td>
<td>CD4- Pacific blue</td>
<td>CD 23 Alexaflour 700</td>
<td></td>
</tr>
<tr>
<td>Tetramers in PE</td>
<td>CD3- ECD</td>
<td>CD5 PerCP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD14 FITC</td>
<td>IgD PE-Cy7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD 10 PE</td>
<td></td>
</tr>
</tbody>
</table>

An ELISA was used to measure the levels of IL-7 and IL-7R in serum and plasma samples of all patients with PTLD and EBV+ (PTLD-) control patients (Rose, Lambotte et al. 2009).

### 3.3 STATISTICAL METHODS

In paper I: Differences in distribution of patients with adenovirus DNA positive were compared by the Chi-square test and the levels of AdV DNA were compared by Mann Whitney U-test.

In paper II: Risk factors were analyzed by creating multivariate logistic regression models. Data were analyzed using Statistica software version 8.0 for Windows.

In Paper III: Analysis of factors influencing the viral loads was done by multiple linear regressions. Univariate and multivariate logistic regression techniques were applied to evaluate the possible risk factors for EBV viremia.

In Paper IV: A permutation test was used to compare p-STAT5 results with levels of IL-2 and IL-7 in patients and controls. Then Monte Carlo analysis was performed.
4 RESULTS AND DISCUSSION

Adenoviruses and HSCT; papers I and II
Detection of adenovirus DNA with RT-PCR from peripheral blood has been reported being an early and good predictor of disseminated adenovirus disease (Runde, Ross et al. 2001; Lion, Baumgartinger et al. 2003; Muller, Levin et al. 2005). However, most studies were performed in children, whom has an increased risk for adenovirus disease compared to adults.

In study I we analyzed the possible predictive value of adenovirus viremia (DNAemia) for development and outcome of adenovirus disease in adults. In study II we attempted a monitoring strategy for early detection of adenovirus DNA positivity with the aim of developing an intervention strategy. In addition, we assessed adenovirus specific T cell response in a subgroup of the study cohort.

Study I:
Poor outcome of adenovirus infections in adult hematopoietic stem cell transplant patients with sustained adenovirus viremia
H. Omar, Z. Yun, I. Lewensohn-Fuchs, L. Pérez Bercoff, C. Örvell, L. Engström, G.-K. Vuong, P. Ljungman. Transplant Infectious Diseases, in press 2010

Characterization of adenovirus infections in adult patients
All patients with AdV viremia, who underwent allogeneic HSCT at Karolinska University Hospital between the years of 2002 and 2006 were included in the study. Patients were retrospectively identified through the records of the virological laboratory and a chart review was performed to possibly identify risk factors for adenovirus disease, the role of antiviral therapy and outcome. Adenoviremia was detected in 4.9% of the patients; 5.4% in adults and 3% in children.

Risk factors associated with adenoviral disease
We were unable to find an increased risk among patients receiving grafts from unrelated donors despite that this has been a finding in several centers including a study from our own center (Lion, Baumgartinger et al. 2003; Muller, Levin et al. 2005;
Gustafson, Lindblom et al. 2008). The explanation for this is unclear. Younger age has been shown to be a significant risk factor for adenovirus disease especially in children less than 5 years (Hakim, Memon et al. 2005). It has been suggested that this is due to activation of a persistent virus (Garnett, Erdman et al. 2002; Hakim, Memon et al. 2005). However, we were unable to reproduce this finding possibly due to the low number of children who had adenoviremia and the transplantation techniques used in our center for example we do not perform haplo-identical transplants. Either CMV or EBV viremia was accompanied by adenoviremia in 70 and 64% of the patients, respectively and both viruses were present at high levels in 36% of the patients. This simultaneous presence of several viruses might be due to a poor T-cell immune reconstitution but also due to that CMV is immunosuppressive in its own right. In fact 16/17 patients had severe lymphopenia at the time of high viral load. This finding is in agreement with results by Lion et al. in a study on a pediatric cohort (Lion, Baumgartinger et al. 2003) and also with a previous study from our center where several different herpesviruses could be simultaneously detected (Wang, Dahl et al. 1996). In paper I we did not look for HHV-6 but that is also an immunosuppressive virus that seems to influence the course of other viral infections and might well have been present as well (Wang, Linde et al. 1999). 13/17 (76%) of patients with adenoviremia had acute GVHD grade II-IV which is a similar finding to many other studies probably due to the severe immunosuppression caused by GVHD and its treatment. (Avivi, Chakrabarti et al. 2004; Myers, Krance et al. 2005; Robin, Marque-Juillet et al. 2007).

