Identification of minor histocompatibility antigens

Milosevic Slavoljub

February 2003

Identification of minor histocompatibility antigens

Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften
an der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

Milosevic Slavoljub

München, 27.2.2003

Angefertigt am GSF-Forschungszentrum für Umwelt und Gesundheit GmbH
Institut für Klinische Molekularbiologie und Tumorgenetik
München

1. Berichterstatter:	**Prof. Dr. Dirk Eick**
2. Berichterstatter:	**Prof. Dr. Elisabeth Weiß**
3. Berichterstatter:	**Prof. Dr. Beate Averhoff**
4. Berichterstatter:	**Dr. Bettina Kempkes**

Tag der mündlichen Prüfung: 1.7.2003

To Laura, Teodor and our mother

Table of Contents:

3. Results

1. Introduction

1.1. General introduction to bone marrow transplantation

The transplantation of bone marrow (BM) is performed to reconstitute diseased or damaged hematopoietic systems in patients with malignant haematological diseases, e.g. leukaemia and lymphoma, congenital genetic disorders, e.g. thalassemia major (Thomas et al., 1982; Lucarelli et al., 1990; Giardini et al., 1999) or sickle cell disease (Bernaudin et al., 1999; Vermylen et al., 1998; Miller et al., 2000), autoimmune diseases, e.g. multiple sclerosis (Fassas et al., 2000; Mandalfino et al., 2000) or systemic sclerosis (Tyndall et al., 2002), and patient with therapy-related toxicity after high-dose radio/chemotherapy to cure malignant solid tumors. While in the latter case and in some patients with haematological malignancy in complete remission, autologous bone marrow from the patient can be removed before, and given back after therapy, allogeneic bone marrow from a different individual has to be used in all other cases. Due to tissue incompatibility, the consequence of genetic differences between BM donor and recipient, such allogeneic bone marrow transplantation (BMT) may lead to severe immunological reactions known as graft-versus-host disease (GvHD). This immune response is a major cause of mortality after bone marrow transplantation. Patients with leukemia undergoing allogeneic as compared to autologous BMT, however, were shown to have a significantly reduced rate of tumor relapse, an effect known as graft-versus-leukemia response (GvL). Targets of GvHD and/or GvL responses are major and minor histocompatibility antigens, in which donor and patient differ. The development of methods to determine the major histocompatibility genotype of patients and volunteer BM donors, and the collection of this information in international registers, has greatly facilitated the rapid identification and selection of compatible bone marrow donors, and reduced the risk of severe immune responses due to disparities in major histocompatibility antigens. Minor histocompatibility antigens (mHA), in contrast, have remained largely unknown, which precludes mHA-genotyping of patients and bone marrow donors. Molecular identification of mHA, furthermore, is required for the development of novel immunotherapeutic approaches to selectively enhance GvL, and reduce GvH responses.

1.2. Historical view on bone marrow transplantation

In 1949, Jacobson and co-workers noticed that mice could survive a normally lethal dose of ionizing irradiation when their spleens were shielded (Jacobson et al., 1949). Subsequently, it was reported that lethally irradiated mice could also survive when spleen or bone marrow cells were infused after irradiation (Lorenz et al., 1951). These experiments indicated that cells of the hematopoietic system are most susceptible to ionizing irradiation, and that the hematopoietic system can be reconstituted by the infusion of spleen or bone marrow cells. Infusion of bone marrow (BM) derived from a genetically different mouse strain, however, could cause an immune attack directed against the host. This immune reaction, which was found to be controlled by genetic factors (Uphoff, 1957), resulted in a wasting syndrome known at that time as "secondary disease" and today as graft-versus-host disease (GvHD) (van Bekkum and De Vries, 1967).

Subsequent studies in dogs provided important informations on applicability and feasibility of BMT in humans. First, it was shown that dogs could survive as much as two to four times the lethal dose of total body irradiation (TBI) (Mannick et al., 1960), when bone marrow cells were taken before and infused intravenously after irradiation. Second, bone marrow could be successfully transplanted in dogs treated with chemotherapy instead of TBI (Storb et al., 1969). Third, hematopoietic cells used in BMT could be obtained from BM as well as peripheral blood (Cavins et al., 1964). Fourth, the importance of leukocyte antigens in BMT, first established in mice, was also demonstrated in dogs. Animals receiving dog leukocyte antigen (DLA)-matched BM become healthy chimeras while DLA-mismatched BMT recipients usually died of graft rejection or GvHD (Epstein et al., 1968; Storb et al., 1971).

These results obtained in preclinical models inferred that successful BMT in humans was crucially dependent on the definition of human leukocyte antigens (HLA). As had been shown in animal models, these antigens may evoke immune responses after BMT. Serological HLA typing of patient and donor greatly reduced the risk of GvH responses. Accordingly, early attempts of BMT in humans failed because the patient died of GvHD (Mathe et al., 1965). The first successful human BMT was performed in a patient with severe combined immune deficiency in 1968 (Gatti et al., 1968). Two similar successful BMTs were reported at almost the same time (Bach et al., 1968; de Koning et al., 1969). Because of the diversity of HLAs, most BMTs were initially performed between sibling donor/recipient pairs. The introduction of immunosuppressive drugs, most

importantly cyclosporine A, ameliorated graft-versus-host immune reactions, and greatly improved clinical outcome of BMTs between unrelated patient/donor pairs (Storb et al., 1988; Storb et al., 1989). In 1990s, molecular techniques were developed to precisely type HLA alleles, which then allowed the selection of HLA-matched unrelated BM donors (Thomas et al., 1999).

1.3. Graft-versus-host (GvH) and graft-versus-leukemia (GvL) responses after allogeneic BMT

The reconstitution of BM in cancer patients treated with high dose chemo/radio - therapy is a major indication for BMT. In these cases, BM is collected from the patient before, and given back after chemo/radio therapy. Because the patient's own bone marrow is transplanted, this so-called autologous BMT is not associated with immunological rejection reactions. Autologous BMT, however, can not be performed in patients with genetic disorders or autoimmune diseases. In these cases allogeneic BM from a healthy donor must be used. With the rare exception of syngeneic BMT, where BM from a genetically identical twin is transplanted, the genetic differences between BM donor and recipient may evoke severe immunological complications after allogeneic BMT, known as graft-versus-host disease (GvHD) and host-versus-graft reaction (HvGR) resulting in BM rejection and graft failure. The main targets of these immune responses are HLA encoded by the major histocompatibility complex (MHC). Alloreactive T cells recognize non-self MHC molecules and may cause acute and often fatal immune reactions. While non-self MHC molecules are the main targets of the immune response after HLA-mismatched BMT, GvHD still occurs in more than half of the patients receiving HLA-identical BM from a sibling (Gratwohl et al., 2002; Sierra et al., 2002). In analogy to major histocompatibility antigens, the targets of the immune reaction under these circumstances have been designated minor histocompatibility antigens (mHA).

Minor HA are gene products of polymorphic genetic loci, in which BM donor and recipient differ (Meadows et al., 1997; den Haan et al., 1998; Dolstra et al., 1999; Pierce et al., 1999; Warren et al., 2000). Peptides derived from mHA are presented by MHC class I and class II molecules on the cell surface where they can be recognized by donor-derived T cells (Fig. 1) (Sahara et al., 2003). Although the frequency of T cells specific for mHA is much lower then the frequency of alloreactive T cells recognizing non-self MHC molecules, and the elicited immune response against mHA is usually not as rapid and not

as vigorous as the allo-response (Goulmy et al., 1997; Warren et al., 1998), GvHD is still the major cause of mortality in these patients (Gratwohl et al., 2002). Removal of the T cells from grafted BM can prevent GvHD. However, such depletion increases the risk of graft failure (Kernan et al., 1989), and delayed immunological recovery (Marmont et al., 1991). Furthermore, this immunological rejection reaction has also beneficial therapeutic effects. It is well documented that the rate of disease relapse is significantly lower in patients with leukemia receiving allogeneic as compared to syngeneic BM (Horowitz et al., 1990). These clinical observations suggest that the immune response associated with allogeneic BMT is also directed against residual tumor cells, an effect called graft-versus-leukemia (GvL) response. This antitumoral effect of allogeneic BMT has recently led to clinical trials for the treatment of other malignancies eg. ovarian carcinoma (Bay et al., 2000), and renal cell carcinoma (Childs et al., 1999; Childs et al., 2000; Rini et al., 2001) with high dose chemotherapy and subsequent allogeneic BMT.

1.4. Antigens involved in GvH and GvL response

Because GvL responses have been observed without any signs of GvHD (Slavin et al., 1990; Van Lochem et al., 1992; Falkenburg et al., 1993), the antigens recognized in these two types of immune responses are unlikely to be identical in all cases. In patients with leukemia receiving HLA-matched BM, three different types of antigens may lead to a GvL response without causing GvHD: leukemia-specific antigens, leukemia-associated antigens and hematopoiesis-specific mHA.

Leukemias often display characteristic chromosomal translocations, which may lead to the formation of tumor-specific fusion proteins. In the case of chronic myeloid leukemia (CML), this translocation involves chromosomes 9 and 22 and leads to the generation of the BCR/ABL fusion protein (Bocchia et al., 1995; Bosch et al., 1996; Berke et al., 2000). Peptides derived from this fusion protein can be presented by MHC class I and class II molecules on the cell surface, where they are recognized by $CD8^+$ cytotoxic T cells (CTL) (Yotnda et al., 1998; Osman et al., 1999) or $CD4^+$ T helper cells (Th) (Bocchia et al., 1996). CTL recognition of BCR/ABL-derived peptides has been shown to eradicate CML cells in vitro (Yotnda et al., 1998; Norbury et al., 2000). Certain HLA molecules bind and present BCR/ABL peptides with high efficiency.

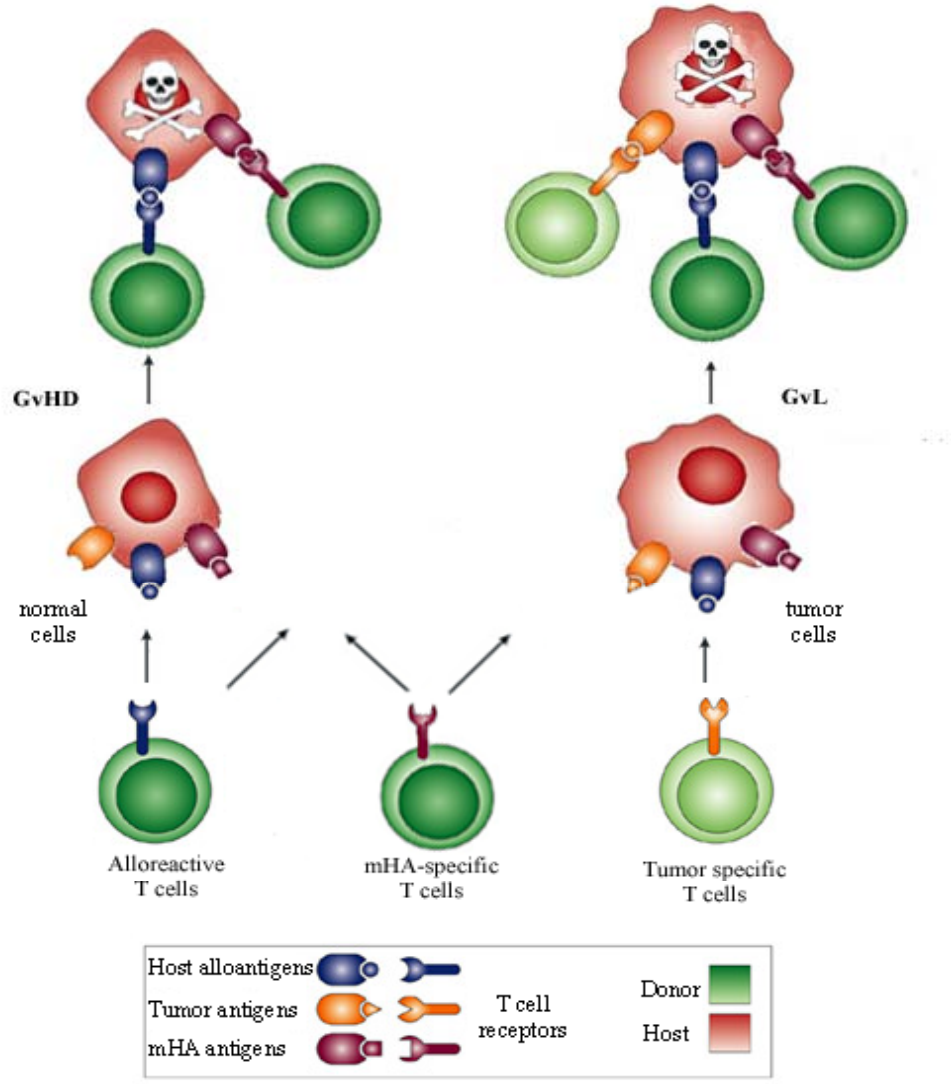


Fig.1

T cells involved in the immune response after allogeneic BMT. Three types of T cells can recognize MHC/antigen complexes presented on the surface of host tissues. First, alloreactive T cells recognize non-self MHC molecules on the surface of host cells and are responsible for the development of severe GvHD. These cells also recognize MHC/antigen complexes on tumor cells and cause their elimination. Second, tumor-specific T cells can recognize antigen exclusively (leukemia-specific antigens) or preferentially (leukemia-associated antigens) expressed on tumor cells. These T cells cause specific destruction of residual tumor cells. Third, mHA-specific T cells recognize antigens expressed on the surface of normal as well as tumor cells. Therefore, mHA-specific T cells may cause GvHD and GvL responses.

Because expression of these HLA has been associated with a lower risk of CML development (Posthuma et al., 1999; Posthuma et al., 2000), this correlation suggests that BCR/ABL-specific T cells are involved in protective immunity against CML. Immune

response against peptides spanning the junction of other fusion proteins, or tumor-specific mutations, have also been observed (Ohminami et al., 1999), but their contribution to immunological tumor control still needs to be established.

Besides leukemia-specific antigens, leukemia-associated antigens may induce T cell responses as well. Tumor-associated antigens are either over- or aberrantly expressed in tumor cells. Proteinase-3 (PR-3), for example, is a serine protease normally expressed during myeloid differentiation and overexpressed in myeloid leukemias. Peptides derived from PR-3 are presented by HLA molecules and induce CTL responses, which preferentially lyse myeloid leukemia cells and specifically inhibit CML progenitor cell outgrowth (Molldrem et al., 1996; Molldrem et al., 1997). PR3-specific T cells have been detected after allogeneic BMT (Molldrem et al., 2000). The appearance of these cells has been associated with a more favourable prognosis, suggesting that donor-derived PR3-specific CTL are involved in GvL responses. Other examples of leukemia-associated antigens include e.g. the products of the Wilm's tumor gene (WT-1), which is overexpressed in different types of leukemia (Ohminami et al., 2000; Oka et al., 2000; Gao et al., 2000), PRAME (Warren et al., 1998; Kessler et al., 2001), and the telomerase reverse transcriptase (TERT) (Minev et al., 2000; Nair et al., 2000).