Sustained adenoviremia (≥ 3 positive RT-PCR samples) was frequent in our studied patients; 12/14 (86%) adult patients had sustained adenoviremia and these patients had also higher viral loads than those with a transient adenoviremia. Furthermore, 5 of twelve (42%) adult patients with a sustained adenoviremia had definite or disseminated adenoviral disease confirmed with biopsy or autopsy and in three patients, adenovirus disease proved to be the cause of death.

65% of adenoviremic patients were also PCR positive in stool and 23% had positive urine samples. However, Lion et al. recently showed in a pediatric allogeneic HSCT cohort that 100% of the patients who had adenoviremia in peripheral blood also were positive for adenovirus DNA in stool. However, this finding may be more applicable to
children as the 3 children with adenoviremia included in our study also were PCR positive in stool (Lion, Kosulin et al. 2010)

Serotyping of positive samples was done in 11 patients and species A, B and C were found in 3, 4, 4 patients, respectively. All patients with serotypes A and C were positive in stool and plasma however 3/4 patients with serotype B showed adenovirus PCR positive in urine. Details of serotypes and sites of diagnoses are mentioned in table (2).

Table (2): Adenovirus serotypes and sites of positive RT-PCR samples

<table>
<thead>
<tr>
<th>Species/serotype</th>
<th>Adenovirus positive specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/35</td>
<td>Urine, BAL, PL</td>
</tr>
<tr>
<td>B/35</td>
<td>Urine, PL</td>
</tr>
<tr>
<td>B/35</td>
<td>Urine, PL</td>
</tr>
<tr>
<td>B/35</td>
<td>BAL, PL</td>
</tr>
<tr>
<td>A/31</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>A/31</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>A/31</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>C/2</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>C/5</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>C/2</td>
<td>Stool, PL</td>
</tr>
<tr>
<td>C/1</td>
<td>Stool, PL</td>
</tr>
</tbody>
</table>

All patients who were treated with cidofovir as preemptive therapy cleared the virus and none died of adenovirus disease. This is in agreement with current management recommendations (Ljungman, Ribaud et al. 2003; Suparno, Milligan et al. 2004; Hakim, Memon et al. 2005; Neofytos, Ojha et al. 2007; Sivaprakasam, Carr et al. 2007; Zaia, Baden et al. 2009). However, when to start preemptive treatment has not been well established due to lack of well designed studies. Cidofovir has important side effects such as nephrotoxicity (Ljungman, Ribaud et al. 2003) and there are also reports of poor efficacy. Therefore, more effective and safer drugs are needed.
Study II


In the previous study we documented a correlation between the presence of sustained adenoviremia and mortality from disseminated adenovirus disease. This has previously been found by other investigators (Suparno, Milligan et al. 2004). The aim of this prospective study was to investigate the effect of early detection of adenoviremia on outcome.

97 patients (77 adults and 20 children) were analyzed. Five of the 97 (5%) patients had positive adenovirus DNA detected at least once; 3 children and 2 adults. Four patients had peak viral loads less than 1000 copies/ml from 1-2 samples while one patient had a sustained elevated viral load for three months with a peak level of 9000 copies/ml. Genotyping of the positive samples showed that the patient with a sustained adenoviremia had genotype 1 and the rest of the strains were of types 2, 3, and 31, respectively. None of these patients had confirmed adenovirus disease and no one received antiviral treatment. Thus, overall the study was negative since the frequency of adenovirus detection was low and we had no possibility to based on these data fulfill our aim.

The study showed that 15% of the children and 3% of the adults were adenovirus DNA positive (p= 0.06). This lower incidence of adenoviremia in adults was also shown in other studies, (Flomenberg, Babbitt et al. 1994; Kalpoe, van der Heiden et al. 2007). However, it contrasts somewhat with what we found in study I. The reason for this difference is unclear but it might be due to the different designs of the two studies: study II was prospective with a clear sample algorithm while study I was retrospective and sampling performed only on clinical suspicion.

Risk factors for adenoviremia have been identified in several studies. These risk factors are younger age, T cell depleted grafts, GVHD, delayed immune recovery grafts from
unrelated donors (Baldwin, Kingman et al. 2000; Bruno, Gooley et al. 2003; Kampmann, Cubitt et al. 2005; Myers, Krance et al. 2005). We could not identify in this study the impact of any of these risk factors on adenoviremia. However, we found that myelodysplastic syndrome is a significant risk factor in this cohort (p=0.002), currently, we have no explanation for this finding that needs to be confirmed in other studies. We also found that bone marrow instead of peripheral blood stem cells was a significant risk factor for adenoviremia. This finding is also unexplained although the center policy was to use bone marrow as the primary stem cell source for children and for patients with nonmalignant disorders such as immune deficiencies.

The results of this study argue against a general application of monitoring for adenovirus in HSCT recipients. However, a monitoring strategy targeting patients with known high risk factors especially children could be contemplated. (Chakrabarti, Mautner et al. 2002; Lion, Baumgartinger et al. 2003). Recently Lion et al. suggested applying the monitoring strategy in stool samples than in peripheral blood as an early predictor of adenovirus disease in children (Lion, Kosulin et al. 2010).

Another possible way to assess the risk for adenovirus disease is to analyze the adenovirus specific immune response. Twelve patients from the prospective study were selected to examine their immune response against adenovirus infection by an ELISPOT assay. Patients were assayed at three time points 4, 8, 12 weeks after allogeneic HSCT. Seven (58%) patients had adenovirus specific T-cell immune response by presence of spot forming units (SFU) ≥20 spots at least once. However, none was adenovirus PCR positive. We also studied 12 other patients not included in the prospective monitoring study. Putting both groups together, the ELISPOT results of these 24 patients showed that 20/24 (83%) patients had positive adenovirus specific T-cell at least at one time point during the first three months; 47% of samples had spots ≥ 20 adenovirus specific T-cells/10^6 PBMCs. Adenovirus specific T-cells tend to decrease with time. The overall responses at 4 weeks were 53%, 55% at 8 weeks and 43% at 12 weeks which was borderline significantly lower compared to at 8 weeks (p=0.058). Patients with PBSC grafts tended to have more adenovirus specific T cells at 8 weeks (p=0.058). However, we did not found any effect of the donor type, the severity of GVHD, conditioning regimens, or T-cell depletion either with alemtuzumab or ATG. The presence of adenovirus specific T-cells may be of
importance in prevention of adenovirus infection early after allogeneic HSCT (Feuchtinger, Lucke et al. 2005).

In conclusion, adenovirus infections are more commonly in children and monitoring strategy could be applied to high risk groups. Most adult patients had adenovirus specific T-cells during the first three months after allogeneic HSCT possibly explaining the low risk for adenovirus infection and disease.

EBV-PTLD in HSCT recipients

In study III we monitored the patients at high risk of EBV-PTLD development. In study IV we examined the role of IL-7 and IL-7R on development of EBV-PTLD

Study III

Targeted monitoring of patients at high risk of post-transplant lymphoproliferative disease by a quantitative Epstein-Barr virus polymerase chain reaction.


The wide variation in the incidence of EBV-PTLD between different centers might depend on the varying presence of recognized risk factors.(Aversa, Tabilio et al. 1998; Hale and Waldmann 1998; Curtis, Travis et al. 1999; Brunstein, Weisdorf et al. 2006; Sundin, Le Blanc et al. 2006; Landgren, Gilbert et al. 2009). Weekly monitoring of EBV DNA by RT-PCR may help to decrease the mortality risk of EBV-PTLD in high risk patients by early detection of EBV viremia and starting preemptive treatment with rituximab (Everly, Bloom et al. 2007; Kinch, Oberg et al. 2007; Styczynski, Einsele et al. 2009; Zaia, Baden et al. 2009). However, the techniques used to monitor EBV, the sample types, and the decisions when to intervene varies greatly between different centers (Weinstock, Ambrossi et al. 2006; Styczynski, Einsele et al. 2009). The aim with this study was therefore to use existing knowledge of risk factors to develop a monitoring strategy targeting patients with risk factors.
131 patients who underwent allogeneic HSCT between July 2005 and June 2007 were included in the study following a predetermined management algorithm. The patients were divided into high risk and standard risk groups. The high risk group included patients with the following risk factors: EBV mismatch between donor and recipient before HSCT, cord blood graft, patients transplanted due to lymphoma, and patients who had EBV disease before allogeneic HSCT. These patients were monitored by EBV RT-PCR once weekly in the first three months after allogeneic HSCT. Rituximab was given when the viral loads were 10,000 copies/ml or there were symptoms suggesting EBV disease. 150 patients transplanted during the period between (January 2003 to June 2005) were used as a control group. During this period patients were sampled for EBV DNA on clinical suspicion and no guidelines existed regarding rituximab therapy. A summary of both the study and control groups are shown in table (3).