The third group of antigens recognized after HLA-identical allogeneic BMT is that of minor histocompatibility antigens (mHA). These include Y-chromosome encoded male-specific antigens, which may evoke immune responses when BM from a female donor is transplanted into a male patient (Goulmy et al., 1997), as well as autosomally encoded mHA, derived from polymorphic genes (Goulmy et al., 1997). These mHA may also evoke immune responses after BMT when the proteins expressed from donor and recipient BM alleles differ in their amino acid sequences. Donor-derived T cells may recognize these differences because mHA, like all other cellular proteins, are presented on MHC molecules (den Haan et al., 1995; Meadows et al., 1997; Brickner et al., 2001). Depending on the tissue expression pattern, mHA may elicit GvHD and/or GvL responses. mHA expressed ubiquitously or in a variety of tissues may evoke immune responses against these tissues, and thus cause the symptoms of GvHD. Hematopoiesis-specific mHA may cause immune responses directed only against host-derived hematopoietic cells including residual tumor cells, and thus may contribute to tumor eradication, residual host hematopoietic cells elimination, and enhanced graft acceptance.

The role of the three groups of antigens in the immune response after BMT has been elucidated by comparing clinical outcome after autologous, syngeneic and allogeneic

BMT. Compared to allogeneic BM recipients, patients undergoing autologous or syngeneic (between genetically identical twins) BMT have a higher risk of developing recurrent leukemia (Champlin et al., 1992). In the latter two cases, no major or minor histocompatibility differences exist, thus antitumoral immune responses are restricted to leukemia-specific and leukemia-associated antigens. The rate of disease relapse after HLA-identical sibling BMT versus syngeneic BMT for haematological malignancies was 25% versus 46% in a study of Horowitz et al. (1990). In fact, in this trial, the relative risk of disease relapse was high for syngeneic BMT (2,09) while significantly lower for patients with HLA-identical sibling BMT (1,76-0,33), depending on T cell depletion (1,76) or evidence of acute (0,68), chonic (0,43), acute and chronic (1,0) or no GvHD (0,33). These results clearly demonstrate that recognition of minor histocompatibility antigens by T cells is most critical for GvHD and GvL responses after HLA-identical allogeneic BMT.

This notion is further supported by the effective treatment of leukemia relapse with the infusion of donor lymphocytes (DLI) (Kolb et al., 1990). In the initial study, DLI treatment of three patients with CML led to a complete tumor remission in all patients, but two of them developed GvHD. The importance of mHA in GvL response was further confirmed by the successful isolation of mHA-specific CTLs from BMT patients (Tsoi et al., 1980; Tsoi et al., 1983; Goulmy et al., 1983; Irle et al., 1985; Niederwieser et al., 1993; Goulmy et al 1997; Warren et al., 1998). These mHA-specific CTLs can effectively lyse leukemia cells and inhibit leukemia progenitor cell colony formation in vitro and in vivo (de Bueger et al., 1992; van der Harst et al., 1994; Dolstra et al., 1997; Bonnet et al., 1999; Nijmeijer et al., 2002). In order to enhance antitumoral responses after allogeneic BMT and reduce incidence and severity of GvHD, much effort has been put into the identification of mHAs. The rationale for their molecular identification is twofold: First, the selection of BM donors with a similar genotype in ubiquitously expressed mHA, and a greatly different genotype in hematopoiesis-specific mHA, is expected to reduce GvHD, and enhance GvL responses. Second, enhancing immune responses against hematopoiesis-specific mHA by vaccination or the adoptive transfer of in vitro expanded T cells specific for these mHA, is expected to augment GvL responses without causing GvHD.

1.5. Identification of MHC class I-restricted mHAs

The development of cell culture techniques for the isolation and cultivation of mHA-reactive T cells has created the basis for the identification and molecular characterisation of the antigens recognized. A number of $CD8^+$ mHA-specific T cell clones have been isolated from peripheral blood of patients after allogeneic BMT by different groups (van der Harst et al., 1994; Goulmy et al., 1997). In one study, 56 $CD8^+$ mHA-specific T cell clones were isolated from ten patients with different hematopoietic malignancies after allogeneic BMT (Warren et al., 1998). These mHA-specific T cell clones recognized 17 different mHAs, of which 5 are expressed in both hematopoietic cells and fibroblasts, whereas 12 are expressed in hematopoietic cells only. Based on recognition pattern, HLA restriction and tissue expression, Goulmy and co-workers characterized $CD8^+$ T cell clones that recognized seven different mHA (HA-1 to HA-7) (Goulmy et al., 1997). These studies indicated that a large number of mHAs with diverse tissue expression patterns and MHC restrictions exists.

mHA-specific T cells have been used to molecularly identify the antigens recognized. Up to now, all described mHA are recognised by $CD8^+$ mHA-specific T cell clones isolated after allogeneic BMT. Two different approaches have been used for the molecular identification of MHC class I-restricted mHA, (i) biochemical peptide fractionation and (ii) a genetic cDNA expression cloning approach.

The biochemical peptide fractionation approach relies on the immune precipitation of the restricting MHC molecule from the surface of the cell line recognized by the mHA-specific T cell clone. Subsequently, the peptides bound to the MHC molecules are eluted and fractionated by high performance liquid chromatography (HPLC). The peptide fractions are then tested in a recognition assay with mHA-specific $CD8^+$ T cells and positive fractions are sequenced by mass spectroscopy. By comparing the sequence of the identified peptide with sequences in the DNA and protein database, the gene encoding the mHA can be identified. This method has been successfully used for the identification of HA-1, HA-2 (Den Haan et al., 1995; Den Haan et al., 1998) and HA-8 (Brickner et al., 2001), SMCY (Meadows et al., 1997) and DFFRY (Pierce et al., 1999; Brickner et al., 2001).

In the second approach, pools of a cDNA library derived from antigen-positive cells are transfected into antigen-negative target cells, together with the cDNA encoding the restricting MHC class I molecule. After expression of the transfected genes, peptides

derived from the gene products are presented by MHC class I molecules on the cell surface. Antigen containing cDNA pools are identified by co-culture of the transfected cell with the antigen-specific T cells. Recognition of the antigen by the T cells leads to cytotoxicity and the secretion of cytokines, both of which can be measured. Positive pools are subdivided and individual cDNAs tested in an identical fashion. This cDNA expression cloning method has been successfully used for the identification of the Y-chromosome encoded UTY gene (Warren et al., 2000) and HB-1, which is selectively expressed in Epstein-Barr virus (EBV) transformed B cells (lymphoblastoid cell line, LCL) and B cell lymphoid leukemia (Dolstra et al., 1997).

1.6. Identification of MHC class II restricted mHAs

MHC class II-restricted $CD4^+$ T cells play a central role in autoimmunity, infectious diseases, and antitumoral immune responses. An increasing number of evidences suggest an important role of $CD4^+$ cells in GvHD and GvL responses after BMT (Russell et al., 2001). Animal models demonstrated that $CD4^+$ T cells are required for sustained GvL responses and tumor eradication (Zeis et al., 2001). Furthermore, in MHC class I deficient mice, $CD4^+$ T cells induce and maintain GvHD after HLA-matched allogeneic BMT (Shenoy et al., 1998). Recently, it has been shown that $CD4^+$ donor lymphocyte infusions elicit potent allogeneic responses mediated by mHA-specific $CD8^+$ T cells in humans (Zorn et al., 2002). Despite their importance in the immune responses after HLA-identical BMT, little is known about the antigens recognized by mHA-specific $CD4^+$ T cells. Except for DBY, which was identified as the antigen recognized by a mHA-specific $CD4^+$ T cell clone by testing a small number of candidate genes (Scott et al., 2000; Vogt et al., 2002), no other MHC class II-restricted mHA has been identified, mostly because generic methods for the molecular identification of these antigens are lacking.

$CD4^+$ T cells recognize peptides bound to MHC class II molecules at the surface of professional antigen presenting cells (pAPCs), like DC, macrophages, and B cells. In contrast to MHC class I bound peptides, peptides bound to MHC class II molecules are heterogeneous in length due to an open peptide-binding groove of these molecules.

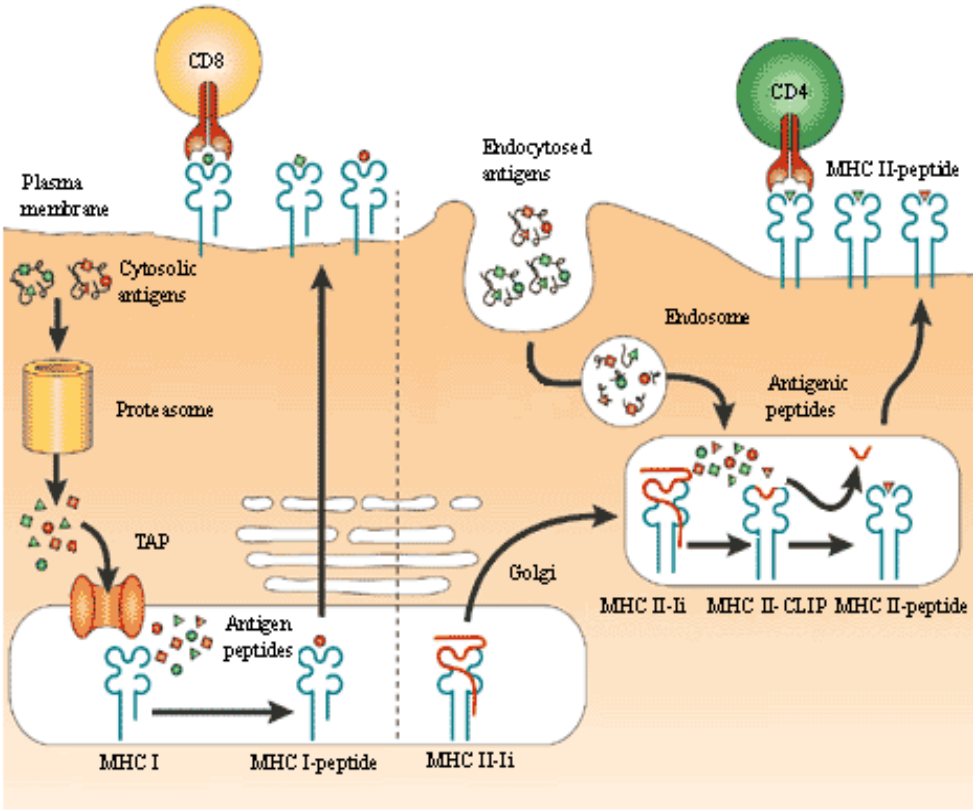


Fig. 2

Antigen presentation on MHC class I and II. Intracellular proteins are degraded by cytoplasmatic proteases e.g. the proteasome into peptides, which are transported by the transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum, where peptides are loaded onto newly synthesized MHC class I molecules. These peptide/MHC complexes are then transported through the Golgi apparatus to the cell surface. Antigens presented on MHC class II are derived from internalised proteins that are degraded in the endosomal/lysosomal system. Newly synthesized MHC class II molecules are prevented from binding peptides in ER through their association with invariant chain, which also directs MHC class II molecules into the endosomal/lysosomal compartment. Within the lytic compartment, invariant chain is degraded, allowing peptides derived from exogenous proteins to bind to MHC class II. These peptide/MHC class II complexes are then transported to the cell surface (taken and modified from Nature Reviews Immunology).

Thus, high performance liquid chromatography (HPLC) followed by mass spectroscopy of eluted peptides, as used for the identification of antigens recognized by $CD8^+$ cells, cannot be applied for the identification of antigens presented on MHC class II molecules. Furthermore, a simple adaptation of the cDNA expression cloning approach used for the identification of MHC class I-restricted antigens is not possible for two reasons: First,

antigens presented on MHC class I are derived from intracellular proteins degraded by cytoplasmatic proteases like the proteasome. While, antigens presented on MHC class II molecules are mostly derived from exogenous proteins taken up by pAPC and cleaved into peptides by proteases in the endosomal/lysosomal compartment (Fig. 2). Second, pAPC are poorly transfectable, and therefore cannot serve as recipient cells for cDNA libraries.

Nevertheless, Wang et al. succeeded in adapting this approach for the identification of MHC class II-restricted tumor antigens (Wang et al., 1999). To overcome the differences in antigen presentation, libraries were constructed where the cDNAs were fused to the invariant chain gene. Due to the signal sequences present in the invariant chain, the resulting fusion proteins are directed into the endosomal/lysosomal compartment where processing and loading onto MHC class II molecules occurs (Wang et al., 1999; Sanderson et al., 1995; van Bergen et al., 1997; Fujii et al., 1998; Malcherek at al., 1998). As recipient cell line for the expression of cDNA libraries, highly transfectable 293 cells (transformed human kidney embryonic cell line) were genetically modified to become artificial pAPC. The cells were stably transfected with cDNAs encoding DRα and DRβ, which code for the appropriate MHC class II restriction element, DMα and DMβ which help binding of peptides to MHC class II molecules, and invariant chain, which directs newly synthesized MHC molecules into the MHC class II processing and loading compartment. Using this approach two antigens recognized by melanoma-specific $CD4^+$ cell lines were identified (Wang et al., 1999).

In addition, two tumor antigens recognized by tumor-specific $CD4^+$ T cells have been identified through the biochemical purification of antigenic proteins from tumor lysates combined with mass spectrometric sequencing. Protein fractions obtained by biochemical separation methods are added to cultures of pAPC, which take-up, process and present these exogenous antigens on MHC class II, where they can be recognized by antigen-specific $CD4^+$ T cells. Positive fractions are sequenced by mass spectroscopy and individual proteins tested separately. Because different proteins may exhibit different biochemical behaviour, this approach has to be tailored for each antigen, and is restricted to abundant antigens (Monach et al., 1995; Pieper et al., 1999).

An indirect approach for the identification of tumor antigens recognized by T helper cells was described in 1995 and is based on the molecular identification of the antigens recognized by antibodies present in the serum of tumor patients (Sahin et al., 1997). This method called SEREX (serological analysis of recombinant cDNA expression libraries) is a modification of a strategy developed earlier to assess humoral responses in

tumor patients (Old et al., 1981). The method is based on the concept that B cells require T cell help for switching their immunoglobulin (Ig) isotype. Antigen bound by IgM on the surface of naive B cells is internalized, processed and presented on MHC class II. If T helper cells recognize the antigen/MHC complex, they provide help to the B cells through the secretion of cytokines and the expression of membrane-bound ligands, which causes activation and differentiation into Ig-secreting B cells. Thus, high titer IgG antibody responses can only occur in the presence of T helper cells specific for the same antigen. In order to identify antigens recognized by serum IgG antibodies, a tumor-derived cDNA library is cloned into a λ phage expression vector. After infection of Escherichia coli, recombinant protein expression is induced and the expressed proteins are subsequently blotted onto nitrocellulose membranes and incubated with the serum of the patient. Serum antibodies bound to the proteins are detected with an enzyme linked secondary antibody specific for human IgG. The identity of the corresponding cDNAs can be obtained by sequence analysis of the λ phage coding for the antigen. It has been shown that many antigens recognized by serum antibodies are also targets of the T cell response and vice versa. For example, specific $CD8^+$ and $CD4^+$ responses have been observed in tumor patients with high titer antibodies against NY-ESO-1, a tumor-associated antigen identified with the SEREX method (Jäger et al., 2000; Zeng et al., 2001). These data suggested that SEREX-defined antigens might be a good indicator for T helper cell responses.

1.7. Aim of the work

Because generic methods for the identification of MHC class II-restricted antigens are not available, the aim of this work is to develop a novel approach for the efficient identification of mHA recognized by T helper cells.