Table (3): Results of the study and control groups:

<table>
<thead>
<tr>
<th>Numbers of Patients</th>
<th>Study Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High risk</td>
<td>Standard risk</td>
</tr>
<tr>
<td>Total</td>
<td>53/131 (44.3%)</td>
<td>78/131 (45.7%)</td>
</tr>
<tr>
<td>PCR tests median</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>EBV positive</td>
<td>21/53 (39.6%)</td>
<td>19/78 (24.3%)</td>
</tr>
<tr>
<td>EBV loads (min- max), median</td>
<td>(50- 1.4 × 10^6), 4150 copies/ml</td>
<td>(50- 2.3 × 10^6), 50 copies /ml</td>
</tr>
<tr>
<td>Received rituximab</td>
<td>9/53 (17%)</td>
<td>3/78 (3.8%)</td>
</tr>
<tr>
<td>Developed PTLD</td>
<td>3/53 (5.6%)</td>
<td>1/78 (1.3%)</td>
</tr>
<tr>
<td>Died from PTLD</td>
<td>1/53 (1.9%)</td>
<td>1/78 (1.3%)</td>
</tr>
</tbody>
</table>

30% of the study group had EBV reactivations; 39.6% among the high-risk patients and 24.3% among the standard risk patients (p=0.009). We found in multivariate analysis that patients with younger age were at a higher risk of EBV viremia (p=0.02) and that a higher number of samples increased the possibility of detecting EBV DNA (p<0.0001). Patients in the high risk group developed higher viral loads. Previous studies have shown that patients with EBV associated PTLD usually have high viral loads (van Esser, van der Holt et al. 2001; Gartner, Schafer et al. 2002; Kinch, Oberg et al. 2007) and measurement of EBV load may co-vary with the tumor burden (Wagner, Wessel et al. 2001; Tsai, Douglas et al. 2008)
Nine of 53 (17%) patients in the high risk group received rituximab according to the monitoring strategy and three (5.6%) developed PTLD. In the standard risk group, three of 78 (3.8%) patients in the study group and one (1.3%) patient had PTLD and died from PTLD. The mortality from PTLD in the high risk and the historical high risk control groups was 1.9 and 5.7%, respectively. Thus, the incidence of PTLD and the mortality rates seemingly did decrease with the management algorithm but this difference is not statistically significant. Furthermore not to monitor standard risk patients seemed to be a safe strategy. Currently, preemptive treatment with rituximab is widely used in transplant centers. However, the threshold EBV PCR value for preemptive treatment is different from centre to centre. This might be one important factor for the wide variation in the PTLD prevalence (van Esser, van der Holt et al. 2001; Annels, Kalpoe et al. 2006; Kinch, Oberg et al. 2007; Meerbach, Wutzler et al. 2008; Ahmad, Cau et al. 2009).

In the study group, only splenectomy was found to be a high risk factor in both univariate (p=0.046) and multivariate analysis (p=0.045). The presence of an EBV positive donor to an EBV negative recipient was significant only in univariate analysis (p=0.001). However, in the control group, multivariate analysis showed that using an EBV positive donor to an EBV negative recipient (p=0.01) and HLA mismatched donor (p=0.01) were significant risk factors on PTLD development. Splenectomy was shown to be a borderline risk factor (p=0.08).

Splenectomy was shown for the first time as a significant risk factor by Sundin et al, and our report confirm this finding which may occur due to impairment of B cell selection due to removal of the spleen (Gaudin, Rosado et al. 2004; Sundin, Le Blanc et al. 2006). It should be recognized that some patients were included in both our study and the study by Sundin et al. Other factors that can increase the risk of PTLD as the use of anti-T-cell antibodies (e.g. ATG), stem cell source, donor type, conditioning regimen and grade of acute GVHD had no significant impact on PTLD development in our cohort, however they were shown to be high risk factors in other centers (Bhatia, Ramsay et al. 1996; Curtis, Travis et al. 1999; van Esser, Niesters et al. 2002; Baker, DeFor et al. 2003; Juvonen, Aalto et al. 2003; Brunstein, Weisdorf et al. 2006; Sundin, Le Blanc et al. 2006)

In conclusion, this monitoring strategy seemingly reduced the risk of PTLD in patients with risk factors. A weakness of our study is the use of historical controls. Therefore, to
prove this positive effect a randomized, controlled trial with large number of patients would be necessary. Such a trial is unlikely to be performed since monitoring today is standard practice in many centres including our own.

Study IV.

**Decreased IL-7 signaling in T-cells from patients with PTLD after allogeneic HSCT**

Hamdy Omar, Raija Ahmed, Andreas Björklund, Åsa Gustafsson-Jernberg, Per Ljungman and Markus J Maeurer.

EBV remains in B-cells in a persistent form and may lead to B-cell transformation after allogeneic HSCT due to prolonged or intensive immune-suppression (Brauninger, Spieker et al. 2003). The production of IL-7 protein, as well as the expression of the IL-7R has been described to be increased in EBV positive B cell lines as compared to EBV–negative B-cells lines (Benjamin, Sharma et al. 1994). IL-7 may induce lymphoma formation, expression of the IL-7R may cause expansion of pre-B-cells (Abraham, Ma et al. 2005; Erlandsson, Licence et al. 2005). In PTLD, all EBV latency proteins are expressed (latency III program) that can be detected by EBV-specific CD8+ T-cells (Brauninger, Spieker et al. 2003). Thus, the IL-7/IL-7R axis appears to be a ‘double-edged sword’: The IL-7R may mediate growth promoting effects in transformed cells and contribute therefore to tumor development. In contrast, IL-7 represents a central T-cell survival factor (Maeurer, Walter et al. 1997; Al-Rawi, Rmali et al. 2004; Cattaruzza, Gloghini et al. 2009). IL-7R expression on immune effector cells (Fry and Mackall 2002; Abraham, Ma et al. 2005) aid to establish and to maintain long-term anti-EBV cellular immune responses that may be able to kill off or contain EBV+ B-cells.

The aim of the study was to examine if the response of EBV-specific CD8+ T-cells is different in patients with PTLD as compared to EBV DNA positive controls, who did not develop PTLD. This could be measured by the enumeration of EBV-specific, tetramer-reactive T-cells followed by a more detailed analysis of the T-cell differentiation / homing (CD45RA/CCR7) (Magalhaes, Vudattu et al. 2008) and degranulation (CD107a) (Chentoufi, Zhang et al. 2008) markers on antigen-specific T-cells. Failure to effectively contain EBV+ B-cells could also be due to impaired
function of antigen-specific T-cells. The IL-7 signaling pathway could be affected, since the IL-7/IL-7R axis represents a key pathway to prevent T-cells from activation-induced cell death associated with the up-regulation of T-cell survival factors (Marsden, Kappler et al. 2006). We measured therefore the expression of the IL-7R on CD4+, CD8+ T-cells, as well as on tetramer-reactive CD8+ T-cells and evaluated the function of the IL-7 signaling pathway defined by the phosphorylation of STAT5. PBMCs from 7 patients had PTLD and 10 controls were used in the study.

Detection of CD8+ EBV –specific T cells in Patients with PTLD and controls

We could detect EBV-specific CD8+ T-cells against epitopes from EBV latent and lytic proteins in blood from patients with PTLD as well as from EBV+ control patients using HLA A*0201 and HLA-A*2402 tetramer molecules loaded with EBV target peptides.