The correlation of humoral and cellular immune response observed in selected tumor patients had suggested that the identification of the targets of the antibody response may be useful to define the antigens against which a T helper cell response had been elicited. However, humoral and cellular immune responses had never been assessed with independent methods in these patients, leaving the question unanswered whether the targets of these two types of immune responses are identical, overlapping, or mostly diverse. Because simple methods to define the targets of the humoral immune response have been developed, the answer to this question has important implications for future

strategies to assess T helper cell responses. If a largely different set of antigens was identified with these two approaches, then indirect serological methods may be inadequate to define the antigens recognized by CD4 T cells, and can not substitute for direct methods to identify MHC class II-restricted antigens recognized by T helper cells. If the antigens identified with these two approaches are identical or overlapping, serological methods may be used for the identification of MHC class II-restricted antigens as long as generic direct methods are not available.

To address this question, humoral and cellular immune responses should be assessed in parallel in a patient undergoing HLA-identical, allogeneic BMT. To identify the targets of the humoral immune response after BMT, the SEREX method originally developed to define the targets of the antibody response in cancer patients should be applied. Because mHA are the products of polymorphic genes, the successful adaptation of this method to BM recipients should result in the identification of antigens encoded by genes that differ between patient and BM donor. These candidate mHA should then be used in a recognition assay to test whether these polymorphic antigens are also recognized by mHA-specific T helper cells. To this end, efficient methods for the isolation and characterisation of the $CD4^+$ mHA-specific T cell lines and clones should be developed to answer the question whether the targets of the humoral and the cellular immune response after BMT are identical or diverse.

2. Materials and methods

2.1. Materials

If not stated otherweise, materials and reagents were obtained from the following companies: Amersham/Pharmacia Bioscience (Freiburg), Applied Biosystem (Weiterstadt), Becton Dickinson/Pharmingen (Heidelberg), BioRad (München), Calbiochem/Novabiochem (Beeston, Nottingham, UK), Costar (Bodenheim), Dianova (Hamburg), Difco (Difco Laboratories, Detroit, USA), Eppendorf (Hamburg), GLW (Würzburg), Greiner (Frickenhausen), ICN Biomedicals GmbH (Eschwege), Integra Bioscience GmbH (Fernwald), Labor Schubert & Weiß GmbH (München), Laborsystem Osvath (Geretsried), Life Technologies - Invitrogen (Karlsruhe), MBI-Fermentas (Vilnius, Lettland), Merck (E. Merck AG, Darmstadt), Millipore (Beedford, USA), NEB (Schwalbach), Neolab (Heildelberg), Nunc (Wiesbaden), Packard Instruments (Karlsruhe), Perkin Elmer/Applied Biosystems (Weiterstadt), Promega (Mannheim), Qiagen (Hilden), Roche/Boehringer (Mannheim), Roth (Karlsruhe), Sartorius (Göttingen), Sigma (Taufkirchen-München), Stratagene (Amsterdam).

2.1.1. Commonly used material

3MM paper	Whatman, Maidstone, USA
Bacteria und cell culture dishes	Greiner, Frickenhausen
Cryotubes	Nunc, Wiesbaden
Cell culture Flasks (50, 250, 600 ml)	Costar, Greiner, Nunc
Elektroporation cuvettes	BioRad, München
Falcon, Multiwell™ 24 well plate	Becton-Dickinson, Heidelberg
Falcon, Multiwell™ 48 well plate	Becton-Dickinson, Heidelberg
Falcon, Microtest™ 96 well plate	Becton-Dickinson, Heidelberg
FACS-tubes	Becton-Dickinson, Heidelberg
JetStar	Genomed, Bad Oeyenhausen
Mikro-reactions tubes	Eppendorf, Hamburg
Parafilm	Dynatech, Denkendorf
PCR-Purification-Kit	Qiagen GmbH, Hilden
Polypropylen tubes (5ml, 14ml)	Becton-Dickinson, Heidelberg
Polypropylen tubes (15ml, 50ml)	Becton-Dickinson, Heidelberg
Qiagen Plasmid Mini Kit	Qiagen GmbH, Hilden
Qiagen Plasmid Maxi Kit	Qiagen GmbH, Hilden
Qiaex II Gel Extraction Kit	Qiagen GmbH, Hilden
RNeasy-Mini-Kit	Qiagen GmbH, Hilden
Sterile filter (0,22µM, 0,45µM)	Millipore, Eschborn

Disposable tissue culture dishes, culture bottles, culture- and test tubes were purchased from Nunc GmbH, Wiesbaden; Beckton Dickinson, Heidelberg; Greiner GmbH, Frickenhausen, Nürtlingen; or Corning Glassworks, NY. Glassware was from Corning Glassworks, NY, and Fisher Scientific, Fair Lawn, NJ. Conical 1,5 ml centrifuge tubes were manufactured by Eppendorf, BioRad or Sarstedt. Other general supplies, including pipets, test tubes, filter papers, syringes, and sterile filtration units were obtained from Fisher Scientific.

2.1.2. Chemicals and biological reagents

Agarose	Life Technologies – Invitrogen, Karlsruhre
Amphotericin B	Life Technologies – Invitrogen, Karlsruhre
Bromophenol Blue	IBI, New Haven, CN, USA
BSA	Sigma –Aldrich, Taufkirchen
Chloroform	Merck, Darmstadt
DMEM	Life Technologies – Invitrogen, Karlsruhre
DMSO	Sigma –Aldrich, Taufkirchen
EDTA	Sigma –Aldrich, Taufkirchen
Ethidium bromide	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Ficoll-Paque	Amersham-Pharmacia, Freiburg
Fetal calf serum, (FCS)	Life Technologies – Invitrogen, Karlsruhre Biochrom, Berlin Bioser, Buenos Aires
L- Glutamine	Life Technologies – Invitrogen, Karlsruhre
Glycerol	Sigma –Aldrich, Taufkirchen
Glycine	Sigma –Aldrich, Taufkirchen
HEPES	Sigma –Aldrich, Taufkirchen
Hybond-C blotting membrane	Amersham-Pharmacia, Freiburg
Hydrochloric acid	Merck, Darmstadt
Isopropanol	Merck, Darmstadt
Magnesium chloride	Sigma –Aldrich, Taufkirchen
Mycoplasma removal agent	ICN Biomedicals GmbH, Eschwege
Phenol	Merck, Darmstadt
Potassium chloride	Fluca Chemie AG, Buchs, CH
Propidium iodide	Sigma –Aldrich, Taufkirchen
RNase A	Roche/Boehringer, Mannheim
RPMI 1640	Life Technologies – Invitrogen, Karlsruhre
SDS	Merck, Darmstadt
Sodium acetate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium hydroxide	Merck, Darmstadt
TEMED	Merck, Darmstadt
Tris	Sigma –Aldrich, Taufkirchen
Trypsine	Life Technologies – Invitrogen, Karlsruhre
Tween 20	Sigma –Aldrich, Taufkirchen
Whatman 3MM-paper	Whatman, Maidstone, England, UK

2.1.3. Solutions, buffers and media

10x STE	1 M NaCl; 200 mM Tris-Cl pH 7,5; 100 mM EDTA in H_2O, autoclaved
TBS	20 mM Tris-Cl pH 7,5; 150 mM NaCl in H_2O
TBST	TBS + 0,05% (v/v)Tween 20
1xTE	10 mM Tris-Cl pH 7,5; 1 mM EDTA in H_2O
x-gal	40 mg/ml x-gal (5-Bromo-4-Chloro-3-Indoyl-β-D Galactopyranosid) dissolved in DMF; sterile filtrated
10× MOPS Buffer	200 mM MOPS (3-[*N*-morpholino]propane-sulfonic acid); 50 mM sodium acetate; 10 mM EDTA. PH was adjusted to 6.5–7.0 with NaOH
50x TAE	2 M Tris Base; 50 mM EDTA pH 8,5 in H_2O
Ethidiumbromide-Solution	10 mg/ml in ddH_2O
Formaldehyde Gel loading buffer	720 µl of formamide; 160 µl of 10× MOPS buffer; 260 µl of 37% formaldehyde; 100 µl of sterile water; 100 µl of EtBr (10 mg/ml); 80 µl of sterile glycerol; 80 µl of saturated bromophenol blue (BPBll) in sterile water
LB Agar	10 g of NaCl; 10 g of tryptone; 5 g of yeast extract; 20 g of agar. Deionized H_2O was added to a final volume of 1 liter and pH adjusted to 7.0 with 5 N NaOH. Medium was autoclaved and poured into petri dishes (~25 ml/100-mm plate)
LB media	10 g of NaCl; 10 g of tryptone; 5 g of yeast extract. Mixture was dissolved in deionized H_2O to a final volume of 1 liter. pH was adjusted to 7.0 with 5 N NaOH, autoclaved, and used with appropriate antibiotics
SM Buffer	5.8 g of NaCl; 2.0 g of $MgSO_4 \cdot 7\ H_2O$; 50 ml of 1 M Tris-HCl (pH 7.5); 5.0 ml of 2% (w/v) gelatin. Deionized H_2O was added to a final volume of 1 liter
20× SSC Buffer	175.3 g of NaCl; 88.2 g of sodium citrate; 800 ml of deionized H_2O. pH was adjusted to 7.0 with a few drops of 10 N NaOH and deionized H_2O was added to a final volume of 1 liter
Super Broth	35 g of tryptone; 20 g of yeast extract; 5 g of NaCl were dissolved in deionized H_2O to a final volume of 1 liter. Upon adjustment of pH to 7.5 with 5M NaOH, medium was autoclaved.
FACS-buffer	BSA (Bovine serum albumine) 0.2% in PBS

2.1.4. Antibiotics

Ampicillin — 100mg Ampicillin (Merck, Darmstadt) was dissolved in 10ml 70% ethanol and used at 100 µg/ml. 10 mg/ml solution was stored at -20˚ C

Kanamycin — 100mg Kanamycin (Merck, Darmstadt) was dissolved in 10ml ddH_2O and used at 50 µg/ml. 10 mg/ml solution was stored at -20˚ C

Tetracyclin — 100mg Tetracyclin (Merck, Darmstadt) was dissolved in 10ml of 70% ethanol and used at 12,5 µg/ml. 10 mg/ml solution was stored at -20˚ C

2.1.5. Oligonucleotides (Primers)

All oligonucleotides were synthesised by Metabion GmBH, Martinsried.

Clone number	Primer	5'→3' orientation
2-1	sense primer I	agacattgctgtctggtg
	antisense primer I	aactggccactgtttgca
	sense primer II	caagagagtggcagacat
	antisense primer II	tgcgattcttccgtgact
2-2	sense primer	gcagaatggctcccgcaa
	antisense primer	cagcgattagttctcatcca
8	sense primer	ttcagcgcatggacaccac
	antisense primer	cacagggatagatttatgcag
	sense primer I	ggacatactacatggatcag
	antisense primer I	cacttgatgaggtactggat
	sense primer II	cctagtggtgactttgacac
	antisense primer II	aggagcctatggttggactt
	sense primer III	gaagcccatcggttgaagaa
	antisense primer III	ttgagttggaccttcctgag
	sense primer IV	ggagaggtccaacaatcaag
	antisense primer IV	tggacttttttccaagccatc
10-2	sense primer	tgtgcttctactgaggactg
	antisense primer	cgctggtcccatgctcag
10-1	sense primer I	tagaagacttccgcgtaacc
	antisense primer I	atggtttcgaagacctcctc
	sense primer II	atctccaagagccagtctct
	antisense primer II	cagatctaattggatcgggc
20-1	sense primer	ttataggtagaggcgacaga
	antisense primer	atgctacctttgcacggtta
21-1	sense primer I	ggccacagccagcatgca
	antisense primer I	aggatgacggtgctgcgta
	sense primer II	gactacaagctccggccat
	antisense primer II	tggtggtggcactgtctca
22-1	sense primer	tctgggtggccatggag
	antisense primer	cttgttcctgccagcatt
22-2	sense primer	aca ggtaccatgaaggtctc
	antisense primer	aggttcaaggactctccatc

22-3	sense new	agagctcagctcctcacca
	antisense new	tctgacactctcaggatgtg
31	sense primer	agagcctagaggatgtttca
	antisense primer	ccatcgcctcagactctg
64-2	sense primer	gaagatggcggacattca
	antisense primer	atgtccagcctcagaact
65-2	sense primer	tcagaagcagtcctttgagc
	antisense primer	aactggctctctcaagctga
69	sense primer I	tcacatcgaacatgtggtgc
	antisense primer I	gataccatctcctgctgctt
	sense primer II	gacagattcaaggccactg
	antisense primer II	ccagtcctaaggaacagag
116	sense primer	cctaggtcacggcaatca
	antisense primer	cctgctgcctcttctaat
120-1	sense primer I	cgataagaagtcctcctg
	antisense primer I	cttcaggagatgagaagc
	sense primer II	gctaaggtaatgctggag
	antisense primer II	aggtccataggacaagct
120-2	sense primer	gcgggattctgacttaga
	antisense primer	caggtggggagtttgac
133-2	sense primer I	gccgagatggggctgc
	antisense primer I	gcctgtagagtgcaactg
	sense primer II	agatcacattccctgcaca
	antisense primer II	aaggaatggtttagcgcca
209	sense primer I	tgaccagccgcaagatgg
	antisense primer I	aagcgactctgggttcttg
	sense primer II	agcccgaccagtaccaga
	antisense primer II	agagaaggcagtatcccag
216	sense primer	atggaagccttggggttt
	antisense primer	tcagcgggatacgatctt
233	sense primer	cagaacacaggtgtcgtgaa
	antisense primer	accgttcttccaccactgat

2.1.6. Peptides

All peptides were synthesised by Metabion GmBH, Martinsried. The purity of the peptides was greater than 90%.

Name	Sequence	BMT
Peptide 10-1	TQSVPVRCPARRRQS	before (patient)
Control peptide 10-1	TQSVPVRRPARRRQS	after (donor)
Peptide 10-2	DVRIWPLDPSLLGEP	before (patient)
Control peptide 10-2	DVRIWPLHPSLLGEP	after (donor)
Peptide 21-1	GWYTYMLLPAALTGL	before (patient)
Control peptide 21-1	GWYTYMLVPAALTGL	after (donor)
Control peptide 22	QQTQVAASGGAGRAA	before (patient)

2.1.7. Antibodies

Antibody	Antigen	
Alkaline Phosphatase-conjugated AffiniPure Goat Anti-Human IgG	IgG, Fcγ Fragment specific	Dianova, Hamburg
PE labelled goat Anti-mouse	IgM and IgG, Fab Fragment specific	
Monoclonal Anti-Human	HLA Class I Antigene (clone W6/32)	Sigma-Aldrich
Purified Anti-Human	HLA-DR (clone L243)	Pharmingen, Becton Dickinson (Heidelberg)
PE labelled Anti-Human	CD4	
FITC labelled Anti-Human	CD8	
Purified Anti-Human	CD16	
	CD45RO	
	CD62L	
	TCR αβ	
	TCR γδ	

2.1.8. Bacterial strains

E. coli XL1-Blue MRF‘ (Stratagene, La Jolla, CA) (Jerpseth et al., 1992) Genotyp: Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac, [F’proAB lacqZ Δ M15 Tn10 (Tetr)]c. Used as host strain for λ-phages in all steps including titration, blue-white-selection, amplification, screening with serum except propagation of filamentous phages.

E. coli SOLR (Stratagene, La Jolla, CA) (Hay & Short, 1992) Genotyp: E14-(McrA-), Δ mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, uvrC, umuC::Tn5, (Kanr), lac, gyrA96, relA1, thi-1, endA1, λR, [F’proAB lacqZΔM15]c. Used as host strain for packed phagmides after excision.