The presence of EBV specific CD8+ T-cells plays an important role in the control of EBV during the period of immune reconstitution early after allogeneic HSCT: PTLD can be prevented and treated by adoptive transfer of EBV specific T-cells (Gustafsson, Levitsky et al. 2000; Heslop, Slobod et al. 2010). We could demonstrate EBV / tetramer+ CD8+ T-cells in blood from PTLD patients. EBV specific T-cells ranged between 0.1-11.8 percent of the entire CD8 + T-cell population and appeared to be associated with the level of EBV viral load ranges (1400- 1.4x 10^6 copies/ml). The control group (with less EBV loads: 50-13000 copies/ml) exhibited a different picture. 4 individuals had EBV reactivation before the time of sampling and 6 experienced EBV reactivation at the time of sampling). We could detect a different number of EBV-specific CD8+ T-cells ranging from 0.01-1.03% in CD8+ T-cells. These data are in line with Annels et al, 2006, who reported the range of tetramer+ CD8+ T-cells in association with EBV reactivation after allogeneic HSCT between 0.1-12% in CD8+ T-cells. Difference in EBV-responses may in part reflect individual differences, they may also be associated with different restricting MHC class I alleles and, not mutually exclusive, with different EBV target epitopes. We have been able to confirm this notion. We were able to examine the EBV-responses in a single (PTLD) patient in greater detail using 8 different MHC class I/EBV tetramer molecules. The number of EBV-specific CD8+ T-cells changed over time after HSCT and this was associated with a different EBV- epitope focus. CD8 + T-cells showed preferential recognition of certain EBV epitopes early after HSCT and this pattern was found to be different several months after HSCT (Table 4). A similar finding has
been reported for the antigen-specific T-cell response in blood from patients with infectious mononucleosis (Hislop, Annels et al. 2002; Annels, Kalpoe et al. 2006). Tetramer-guided analysis of EBV-specific T-cells does not reflect T-cell function. The simultaneous \textit{ex vivo} detection of the degranulation marker CD107a on antigen-specific T-cells suggested that these EBV-reactive T-cells were cytotoxic. They also expressed the IL-7R and could therefore receive T-cell survival signals. Thus, we could detect the presence of EBV-reactive T-cells in patients with PTLD. Yet, these T-cells may be functionally impaired. This has been shown to be true concerning perforin expression or decreased \text{INF} \gamma production in EBV reactive T-cell with PTLD (Guppy, Rawlings et al. 2007; Pietersma, van Dorp et al.).

Table (4): Detection of Antigen-specific T-cells by tetramer-guided analysis in blood from patients with PTLD and control patients

<table>
<thead>
<tr>
<th>HLA-A*2402</th>
<th>BRFL1-Lytic (DYCNVNLKENF)</th>
<th>EBNA3-Latent (RYSIFFDY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTLD patient 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W0</td>
<td>3,93</td>
<td>2,04</td>
</tr>
<tr>
<td>W12</td>
<td>1,81</td>
<td>0,2</td>
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<tr>
<td>W52</td>
<td>6,5</td>
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<td>2,15</td>
</tr>
<tr>
<td>W104</td>
<td>8,71</td>
<td>2,28</td>
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</table>

<table>
<thead>
<tr>
<th>HLA-A*0201</th>
<th>BRFL1-Lytic (GLCTLVAML)</th>
<th>LMP2-Latent (CLGGLLTLML)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTLD patient 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W8</td>
<td>4,97</td>
<td>2,67</td>
</tr>
<tr>
<td>W12</td>
<td>2,06</td>
<td>2,52</td>
</tr>
<tr>
<td>W16</td>
<td>5</td>
<td>2,47</td>
</tr>
<tr>
<td>Control pt 1</td>
<td>0,35</td>
<td>0,88</td>
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<tr>
<td>Control pt 3</td>
<td>0,43</td>
<td>0,22</td>
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<td>Control pt 4</td>
<td>0,019</td>
<td>0,012</td>
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<td>Control pt 5</td>
<td>0,83</td>
<td>0,14</td>
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<td>Control pt 6</td>
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<td>Control pt 7</td>
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<tr>
<td>Control pt 8</td>
<td>0,5</td>
<td>0,012</td>
</tr>
<tr>
<td>Control pt 10 W8</td>
<td>0,019</td>
<td>0,046</td>
</tr>
<tr>
<td>Control pt 10 W12</td>
<td>0,12</td>
<td>1,03</td>
</tr>
</tbody>
</table>
Impairment of STAT5 phosphorylation in T-cells from PTLD patients

STAT5 phosphorylation in response to IL-7 and IL-2 was measured in CD4+ and CD8+ T-cells in blood from PTLD patients and controls. CD4+ T-cells from the EBV-PCR positive controls exhibited strong STAT5 phosphorylation in response to IL-7 (ranging from 27.4 to 89.9 % responding cells, mean: 71) with a similar pattern for IL-2 (ranging from 66.5 to 87.4 %, mean: 79.6). However in PTLD patients, STAT5 phosphorylation in response to IL-7 was decreased in CD4+ T-cells (0.33 to 46.8 % responding cells with a mean 19.1%, p< 0.0001) and also to IL-2 (ranging from 0.24 to 51.7 %, mean 22.3, p< 0.0001). CD8+ T-cells from the control patients showed strong responsiveness to IL-2 (ranging 85.4 -99.7 %, mean: 94.2%) and a lower response to IL-7 (6.53-83.8 % of CD8+ T-cells mean: 32.4). In PBMCs obtained from PTLD patients, the responsiveness to IL-7 was decreased as compared to controls (0.52-17.5 % with a mean of 5.2, p=0004) in CD8+T-cells and the same was found to be true for IL-2-induced STAT5 phosphorylation (1.86 to 93.2% with a mean of 39.4, p < 0.0001). No differences were found in the levels of IL-7R on CD4+ and CD8+ T-cells either in PTLD patients or the control patients. We found that the defect in IL-7 response, defined by STAT5 phosphorylation, was not due to the absence of the STAT5 protein. A defect in IL-7 response could be due to a defect in the functional signal pathway, i.e. the Jak-STAT, PI3K or AKT pathway. Impaired functions have been associated with malignant transformation in epithelial tumor cells (Al-Rawi, Rmali et al. 2004) and Hodgkin cells (Cattaruzza, Gloghini et al. 2009). A previous study showed a similar reactivity pattern in PBMCs from patients with breast cancer (prior to any chemotherapy). Despite the fact that PBMCs from patients with breast cancer lesions showed strong IL-7R expression, they failed to signal properly in response to IL-7 defined by STAT5 phosphorylation, this correlated also with a defective cytokine production (IFNγ) which has not been determined in the current study due to the limited numbers of T-cells after HSCT (Vudattu, Magalhaes et al. 2007). A number of other T-cell defects have been reported in patients with cancer, e.g. defect phosphorylation of the TCR zeta-chain (Whiteside 2004), or immune- ‘exhaustion’ reflected by PD1-expressing immune cells (Barber, Wherry et al. 2006; Radziewicz, Ibegbu et al. 2007; Wang, Lau et al. 2009). It is an open question whether an underlying defect in T-cell signalling contributes to the development of PTLD, or whether EBV+ B-cells produce factors that induce ‘immune-suppression’ which in turn contributes to the failure of the immune system to contain EBV- B-cells. A number of such factors have been reported, e.g. production of IL-10 (Samanta, Iwakiri et al. 2008;
Iwakiri and Takada 2010). A more detailed analysis of the IL-7 signalling pathway, as well as the prospective sampling of patient’s PBMCs may aid to address the sequence of events, which lead to the loss of immune surveillance and uncontrolled growth of (EBV) transformed cells.

Figure (5): STAT5 phosphorylation in CD4+ and CD8+ in PTLD patients and EBV+ control patients after HSCT.

CD4+ T cells

<table>
<thead>
<tr>
<th></th>
<th>PTLD</th>
<th>Control</th>
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<tbody>
<tr>
<td>IL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td></td>
<td></td>
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<tr>
<td>IL-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td></td>
<td></td>
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<tr>
<td>Constitutive p-STAT5</td>
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</table>

CD8+ T cells

<table>
<thead>
<tr>
<th></th>
<th>PTLD</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>IL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P=0.0004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constitutive p-STAT5</td>
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<td></td>
</tr>
</tbody>
</table>

No differences in the patient characteristics or in the transplantation procedures between patients and controls could be found. All patients were adults and all had received unrelated donor grafts and similar GVHD prophylaxis. however it seems that patients with PTLD have a lower risk of severe GVHD; 1/7 patients with PTLD had GVHD grade II which may be due to decreased IL-7 responsiveness compared to the control group. Rituximab (anti-CD20 antibody) therapy acts by eliminating B cells (Dean, Fry et al. 2008; van Dorp, Pietersma et al. 2009; Alousi, Uberti et al. 2010).