2.1.9. Bacteriophages

Lambda ZAP® II (Stratagene, La Jolla, CA) (Short et al., 1988) Expression vector system with capacity to accommodate DNA inserts up to 10 kb in length in 21 unique cloning sites inside of lacZ-gene. The vector system is suitable for library screening with DNA probes or antibodies and allows in vivo excision of the pBluescript phagemide together with inserts.

ExAssist™ Interference-Resistant Helper Phage	(Stratagene, La Jolla, CA) (Hay & Short, 1992) The helper phage carrying amber mutations and is used for pBluescript-plasmids excision from λ-ZAP® II-library.

2.1.10. Enzymes

If not stated otherwise, all enzymes including T4-DNA-polymerase, CIP, T4-DNA-ligase, reverse-transcriptase and adequate buffers were from New England Biolabs (Schwalbach) and MBI Fermentas (Vilnius, Lettland). Taq, PFU polymerase and corresponding buffers were from Promega (Mannheim).

2.1.11. DNA standard

Gene-Ruler (1kb DNA Ladder) standard (MBI Fermentas) has been used for DNA-agarose-gelelectrophoresis.

2.1.12. Software

Clone manager 6 program (Scientific and educational software) was used for DNA sequence and plasmide analysis. After FACS measurements Cell quest software (Becton-Dickinson) and WinMDI 2.8 (J. Trotter; Scripps Research Institute, LA Jolla, CA; http:/facs.scripps.edu/) have been used for analysis of the obtained results. For optical density measurements in ELISA assays Xread Plus (Tecan Deutschland GmbH, Creilsheim) and Microsoft Excel (Microsoft, USA) were used. Processing and data compilation were performed on a PC computer using Microsoft Word programme.

2.1.13. Equipment

The CO_2 incubator used in these studies was manufactured by Heraeus. Sorvall and Beckman refrigerated floor model centrifuges were used for moderate speed centrifugations. Hettich centrifuges were used for low speed cell pelleting. An Eppendorf microcentrifuge was utilized in nucleic acid manipulations and for other experiments using 1.5 ml conical test tubes. General-purpose water baths and shaker tables were used in various functions. Beckman spectrophotometer was used to determine nucleic acid

concentrations, as well as bacterial optical density. FACS analysis was performed on the FACScan, supplied by Becton Dickinson. Microliter pipets manufactured by Gilson were used throughout this work.

Bacteria-incubator	Haereus, Osterode
Bacteria shaker	New Brunswick Scientific, Edison, USA
Cell counting chamber	GLW, Würzburg
Centrifuge 5415C	Eppendorf, Hamburg
Centrifuge	Sepatech Variofuge Haereus
Centrifuge 2K15	Sigma –Aldrich, Taufkirchen
CO2-Incubator, Haereus 6000	Haereus, Osterode
Elektroporation equipment Gene Pulser II	BioRad, München
ELISA-Reader	Tecan Deutschland GmbH, Creilsheim
FACS-Scan equipment	Becton-Dickinson, Heidelberg
Gel electrophoresis chamber	Life Technologies – Invitrogen, Karlsruhre
Light-optical microscope	Axiovert 135, Carl Zeiss, Stuttgart
Milli-Q water preparation equipment	Millipore, Essen
pH-Meter Multi-Calimatic 763	Knick, Engelsbach
Pipettes (20, 200, 1000µl)	Gilson, Netherlands
Pipett Boy	Integra Bioscience GmbH, Fernwald
Power supply Power-Pac 300	BioRad, München
Scanner	Epson GT 9600, Epson, Düsseldorf
Spectrophotometer (BioPhotometer)	Eppendorf, Hamburg
Sterile bench (Lamina Flow Hood)	Bio Flow Technik, Meckenheim
UV-Transilluminator	UVP Inc., San Gabriel, USA
Vortex Genie 2	Bender & Hobein, Ismaning

2.2. Methods

The following molecular biology methods were performed according to the standard protocols decribed by Sambrook, and Russel (2001) and Ausubel et al. (1993): Plasmid-minipreparation from E. coli (STET-Minipreparation), phenol extraction, ethanol precipitation and agarose gelelectrophoresis. Corresponding kits were used for other standard molecular biology method according to manufacturer's instructions: JetStar (Genomed, Bad Oeyenhausen) for plasmid-maxipreparation, Qiaex II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) for DNA fragment isolation from agarose gel and PCR-Purification-Kit (Qiagen GmbH, Hilden, Germany) for PCR fragment purification from agarose gel.

2.2.1. Molecular biology methods

2.2.1.1. Culturing and storage of bacteria

Escherichia coli strains XL-1 Blue MRF' and SOLR were cultured in a bacteria incubator or, as liquid culture, in a bacteria shaker (New Brunswick Scientific Co., Edison, USA) at 37 °C. Appropriate concentrations of antibiotics (ampicilin 100 µg/ml, tetracycline 12,5 µg/ml, and kanamycin 50µg/ml) were used for bacteria culturing. For long-term storage of bacteria, 1 ml of a dense bacteria culture (OD 1,0) was centrifuged at 4000 rpm for 10 min, the bacteria resuspended in 1ml of storage medium (10% (w/v) glycerin in LB-medium), transferred in cryotubes (Nunc, Wiesbaden) and frozen at – 80 °C.

2.2.1.2. RNA isolation, cDNA preparation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from $1x10^7$ cells using RNeasy-Mini-Kit (Quiagen GmBH, Hilden) according to manufacturer's instruction. Purified RNA was resuspended in RNase-free water and aliquots were stored at –80 ˚C. cDNA was prepared from RNA by using MuMLV reverse transcriptase (New England Biolabs, Schwalbach) and Taq Polymerase (Promega, Heidelberg) accoding to the guidelines of the manufacturers in a total volume of 20 µl. For 3 µg total RNA 1 µl oligo (dT) (Roche, Mannheim) and H_2O to a volume of 14 µl were added. Mixture was heated to 70°C for 10 min and chilled on ice for 30 sec. The following components were added:

4 µl 5x Reverse transcriptase buffer
1 µl Reverse transcriptase
1 µl 10 mM dNTP mix

The reaction was incubated at 37˚C for 1 hour. The dNTP mix was purchased from Amersham Pharmacia, USA. All other reagents used were from Promega, Heidelberg. Each PCR reaction was set up in a total reaction volume of 50 µl using 3 µl of RT product and 1 U of Taq DNA polymerase (Promega, Heidelberg). After initial denaturation at 95°C for 5 min, 40 cycles were performed. Each cycle consisted of a denaturation step at 95°C (1 min), followed by a primer annealing step (1 min) at the annealing temperature (AT) between 56 and 62 °C depending on the primer pairs used, and an extension step at

72°C (2 min). Primer pairs were designed to amplify sequences specific for cDNAs of identified antigens. After size separation by gel electrophoresis (1% agarose in TAE buffer), PCR products were purified using Qiaex II Gel Extraction Kit (Qiagen, Hilden).

2.2.1.3. Separation of DNA fragments by agarose gel electrophoresis

Separation of DNA fragments was performed on 0.8-1.2% agarose gels with 0.4 µg/ml ethidium bromide in 1 x TAE at 5-10 V/cm. DNA samples were mixed with 6x concentrated gel loading buffer (0,25% (w/v) bromophenol blue, 0,25% (w/v) xylene cyanol, 30% 0,25% (v/v) glycerol) and loaded into the gel slots. Electrophoresis was performed in the horizontal position with 1x TAE running buffer. 1 Kb DNA Ladder (MBI Fermentas) was used as size standard. For the isolation of DNA fragments from agarose gels, DNA was run on an agarose gel, the desired fragments cut from the gel and DNA isolated using a Qiaex II Gel Extraction Kit (Qiagen, Hilden) according to manufacturer's instructions.

2.2.1.4. Phenol-chloroform extraction and precipitation of DNA

To remove contaminants from DNA, an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (proportions 25/24/1) was added to the DNA solution to be cleaned. The mixture was centrifuged for 10 min at 13000 g to separate the phases. The aquous upper phase was taken and DNA precipitated by the addition of 2 vol. 100% ethanol and 0.1 vol. 3 M sodium acetate. After centrifugation at 13000 g the pellet was washed in 70% ethanol, dried and dissolved in H_2O. DNA concentration was measured by UV-spectrophotometer.

2.2.1.5. DNA restriction

2-4 U of the desired restriction enzymes were added to 1 µg of DNA in the appropriate restriction buffer. The total volume of digestion was 10 x the volume of enzyme added, as glycerol in the enzyme storage buffer may inhibit the reaction. The restriction mix was incubated for 2 h at 37°C.

2.2.1.6. Ligation

DNA fragments with compatible ends were ligated with T4 DNA ligase. The amount of insert used was 3-6 times that of dephosphorylated vector. 1 U enzyme in 20 µl ligation solution (Boehringer, Mannheim) was added to 200 ng DNA and the reaction was incubated overnight at 16°C.

2.2.1.7. Mini- and Maxi-preparation of plasmid DNA

A single bacterial colony was inoculated into 5 ml LB medium containing the appropriate antibiotic and incubated overnight in a shaker at 37°C. The overnight culture was then spun down at 3000 rpm, the supernatant discarded and the bacterial pellet was resuspended in 100 µl of solution 1. The cells were lysed by the addition of 100 µl of solution 2 and incubated at RT for 5 min. The suspension was neutralized with 100 µl of solution 3 and centrifuged 10 min. at 13000 g. The supernatant was transferred into new tube and 700 µl of 100% ethanol was added for DNA precipitation and the solution was centrifuged for 20 min at 13000g. Supernatant was poured off and DNA was air dried for 15 min. DNA was resuspend in 100 µl H_2O.

Solution 1: 50 mM Tris-HCl (pH 8) ; 10 mM EDTA ; 100 µg/ml RNase A
Solution 2: 200 mM NaOH ; 1 % SDS (w/v)
Solution 3: 3 M potassium acetate, pH 5.5

Large amounts of plasmid DNA were obtained from 500 ml LB overnight culture using JetStar (Genomed, Bad Oeynhausen), according to manufacturer's instruction.

2.2.2. SEREX methods

2.2.2.1. Construction of the cDNA library

PBL were obtained 3,5 months after BMT and frozen in liquid nitrogen. PBL were stimulated four times in T cell medium containing 0,25 µg/ml PHA (Sigma Chemie, München) in order to expand T cells. Total RNA was isolated by the phenol/chloroform method. Poly $(A)^+$RNA was isolated using an mRNA isolation kit (Stratagene, La Jolla, USA). 5 µg mRNA was used for cDNA library construction, with the help of the ZAP express cDNA synthesis kit and Gigapack III Gold cloning kit (Stratagene, La Jolla, USA). Briefly, first-strand synthesis was performed using an oligo(dT) primer with an

internal XhoI site, 5-methyl-dCTP, dNTPs, and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). After second-strand synthesis, EcoRI adapters were ligated to the cDNA and digested with XhoI. Sepharose CL-2B gel filtration was performed for size fractionation of the cDNA samples. The fractionated cDNA was ligated into the ZAP express vector. The cDNA expression library was constructed, packaged and used to infect Escherichia coli (E.coli) XL-1 Blue MRF' according to instructions to the λZAPII cDNA Gold cloning kit (Stratagene, La Jolla, USA), resulting in more than 1,2 x 10^6 primary recombinants. To obtain a stable quantity of a high-titer stock of phages, the library was amplified.

2.2.2.2. Preadsorption of sera

Sera from a patient undergoing allogeneic BMT was obtained after BMT and stored at -80°C. To remove antibodies reactive with antigens related to the vector system, each serum was preadsorbed to XL-1 and to non-recombinant λZAPII phage particles by the following procedure: Preadsorption columns were prepared by using glutaraldehyde-activated affinity adsorbens (Roche Diagnostics, Mannheim) and XL-1 either sonicated or lysed by λ-phage infection. Preadsorption nitrocellulose membranes were prepared as plaque lifts from XL-1 cultures lytically infected with λ-phages and grown on Luria-Bertani (LB) agar plates. For preadsorption, 2 ml of serum was diluted 1:10 with Tris-buffered saline (TBS), incubated subsequently with column bound proteins of sonicated and lysed XL-1 (over night, 4°C) and then with 6 plaque lifts (3 hrs each, room temperature). Sera were then further diluted 1:10 in TBS (0,2% low-fat dried milk) stabilised by addition of 0,0125% sodium acide and thimerosale and stored at 4°C.

2.2.2.3. Immunoscreening of cDNA libraries

For library screening, a total of $1{,}2\times10^6$ recombinant clones was tested as described (Sahin et al., 1995) with the modification that serum was primarily diluted only 1:100 as reported by Scanlan et al. (Scanlan et al., 1999). Briefly, λ-phage infected XL-1 were plated on LB-agar ($3{,}5 \times 10^3$ plaque forming units per 15 cm plate) and expression of recombinant proteins induced with isopropyl β-D-thiogalactoside (IPTG). Following overnight incubation, proteins were transferred to nitrocellulose membranes, which were washed with TBS-T (TBS plus 0,5% Tween 20), blocked with 5 % low-fat dried milk in TBS and

then incubated with preadsorbed serum over night at room temperature. Next day, membranes were incubated with an alkaline phosphatase-conjugated anti-human IgG secondary antibody (Dianova, Hamburg) and stained with 5-bromo-4-chloro-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma–Aldrich, Taufkirchen). Staining membranes without prior serum exposure identified recombinants expressing IgG. Positive clones were picked, subcloned to monoclonality by using the same plaque assay procedure and stored in SM buffer.

2.2.2.4. Single clone excision and plasmid extraction

E. coli (XL1-Blue MRF) was infected with isolated positive clone and ExAssist helper phages (1 million PFU, Stratagene, La Jolla, USA). After cell lysis and removal of cell debris, solutions containing the excised pBluescript phagemid vector packaged as filamentous phage particles were incubated with E. coli (SOLR strain). This allows only the excised phagemid to replicate in the host. The cell mixture was plated on LB-kanamycin agar plates, and single clones were picked and grown in kanamycin-containing LB medium. Plasmid DNA was purified over JetStar columns (Genomed, Bad Oeynhausen).

2.2.2.5. Sequencing and homology search

Monoclonal plasmid DNA was sequenced with T3 (5' AATTAACCCTCACTAAAGGG 3') and T7 (5' GTAATACGACTCACTATAGGGC 3') primers. After sequencing of the cDNA inserts, GenBank databases (http://www.ncbi.nlm.nih.gov/) were screened for homologies. Corresponding genomic DNA sequences were analysed using the Internet address http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html (programs: FGENES, FEXH, HSPL, HEXON, TSSG, TSSW and NNP). Open reading frames were predicted by computer programs (Clone Menager 6, Genetool).

2.2.3. Cell biology methods

2.2.3.1. Patient/donor pair

A pediatric patient with β-thalassemia major (BM recipient) and her HLA-identical sister (BM donor) were enrolled in this study which had been approved by the ethical review board of the Technical University of Munich. HLA-identity was demonstrated by genetic

and immunological methods in the National reference laboratory for tissue typing (Prof. Dr. med. E. Albert, Labor für Immungenetik, Poliklinik der Ludwig-Maximilians Universität, München). The conditioning regimen included busulfan, cyclophosphamide and antithymocyte globulin and was well tolerated. For prophylaxis of GvHD, the patient received cyclosporine and methotrexate. 5 x 10^8/kg unfractionated mononuclear cells of donor BM were infused. Three weeks after BMT, the patient developed mild GvHD in liver, skin and gut. Immunosuppressive therapy was given for three months after BMT. Blood samples were collected from the patient one month before BMT as well as three and three and a half months after BMT (d90 and d100). A blood sample from the donor was obtained at the time of transplantation. Chimerism analysis at any time after BMT proved that all PBL collected from the patient after BMT were of donor type (PD Dr. med. P. Bader, MRD/Chimerismuslabor, Abteilung I: Pädiatrische Hämatologie/Onkologie, Universität Tübingen).

2.2.3.2. Retrieval and storage of the cells

PBL were obtained in syringes and upon arrival, immediately processed. Density gradient centrifugation was performed using Ficoll-Paque. For this separation, 20 ml of PBL were transferred into 50 ml Falcon tubes and under layed with 14 ml Ficoll-Paque. PBL were centrifuged for 30 min, 2000 rpm, 4 ˚C. The same procedure was used for the preparation of feeder cells from “Buffy Coats”. After separation, leukocytes were collected from the interphase, transferred to a new tube, filled up with RPMI-1640 to 50 ml and centrifuged at 1500 rpm for 10 min. This wash step was repeated, then cells were resuspended in an appropriate volume of media and further cultivated in 24-well plates. Cells were expanded and aliquots frozen and stored in liquid nitrogen. Cells were frozen in fetal calf serum supplemented with 10% DMSO. When needed, frozen cells were thawed rapidly in a 37 ˚C water bath, resuspended in appropriate medium, and further cultivated in appropriate cell culture plates or flasks.

2.2.3.3. General cell culture conditions and maintenance of cell lines

All mediums were stored at 4 ˚C for no longer than three months, and were used within two weeks following addition of serum. Serum was stored at -20 ˚C for no longer than six months. Supplements were kept at 4 ˚C. Cell manipulations were performed in a biological containment cabinet (hood) using autoclaved or plastic pipets with cotton plugs

and strict aseptic technique. The cells were incubated in an atmosphere of 5% CO_2 and at a temperature of 37 ˚C. Cells cultures were refed with fresh growth medium every 3 to 5 days. Cultures were kept growing only when they were required for experimentation. Cells were ordinarily expended and refrozen to maintain a frozen inventory. Thawed cells were treated for two weeks with mycoplasma removal agent to avoid mycoplasma contamination. T cell lines and clones were grown in the same tissue culture conditions in 24-, 48-, or 96 well plates in T cell medium (RPMI 1640 supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 100 U/ml penicillin und 0,1 mg/ml streptomycin and HEPES buffer (10mM)).

2.2.3.4. Generation of Epstein-Barr virus (EBV)-transformed B cell lines (LCL)

Several LCLs were generated during this work including LCL patient and LCL donor, and from 17 members of one family that shares all HLA class II alleles with the patient/donor pair. In all cases, EBV-transformed B cells were obtained by incubating PBL ($1x10^6$) from each donor with supernatant from EBV-producing B95-8 Marmoset cells, cyclosporin A 1µl (100 µg/ml solution), and feeder cells ($1x\ 10^4$ Wi38 human fibroblasts cell line) in total volume of 200 µl/well in 96-well plate. LCL lines were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 0,1 mg/ml streptomycin and 10% heat-inactivated FCS. LCLs from all individuals were HLA class I and II typed and used to define the HLA restriction elements.

2.2.3.5. PHA blast generation

PHA blasts from patient and donor were generated by culturing PBL for three days in T cell media (RPMI 1640 supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 100 U/ml penicillin und 0,1 mg/ml streptomycin and HEPES buffer (10mM)) containing 0,25µg/ml Phytohemagglutinine (PHA) (Abbott Murex, Wiesbaden). After three days the medium was removed and PHA blasts were further cultured in T cell medium with IL-2. PHA blasts were used as APC in ELISPOT assay and for the isolation of RNA.

2.2.3.6. Generation of mHA-specific T cell lines

Lymphocytes were purified from PBL obtained from the patient three and half months after BMT using Ficoll-Hypaque (Amersham Bioscience, Uppsala, Schweden) density

gradient centrifugation. T cell lines specific for mHA were generated in 24-well plates (Falcon, Multiwell™, Becton Dickinson/Pharmingen, Heidelberg) by stimulating $1x10^6$ lymphocytes obtained after BMT with $1x10^6$ γ-irradiated (80Gy) LCL patient in a total volume of 2 ml/well. Stimulation was performed in T cell medium. After 24 hours 10 U/ml IL-2 was added. After each stimulation 20U/ml IL-2 were added 24 hours later. After three rounds of stimulation reactivity against LCL obtained from PBL before BMT was measured in GM-CSF release assay and used for the generation of mHA-specific T cell clones.

2.2.3.7. Generation of mHA-specific T cell clones

T cell lines with specificity for LCL patient were cloned by limiting dilution in 96-well round-bottom plates (Falcon, Microtest™, Becton Dickinson/Pharmingen, Heidelberg). Each well received $1x10^4$ γ-irradiated (80 Gy) LCL patient, $1x10^5$ γ-irradiated (40 Gy) feeder cells (mixture of three PBLs obtained from unrelated donors) and 200 µl T cell medium containing 20 U/ml IL-2 and 0,25µg/ml PHA and T cells from previously generated mHA-specific T cell lines. Three independent dilutions were made containing 10, 3, and 1 responder T cell from mHA-specific T cell lines per well. Three days later medium was removed and fresh T cell medium containing 20 U/ml IL-2 was added. After 10 days the cultures were restimulated. Wells exhibiting T cell growth were identified by microscopy, and transferred into 48-well plates for further cultivation. All further stimulations were performed in the same fashion.

2.2.3.8. Determination of IL-2, IL-4 and GM-CSF by cytokine ELISA and antibody blocking studies

Specific recognition assays in this work were performed by measuring cytokine release into the media after specific recognition of target cells by T cells. The measurement of cytokines in T lymphocyte culture supernatant was performed by ELISA. The plates were coated with an anti-cytokine antibody, and bound cytokines were revealed by a second, biotinylated anti-cytokine antibody. Assays were performed according to manufacturer's instructions (kits from R & D Systems and Endogen). Briefly, ELISA plates (Stripwell™ Plate, Costar, USA) were coated overnight at 37°C with 100 µl/well capture mouse anti-human cytokine antibody in PBS. Plates were washed three times with PBS/0.05% Tween-20, blocked with 300 µl/well PBS supplemented with 1% bovine serum albumin

(BSA) (ICN Biomedicals Inc., Ohio, USA), 5% sucrose and 0,02% Na-acide for 1 hour at room temperature, and washed three times with PBS/0.05% Tween-20. 100 µl/well of the samples were added to ELISA plates and incubated overnight. Next day, plates were washed three times and incubated for two hours with detection antibody (biotinylated mouse anti-human cytokine). The plates were then washed three times and streptavidine conjugated to horseradish-peroxidase was added and incubated in the dark for 20 min. After 3 washes, 100 µL/well substrate solution (1:1 colour reagent A (H_2O_2) and colour reagent B (tetrametilbenzidine), R&D System, USA) was added and incubated at room temperature for 20 minutes. After addition of 50 µl/well stop solution (2N H_2SO_4), absorption was measured at 450 nm using a reference wavelength of 690 nm. The colorimetric measurement was performed using an ELISA Reader Tecan (Tecan, Crailsheim) using Xread Plus software for quantification (Tecan, Crailsheim). Some assays were performed by preincubating the target cells for 30 min at 37˚C in the presence of 25 µg blocking antibodies (monoclonal anti-human HLA class I antigene, clone W6/32), (monoclonal anti-human HLA-DR, L243) per 10^5 cells in a total volume of 200 µl/well. Cytokine secretion by T-cell lines and clones was assessed after $1x10^5$ effector cells were cultured in 96-well microplates with $1x10^5$ stimulator cells in 200 µl of T-cell medium. After 24-h incubation, 100 µl/well supernatant was harvested, and the release of cytokine was determined using an ELISA kit.

2.2.3.9. IFN-γ ELISPOT

The frequency of T lymphocytes specific for antigens 10, 10-2 and 21 in the T cell lines generated from PBL post BMT was determined with an IFN-γ-specific ELISPOT assay. Assays were performed in 96-well nitrocellulose-lined microtiter plates (Millipore, Mana S45, Germany) coated with anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden) overnight at 4°C. The plates were washed three times with PBS, and then blocked with RPMI supplemented with L-glutamine, penicillin, and 10% heat-inactivated pooled human AB serum for 1 h. The plates were again washed three times with PBS and T cell lines were tested in the assay as follows: Freshly prepared PHA blast cells were γ-irradiated (40Gy) and plated at $1x10^5$ cells/well in serum-free medium and 1µl/well peptides (1mg/ml solution) was added directly to Ab-coated ELISPOT plates in a volume of 100 µl. $1x10^5$ T cells were added to each well and incubated overnight at 37°C. The final volume in each well in the ELISPOT plate was 200 µl. After 24 h the cells were removed

by washing three times with PBS and four times with PBS containing 0.05% Tween (PBST). Next, 100 µl of 1 µg/ml of the biotinylated anti-IFN-γ mAb 7-B6-1 biotin (Mabtech, Stockholm, Sweden) were added. After 3 h of incubation, plates were washed six times more and a 1:1000 dilution of Sigma ExtraVidin peroxidase conjugate (Sigma–Aldrich, Taufkirchen) was added to the wells and the plates incubated at room temperature for a further 2 h. Then, wells were again washed 6 times and 100 µl of Sigma Fast DAB (3,3'-diamino benzidine, urea H_2O_2 in 10 ml deionized H_2O) filtrated solution was added. After 30 min, the colorimetric reaction was terminated by washing with tap water and plates were air dried. Next day the plates were analysed using light microscopy. The frequency of Ag-specific cells was calculated as the number of spots per well.

2.2.3.10. Flow cytometric analysis of mHA-specific T cell clones

T cell lines and clones were analyzed by two-color flow cytometry for expression of CD4, CD8, CD16, CD45RO, CD62L, HLA class I, HLA-DR, TCRαβ, TCRγδ using phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 antibodies. All other antibodies were unlabeled and detected using (FITC)-conjugated mouse anti human IgG secondary antibody (Becton Dickinson/Pharmingen, Heidelberg). Briefly, about $3x10^5$ cells were centrifuged (5min, 500xg, 4˚C), in 100µl FACS buffer (PBS, 2% FCS), resuspended and incubated 30 min on ice with primary antibodies in the dark. After washing three times in 1 ml FACS buffer, cells were incubated 20 min on ice with FITC-conjugated secondary antibody. Cells were washed three times with 1 ml of FACS buffer and resuspended in 400 µl FACS buffer with 2 µl/ml propidium iodide. Analysis was performed on a FACScan flow cytometer with CellQuest software (Becton Dickinson) and WinMDI 2.8 (J. Trotter; Scripps Research Institute, LA Jolla, CA; http:/facs.scripps.edu/).

2.2.3.11. Isolation of peptide specific T cells: MACS Cytokine Secretion assay

In this study, IFN-γ secreting cells were isolated from the T cell line generated from PBL obtained from the patient after BMT. Three fractions of the T cell lines each containing $5x10^6$ cells were incubated for 16 hours (at 37˚C, 5% CO_2) with 10µl of peptides 10, 10-2 and 21 (1µg/µl solution). After 16 hours cells were spun down (1500 rpm, 10 min, 4˚C), washed with 10 ml of cold buffer and again centrifuged at 1500 rpm for 10 min at 4˚C.

Subsequently, the cell pellet was resuspended in 80 µl of cold buffer, 20 µl of IFN-γ catch reagent (20 µl/10^7 total cells) was added, mixed, and incubated for 5 min on ice. After incubation, 10 ml warm medium (37˚C) was added to dilute the cells, then incubated for 45 min (37˚C, 5% CO_2), and mixed every 5 min. The samples were centrifuged at 1500 rpm for 10 min at 4˚C, the supernatant was removed completely, and the cells were washed twice. The cell pellet was resuspended in 80 µl of cold buffer, 20 µl of IFN-γ detection antibody (PE) was added, mixed, and incubated for 10 min on ice. Cells were washed with 10 ml cold buffer two times, centrifuged at 1500 rpm for 10 min at 4˚C. For magnetic labeling, the cell pellet was resuspended in 80 µl of cold buffer, then 20 µl of anti-PE microbeads were added, mixed, and incubated for 15 min at 4˚C followed by washing the cells two times with cold buffer (1500rpm, 10 min, 40C) and removing the supernatant completely. The cells were resuspended in 1 ml of cold buffer. For magnetic separation, LS columns were put in the magnetic field of the MACS separator and rinsed 3 times with 3 ml of cold buffer. Magnetically labeled cells were loaded onto the columns. The columns were washed with 3 x 1 ml cold buffer, and the effluent was collected as negative fraction. Three ml of cold buffer was pipetted on top of the column. Using a plunger, the retained cells were pushed out from the column and harvested by centrifugation.

Cold buffer:	PBS pH 7.2, containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA, 4 ˚C
Medium:	RPMI 1640 containing 5% human serum

3. Results

3.1. Strategy for mHA identification

It has been shown that $CD4^+$ T cells play an important role in GvHD (van Els et al., 1989; van Els et al., 1990; Shenoy et al., 1998) and GvL responses (Faber et al., 1995; Alyea et al., 1998; Alyea et al., 2001) after allogeneic BMT. Thus, for separating GvL from GvHD responses, it is important to know the antigens recognized by $CD4^+$ T cells. However, current methods for the identification of antigens presented on MHC class II are complicated and time-consuming procedures. It is known that B cells require T cell help for switching their isotype and the production of highly specific antibodies. Therefore, two approaches for the identification of mHA presented on MHC class II should be taken in parallel (Fig.3). First, mHA-specific $CD4^+$ T cell clones involved in the cellular immune response after BMT should be isolated. Second, the targets of antibody response after BMT should be identified with the SEREX method. Polymorphisms should be identified by comparing the sequences of the genes encoding the antigens in patient and donor (before and after BMT). Polymorphic antigens would be candidate mHAs. These antigens should then be tested in a recognition assay with mHA-specific T cell clones isolated from the patient after BMT. This strategy should give an answer to the question, whether the humoral and cellular immune responses are directed against the same or different antigens. The specific recognition of these candidate mHAs by the $CD4^+$ mHA-specific T cell clones would provide evidence that these antigens truly represent mHAs, and that the serological approach can be used to identify MHC class II-restricted antigens recognized by $CD4^+$ T cell clones.The role of these antigens in the immune response after BMT should additionally be examined by measuring the frequency of the T cells specific for these antigens before and after BMT.

3.2. Cellular immune response after BMT

3.2.1. Generation of mHA-specific T cell lines

The pediatric patient analyzed in this work suffered from β-thalassemia and received BM from an HLA-identical sister. Approximately 30 days post BMT the patient developed mild symptoms of GvHD. Peripheral blood leukocytes (PBL) were collected from the patient before, and one hundred days after BMT. Since it was not possible to

collect enough material to generate dendritic cells (DC), LCL were used as antigen presenting cells (APC).