More detailed analysis of B cells showed expression of IL-7Rα on CD20+ B cells in 3/7 PTLD patients (0.01% to 25.3%, mean 2.3%) while CD20+ CD127+ B-cells were only found to be positive in 1/10 of control patients (range 0.03 to 3.38%, mean 0.735, p=0.81). Peripheral B-cells do not express CD127, yet some EBV+ B-cells (Benjamin, Sharma et al. 1994) as well as adult pre-B-cell acute lymphoblastic leukemia cells and other B-cell derived malignancies (Sasson, Smith et al. 2010) express the IL-7R. Future studies will need to address whether CD127 on B-cells contribute to malignant transformation in PTLD.
5 CONCLUSION

We showed in study I that most of adult patients who were adenovirus positive by RT-PCR had sustained adenoviremia and adenovirus can take several weeks to cause death from adenovirus disease. CMV and EBV infections did frequently occur with an adenovirus infection and they can complicate the diagnosis and treatment. Cidofovir is a good treatment option for adenovirus infections in comparison to other treatment options as ribavirin. Preemptive treatment with cidofovir may have a role to decrease the mortality rate of adenovirus disease.

Study II showed that incidence of positive adenovirus DNA was 5% and no one developed adenovirus disease or received antiviral treatment. Children have a higher incidence of infection. Patients with myelodysplastic syndrome before allogeneic HSCT and patients who had a bone marrow graft were found to be at a higher risk of adenoviremia in the studied population. A surveillance strategy of adenovirus should be applied to children and high risk patients. Adenovirus specific T-cells were found in adult patients in the first three months after allogeneic HSCT that may be of importance in preventing adenovirus infections.

The monitoring strategy to control EBV-PTLD by weekly measurements of EBV DNA by RT-PCR was safe to be applied and was helpful to reduce the incidence of PTLD in high risk patients. Splenectomy was confirmed to be a high risk factor for EBV-PTLD in the prospective cohort.

In paper IV, we were able to demonstrate the presence of EBV-specific and MHC class I-restricted T-cells in blood from patients with PTLD, as well as in blood from patients with EBV+ after HSCT (who did not develop PTLD). Such EBV-reactive T-cells exhibited CD107a expression, a marker for T-cell degranulation, and they also expressed the IL-7R. Thus, they could receive T-cell survival signals. Functional analysis revealed an impaired response of T-cells to IL-7, defined by STAT5 phosphorylation, in patients with PTLD. In contrast, this was not observed in EBV+ individuals after HSCT. The measurement of IL-7 responsiveness by a STAT5 phosphorylation assay may help to identify patients at risk for PTLD development.
6 FUTURE PERSPECTIVES

Adenovirus infections usually occur with other viral infection at a similar time after HSCT as CMV and EBV infections. The viral infections are usually due to severe immunosuppression and they almost have the same risk factors. A multiplex RT-PCR may be a helpful method to diagnose several viral infections in the same sample. It might allow a diagnosis of multiple viruses early before they result in end-organ disease and might also be helpful to reduce the cost and decrease the burden on the patients from repeated sampling. However, a well-designed study is needed to study all of these different points.

The preemptive treatment of adenovirus disease with cidofovir might reduce the risk for adenovirus disease. However, the level of viral load to start treatment has not well established. A well-designed study is needed to settle that point.

The adoptive transfer of adenovirus specific T-cells from the donor origin has been shown to be effective in small numbers of allogeneic HSCT patients. A study on the effect of adenoviral-specific T-cell therapy on a large cohort is needed. Multi-specific T cells against CMV, EBV and adenovirus therapy might be a good treatment option for treatment of several viruses but needs prospective studies to prove efficacy.

IL-7 is important in T-cell survival, proliferation and memory formation. Its role in control of different viral or fungal infections after allogeneic HSCT is not well studied. This notion is underlined by a recent study which showed that IL-7 expands antigen-specific Th17 cells – which may be crucial in a number of bacterial, viral and fungal infections. Examination of IL-7 functions by robust functional assays may help to learn more about the clinical relevance of IL-7 in infections after HSCT.

IL-7 can increase the risk of GVHD, a situation which may require more detailed studies. An administration of anti-IL-7 antibodies, or appropriate IL-7 antagonists, may be of biological relevance and may present an interesting treatment option in preventing GVHD.

IL-7 isoforms, generated by alternative splicing, are not well studied in the transplant setting; an IL-7 isoform, lacking exon 5, has been shown to act as a superagonist
concerning human thymocyte development and T-cell phosphorylation, IL-7 isoforms may represent interesting targets concerning immune-reconstitution and GVHD development.

Defects in the IL-7/IL-7R axis function may be a risk factor for development of PTLD. The growth promoting effect of IL-7 on EBV+ B-cells requires more detailed analysis. These studies would benefit from the in situ examination of IL-7 and IL-7R expression on tumor cells as well as on immune cells by immunohistochemistry.
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