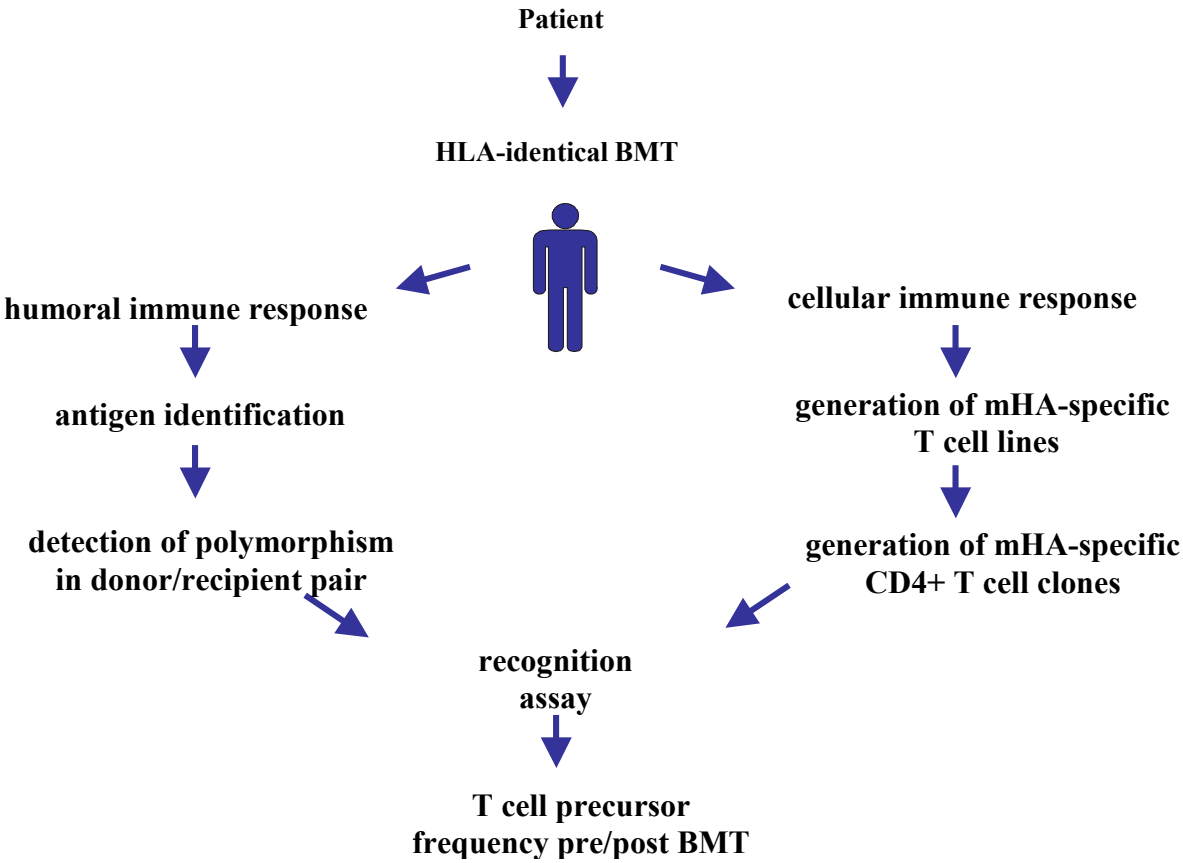


Fig.3

Strategy for mHA identification. In a patient receiving HLA-identical BM, both the cellular and the humoral immune response after BMT should be assessed in parallel. The targets of the humoral immune response should be identified, and in order to detect any polymorphisms in these antigens, their sequences should be compared between patient and donor. mHA-specific $CD4^+$ T cells lines should be generated from PBL of the patient after BMT, and cloned by limiting dilution. The antigens identified as the targets of antibody response should then be tested in a recognition assay, to determine whether the mHA-specific $CD4^+$ T cell clones recognize these or different antigens. Furthermore, the precursor frequency of the T cells recognizing the targets of the humoral immune response should be determined before and after BMT.

The PBL collected before BMT were used to generate a LCL of recipient type and therefore designated “LCL patient”. Chimerism analysis at different time points after BMT revealed that all PBL were derived from the BM donor. Thus, the PBL collected from the patient after BMT are of donor origin, and the LCL generated from these PBL are designated “LCL donor”. In order to generate mHA-specific T cell lines, T cells from PBL taken 100 days after BMT were stimulated with irradiated LCL patient. In a 24-well plate, 1 x 10^6 PBL obtained from the patient after BMT were stimulated with 1 x 10^6 irradiated LCL patient/well in 2 ml of T cell media. After 24 hours, 10 U/ml IL-2 was added. Depending on cell growth, cells were split and after ten days restimulated in an identical fashion. The specificity of the three T cell lines generated, designated I, II and III, was tested by comparing the reactivity of T cell line against LCL patient and LCL donor in a GM-CSF release assay (Fig.4). Because EBV-transformed B cells present self and EBV-antigens on MHC class I and II molecules, mHA-specific as well as EBV- or autoantigen-specific T cells were generated.

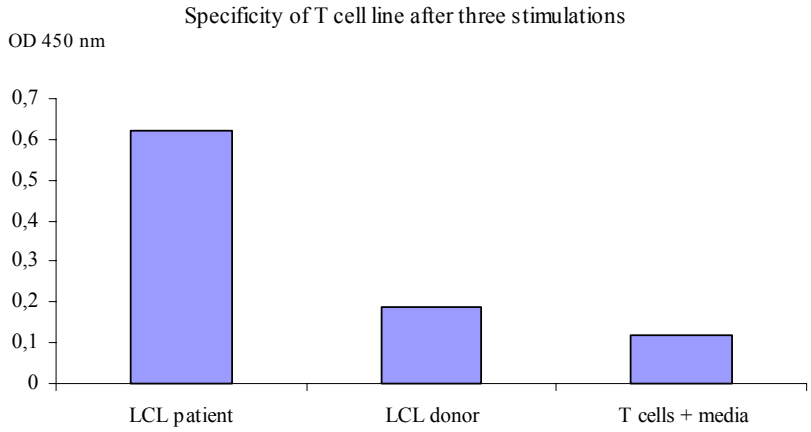


Fig.4

Specific T cell recognition of a representative T cell line (I) generated from PBL of the patient after BMT. Upon recognition of antigen, T helper cells secrete cytokines into the cell culture supernatant. The amount of cytokine in the supernatant can be quantified by a sandwich ELISA-based color reaction, which is measured at 450 nm (OD 450 nm). Release of granulocyte-macrophage colony-stimulating factor (GM-CSF) was measured in this work. PBL obtained from the patient three and a half months after BMT were stimulated three times with LCL patient. mHA specificity of the generated T cell line was tested by comparing reactivity of the line against LCL patient and LCL donor.

The reactivity of T cells against either group of antigens was assessed by testing the T cells against LCL patient and LCL donor. Several mHA-specific T cell lines, that preferentially recognize antigens expressed on LCL patient but not on LCL donor, were generated by this protocol.

3.2.2. Generation of mHA-specific T cell clones

mHA-specific T cell clones were isolated from these mHA-specific T cell lines by limiting dilution cloning. Three different T cell lines, stimulated three, four or six times with LCL patient were used. Clones obtained from these lines are denoted by the appendix I, II and III. Limiting dilution cloning was performed in 96-well round-bottom plates by seeding 1, 3 or 10 T cells per well in 200µl T cell medium supplemented per ml with 250 ng PHA, 20U IL-2, $5x10^5$ irradiated feeder cells and $5x10^3$ irradiated LCL patient.

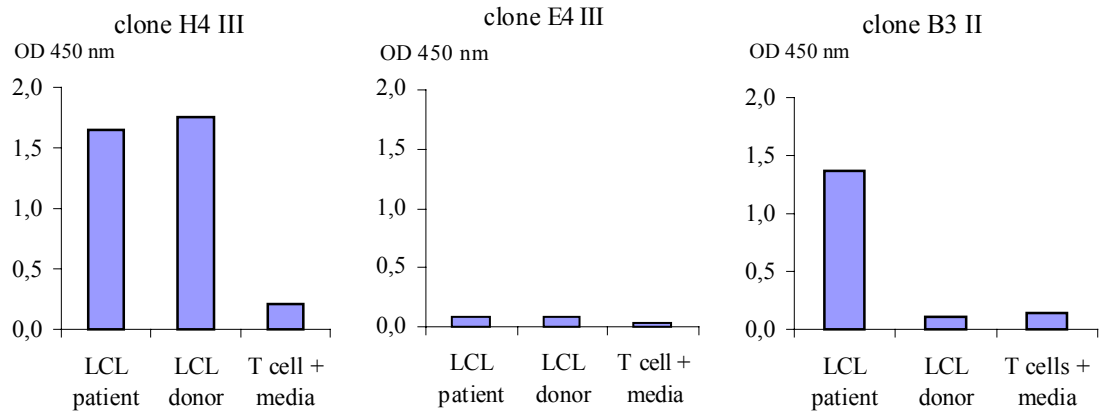


Fig. 5

Recognition pattern of representative T cell clones. The generated T cell clones showed three types of specificities when tested for recognition of LCL patient and LCL donor. Examples of three clones representing these three recognition patterns are shown. Clone H4 III reacts against LCL patient and LCL donor and therefore recognizes an EBV- or autoantigen. The specificity of clone E4 III is unknown, because neither LCL patient nor LCL donor were recognized. Clone B3 II shows the typical recognition pattern for mHA-specific clones. This clone recognizes LCL patient but not LCL donor.

Sixty-seven clones were isolated, expanded, and their specificity tested (Fig.5). T cell clones with three different types of specificities were isolated. The specificity of the first group is directed against EBV- or auto-antigens because antigens presented on both LCLs were recognized. T cell clones in the second group neither responded to LCL patient nor LCL donor. The specificity of these clones is unknown. The recognition pattern of the third group is characteristic for clones with specificity for mHA. LCL patient were highly recognized whereas LCL donor were not. The majority of the T cell clones were mHA-specific.

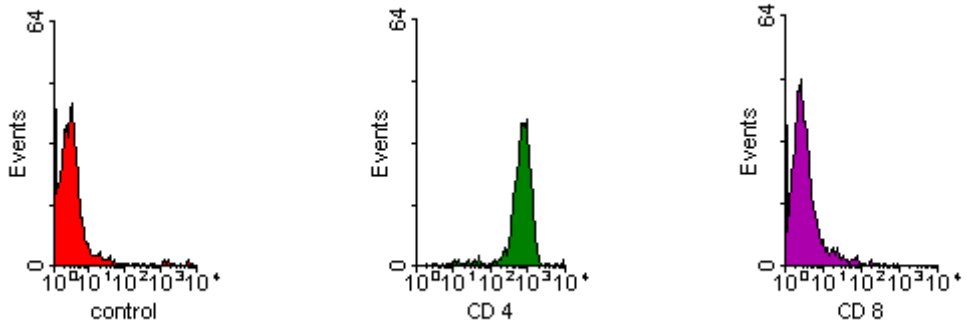
These mHA-specific T cell clones were further characterized and the phenotype determined. Antibodies to the cell surface molecules CD4 and CD8 were used in fluorescence-activating cell sorting (FACS) analysis to determine the T cell subtype of the clones. As illustrated in the Figure 6, mHA-specific T cell clones generated from all three T cell lines (I, II and III) were $CD4^+$ and $CD8^-$. Thus, only $CD4^+$ but no $CD8^+$ T cell clones were established under these in vitro culture conditions.

3.2.3. Determination of the restriction element

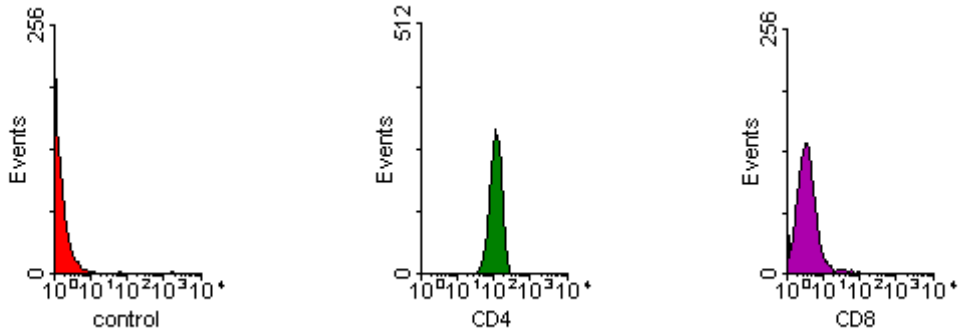
$CD4^+$ T cells recognize antigenic peptides bound to the binding groove of MHC class II molecules. As every individual inherits three different HLA class II molecules from mother and father, usually six different MHC class II molecules are expressed. Depending on the HLA genotype, up to two additional HLA-DRβ chaines can be expressed. To determine which of these MHC class II molecules presented the antigen to the $CD4^+$ T cell clones, a panel of allogeneic LCL, which share one or more HLA molecules with the patient, was tested. Because LCLs have to express the mHA in addition to the restricting MHC class II molecule, the determination of the restriction molecules for mHA-specific $CD4^+$ T cell clones is complicated. Therefore, a large panel of allogeneic LCLs that share one or more HLA class II molecules with the patient/donor pair was tested for recognition by the mHA-specific T cell clones. In the same experiment, blocking studies were performed using antibodies directed against HLA class I (W6/32) (anti-HLA A, B, and C) and HLA class II (L243) (anti-HLA-DR) molecules. A representative example of this analysis is shown in the Figure 7A. Recognition of autologous LCL by clone E8 II was blocked with an antibody against HLA-DR but not

with an antibody against HLA class I molecules, showing that this clone is HLA-DR restricted.

clone D5-2I



clone B5II



clone B10III

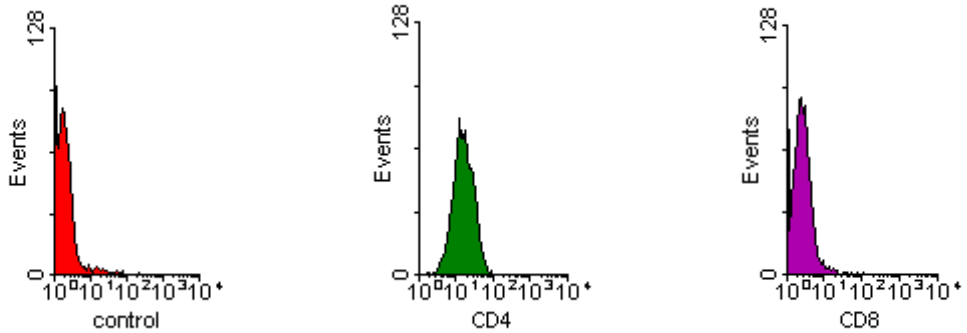
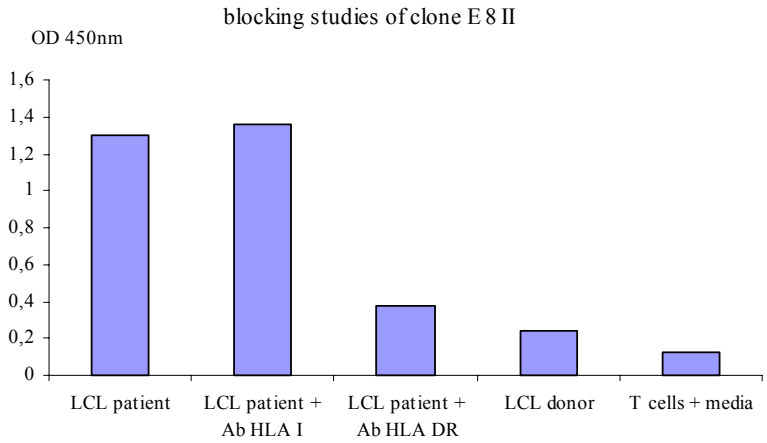


Fig. 6

FACS analysis of mHA-specific T cell clones. Representative examples of three clones D5-2 I, B5 II and B10 III established from three T cell lines (I, II and III) are shown. All isolated mHA-specific T cell clones are $CD4^+$ and $CD8^-$. Control represents staining with propidium-iodide for dead cells.

A)



B)

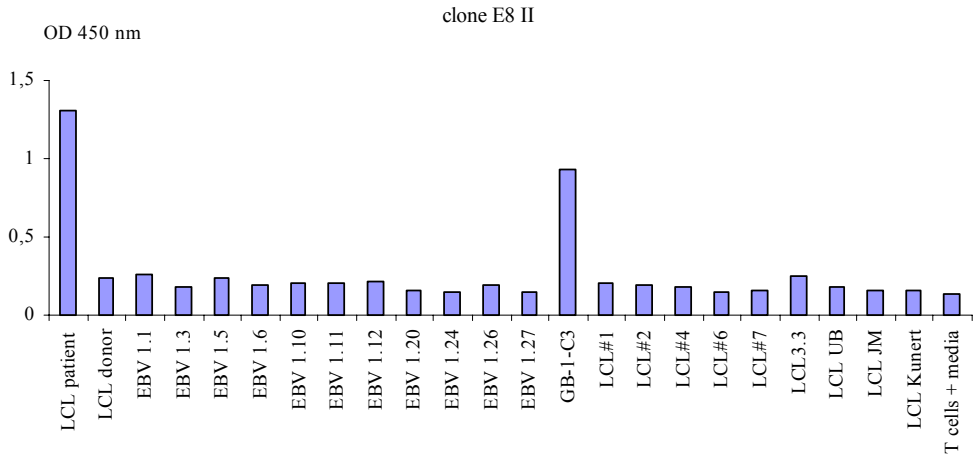


Fig. 7

A) Blocking experiment performed with clone E8 II. In order to determine the MHC class II restriction element for clone E8 II, blocking antibodies were used in the recognition assay. Recognition of autologous LCL could be blocked with an anti-HLA-DR antibody but not with an anti-HLA class I antibody, indicating that this clone is HLA-DR restricted.

B) Recognition of allogeneic LCLs by mHA-specific T cell clone E8 II. A panel of allogeneic LCLs established from unrelated donors which share one or more HLA class II molecules with the patient were used in a cytokine secretion assay. Only one LCL that shares HLA-DR and DQ with the patient/donor pair was recognized, therefore it was not possible to determine the restriction molecule.

Besides the LCL patient, only one allogeneic LCL was recognized by clone E8 II. The HLA class II type of this LCL was identical with the HLA type of the patient/donor

pair. All other LCLs share one or more HLA class II alleles with the patient/donor pair, but none of them was recognized, most likely because these LCLs do not express the mHA recognized by the T cells.

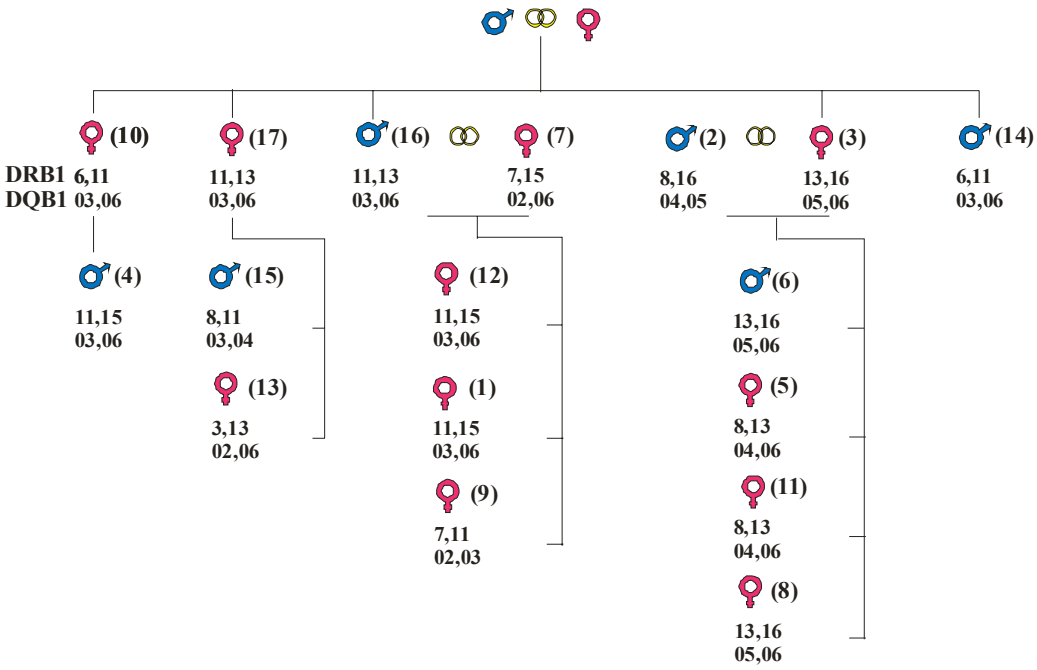


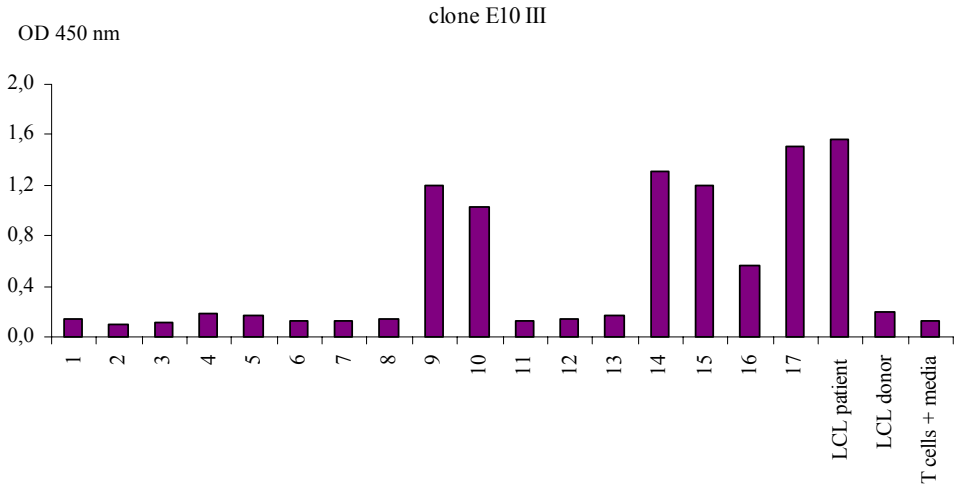
Fig. 8

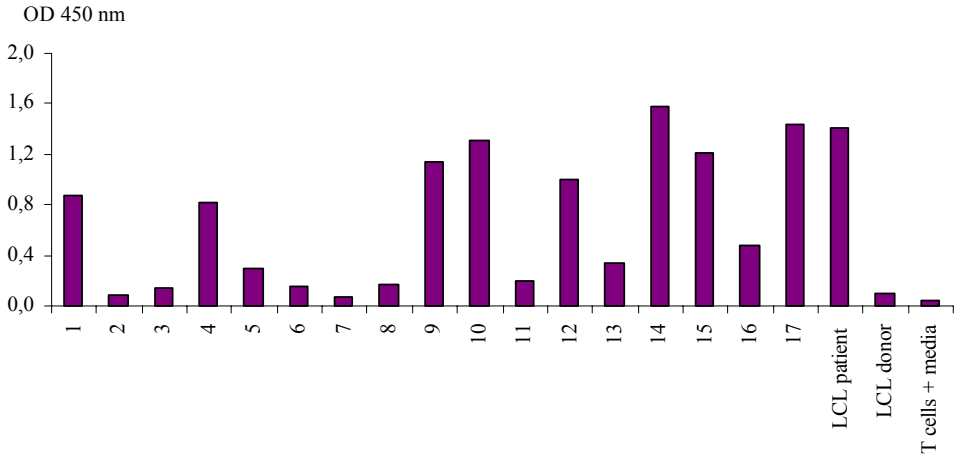
Family tree of donor GB-1-C3. In order to identify the restriction elements of the mHA-specific $CD4^+$ T cell clones, LCLs from seventeen members of the family from which LCL GB-1-C3 originated, were generated and HLA typed. GB-1-C3 shares all tested HLA class II alleles (DR, DQ) with the patient/donor pair and expresses the mHA recognized by T cells. For reasons of lucidity, numbers are given instead of names for each of the generated LCLs. The HLA genotypes for HLA-DR and HLA-DQ alleles are given below the symbol for the gender of each family member.

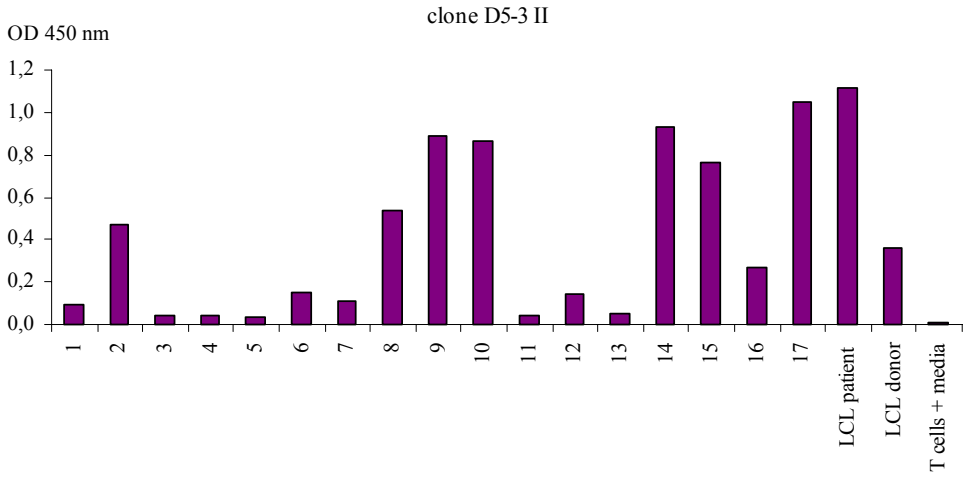
Because only LCL GB-1-C3 was recognized, it was not possible to determine the HLA restriction molecule. These results indicated that the mHA recognized by the T cell clone is expressed in a minority of the LCLs only. Similar results were obtained with other T cell clones. Therefore, instead of generating and testing a larger number of LCL from unrelated donors, several family members of the donor from whom LCL GB-1-C3 originated were analyzed. Within this family, the mHA recognized by the T cells is

expressed. In addition, LCL GB-1-C3 shares all tested HLA class II alleles with patient, implying that other family members may share at least one or more HLA class II alleles with the patient.

It was a lucky case that the donor, from which LCL GB-1-C3 had been generated, is a member of a big family from which PBLs were available. LCLs from 17 family members were generated and HLA typed (Fig. 8). All mHA-specific T cell clones were then tested in a GM-CSF release assay for specific recognition of these 17 LCLs including LCL patient and LCL donor as control. Four recognition patterns were observed (Fig. 9). Most of the T cell clones showed a pattern of recognition as exemplified for clone E10 III. This clone recognizes LCLs 9, 10, 14, 15, 16 and 17. All these LCLs express DR11 and DQ03 alleles. Blocking experiments had shown that this clone is DR and not DQ restricted. Therefore, the restriction element for this clone is DR11. Because most of the generated clones showed the same recognition pattern as clone E10 III, the antigen recognized by this clone probably is immunodominant. LCLs 1, 4 and 12 were not recognized by these clones, although they are HLA-DR11 positive. Thus, these LCLs do not express the mHA recognized by this clone. This pattern of recognition further confirms that the clones are mHA-specific. The inheritance of mHAs follow the Mendelian rules (den Hann et al., 1998), and therefore one can expect that the specific T cell recognition of the LCLs generated from one family will also follow the expression of the specific allele through this family. This prediction did hold true for the mHAs recognized by clones E10 III and C3 III. Clone E10 III recognizes LCLs from the father and a daughter (LCLs 9 and 16), but LCLs from the mother and the two other daughters are not recognized (LCLs 1, 7 and 12). In contrast, clone C3 III recognizes LCLs from the father (LCL 16) and all three daughters (LCLs 1, 9, and 12), showing that the antigen recognized by this clone is transmitted from the father to all daughters. The antigens recognized by the T cells are not sex chromosome encoded, because LCLs generated from female and male donors were equally recognized. Clone C3 III recognizes all LCLs that are HLA-DR11^{+}. Although clone E10 III and C3 III both recognize an antigen presented on HLA-DR11, this pattern of recognition indicates that they recognize different antigens (Fig. 9). So far, it was not possible to determine the restriction element for clone D5-3 II.


clone E10 III
OD 450 nm
2,0
1,6
1,2
0,8
0,4
0,0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
LCL patient
LCL donor
T cells + media


clone C3 III
OD 450 nm
2,0
1,6
1,2
0,8
0,4
0,0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
LCL patient
LCL donor
T cells + media


clone D5-3 II
OD 450 nm
1,2
1,0
0,8
0,6
0,4
0,2
0,0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
LCL patient
LCL donor
T cells + media

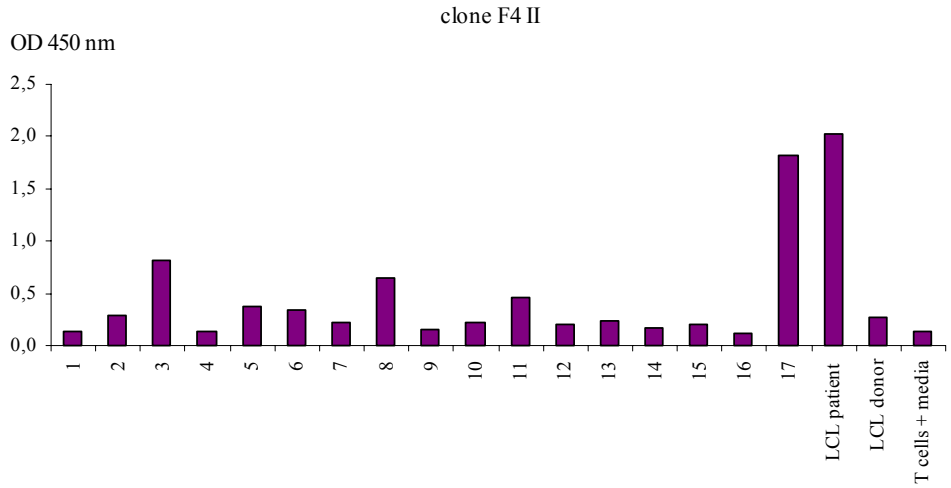


Fig. 9

mHA-specific T cell clones were tested in a GM-CSF release assays for recognition of LCLs generated from 17 members of one family. Based on the recognition patterns, four different specificities were detected. Clone E10 III recognizes LCLs 9, 10, 14, 15, 16 and 17. This recognition pattern shows that this clone is HLA-DR11 restricted. LCLs 1, 4 and 12 are also HLA-DR11^{+}, but they are not recognized probably because they do not express the mHA for which this clone is specific. Clone C3 III recognizes all DR11^{+} LCLs. Therefore, this T cell clone is specific for an antigen presented on HLA-DR11. From the recognition pattern of clone D5-3 II, it was not possible to determine the restriction molecule. However, additional typing showed that recognized LCLs express HLA-DR52 and that clone is HLA-DR52 restricted. The recognized LCLs express different HLA class II alleles. Clone F4 II recognizes an antigen presented on HLA-DQ06.

Blocking experiments indicated that the clone is HLA-DR-restricted, because recognition could be blocked with an antibody directed against this molecule (data not shown). Depending on the HLA-DRB1 allele expressed, additional HLA-DRB alleles may be expressed in some individuals. Therefore, additional HLA typing of HLA-DRB3, 4 and 5 alleles has been performed. LCL patient/donor as well as most LCLs from the family members expresses DR52. Therefore, additional experiments showed that this clone is DR52 restricted. The common restriction element expressed by all LCLs recognized by clone F4 II is HLA-DQ06 (Fig. 9). Together with the antibody blocking results, these data show that this clone is HLA DQ06 restricted.

3.2.4. Determination of T helper cell subtype and cell surface phenotype

In order to determine the T helper cell (Th) subtype of the mHA-specific T cell clones, cytokine secretion assays were performed. Th cells can be subdivided into Th-1 and Th-2 cells, depending on the pattern of cytokines they secret upon stimulation. IFN-γ and IL-4 are regarded as typical cytokines for Th-1 and Th-2 cells, respectively. Therefore, secretion of these cytokines upon antigen stimulation was determined for all T cell clones. As shown for T cell clone E10 III, all generated clones secreted IFN-γ, but not IL-4, and therefore belong to Th-1 type of T helper cells (Fig.10).

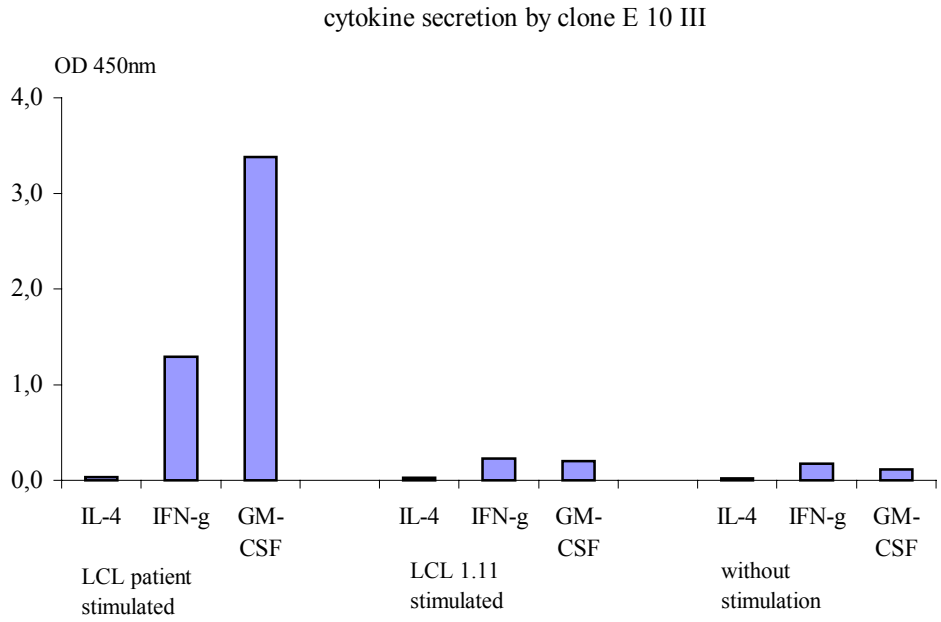


Fig. 10

Determination of Th subtype. IL-4 and IFN-γ release were measured after stimulation of clone E10 III with LCL patient, irrelevant LCL 1.11 and without stimulation. Secretion of IFN-γ but not IL-4 showed that this clone is of Th-1 phenotype.

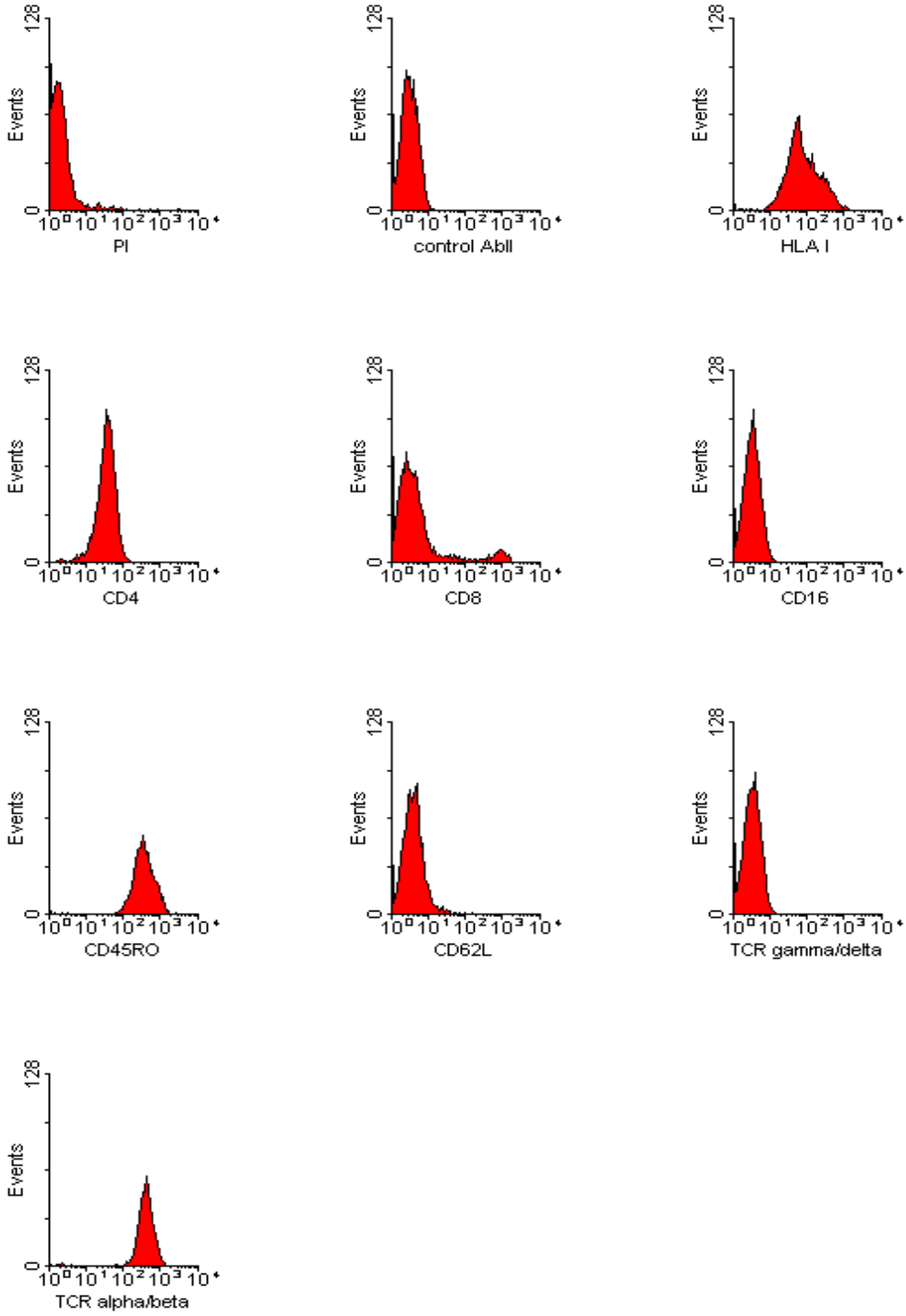


Fig. 11

Phenotypic analysis of the mHA-specific clone E10 III. Propidium iodide (PI) staining was used to exclude dead cells, and background fluorescence was determined by staining with secondary antibody only (AbII). Cells were tested for expression of HLA class I, CD4, CD8, CD16, CD45RO, CD62L, TCR α/β and TCR γ/δ. The phenotype of clone E10 III is typical for antigen experienced $CD4^+$ α/β T cells.

In order to further characterize the mHA-specific $CD4^+$ T cell clones, immunophenotype analysis was performed using fluorescence-activating cell sorting (FACS). Cells were stained with antibodies directed against CD4, CD8, natural killer (NK) cell marker CD16, memory cell marker CD45RO, CD62L (L-selectin) receptor involved in lymphocyte homing after activation, T cell receptor (TCR) α/β and γ/δ (Fig 11). As exemplified by clone E10 III, all mHA-specific T cell clones are $CD4^+$ and $CD8^-$. The small peak that appears in anti-CD8 staining probably represents $CD8^+$ cells from feeder cells that have been used for restimulation. The cells are negative for NK cell marker CD16, positive for memory marker CD45RO, and negative for CD62L. All T cells are TCRα/β positive and TCRγ/δ negative.

3.3. Humoral immune response

3.3.1. Expression library construction and antigen identification

In order to identify the antigens against which the humoral immune responses after BMT are directed, the SEREX methodology had to be adapted. With the SEREX method antigens can be identified, against which high titer antibodies are present in the serum of the patient (Fig. 12). A cDNA expression library was constructed from the same patient with β-thalassemia mentioned above who received allogeneic BM from an HLA-identical sister. The SEREX method is based on the recognition of proteins from an expression library by high titer IgG antibodies present in the serum of the patient. Bound antibodies are detected with an alkaline phosphatase conjugated goat anti-human IgG secondary antibody. One of the difficulties in antigen identification is the contamination with IgG genes derived from B cells in the target tissue used for library construction. Secondary antibodies directly recognize IgG proteins expressed in E. coli. To avoid this problem and to identify antigens specifically expressed in hematopoietic cells, PBL from the patient taken before BMT were stimulated with phytohemagglutinin (PHA), which non-specifically activates T cells and induces their proliferation. After several rounds of stimulation, a pure population of T cells was obtained and the mRNA isolated. This mRNA was reverse-transcribed into cDNA, cloned into the λ ZapII expression vector, and packaged into λ-phage particles. These were used to infect E.coli and recombinant protein production was induced with IPTG.

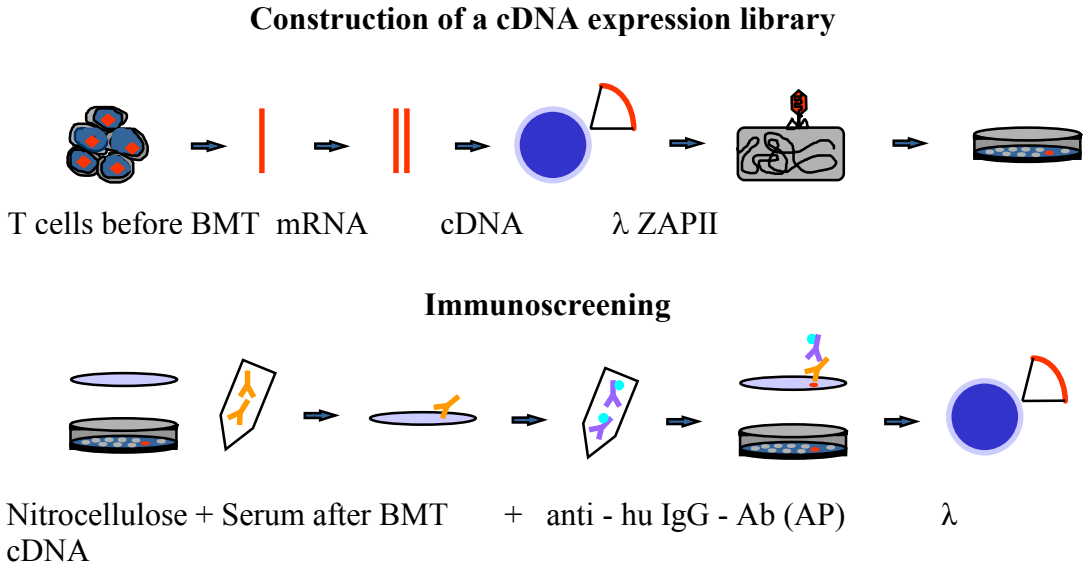


Fig. 12

Antigen identification by the SEREX method. mRNA is extracted from the tissue under investigation, here from T cells, reverse-transcribed into cDNA and ligated into a λ-phage expression vector. The recombinant vector is then packaged, and the recombinant lambda phages used to lytically infect E. coli. The recombinant protein expression is induced by IPTG, and proteins are blotted onto nitrocellulose membranes, which are then incubated with serum from the patient. Serum antibodies bound to recombinant proteins in lytic plaques can be detected with an enzyme-conjugated anti-human IgG secondary antibody, and the positive recombinant λ-phage can be isolated from the respective agar plate. After in vivo excision of recombinant Bluescript vector the cDNA encoding the antigen can be sequenced.

A total number of 1,2 x 10^6 recombinant clones were screened with serum obtained three months post BMT. The serum was preadsorbed against λ phages proteins to reduce background. In the first screening, 262 positive clones were isolated. Due to the high number of positive clones some of them were randomly chosen, excised in vivo and propagated in the E.coli strain SOLR. Surprisingly, sequencing of this randomly chosen clones showed that most of the cDNAs encoded IgG heavy chains. It is not clear at the moment, whether these Ig mRNAs stem from surviving B cells or were the result of basal Ig heavy chain transcription in activated T cells. However, in order to test all primarily positive clones for direct binding of secondary antibody they were incubated with buffer instead of serum. Using this procedure, false positive clones can be excluded. Twenty-one positive clones, recognized by the serum of the patient after BMT, were included in further analysis. After in vivo excision and amplification of the resulting phagemide in the

Clone	Accession number	GeneBank entry
2-1	XM_028710	Hematopoietic protein 1 (HEM-1), mRNA
2-2	NM_000993 NM_005617	Ribosomal protein L31 (RPL31), mRNA Ribosomal protein S14 (RPS14), mRNA
8	NM_001271	Chromodomain helicase DNA binding protein 2 (CHD2), mRNA
10	AK024488 XM_029084 BC030281	FLJ00087, mRNA FLJ21438, mRNA FLJ21438, mRNA, splice variant
20-1	AF346991	Mitochondrion, complete genome
21-1	AK026923 AK074088	FLJ23270, cDNA FLJ00159, mRNA
22-1	AK056934	FLJ32372, cDNA
22-2	NM_002985	Small inducible cytokine A5 (RANTES), mRNA
22-3	AY007165	CDABP0163 (ICAP1), mRNA
31	NM_016208	Vacuolar protein sorting 28 (VPS28), mRNA
42-2	M62378	T cell receptor beta chain V beta region, mRNA
64	NM_001015	Ribosomal protein S11 (RPS11), mRNA
65-2	BC001773	Similar to ribosomal protein L34, mRNA
69	XM_031425	Similar to KIAA1523, mRNA
116	AK027092	FLJ23439, cDNA
120-1	AL365277	Clone RP11-69E11, genomic DNA
120-2	U13369	Ribosomal DNA
133-2		
209	X98411	Myosin-IF, partial mRNA
216	AC006323	RP5-1151M5, genomic DNA
233	XM_004398	Similar to eukaryotic translation elongation factor 1 alpha (EEF1A1), mRNA

Table 1

cDNA clones identified by screening the λ-phage expression library. The expression library was derived from T cells taken from the patient before BMT, and screened with serum from the patient taken three months after BMT. Twenty different

antigens were identified. The number of the isolated clones, the GenBank accession numbers and the respective human GenBank entries are given in the table.

SOLR E.coli strain, plasmids were extracted and all cDNA inserts characterized by restriction enzyme digestion and DNA sequence analysis. The size of the cDNAs varied from 150 base pairs to 1500 base pairs. DNA sequences were determined and aligned to sequences in the GenBank using BLASTN algorithm. The results from these alignments are summarized in the Table 1.

3.3.2. Sequence analysis of the clones involved in the humoral immune response

mHAs represent products of polymorphic genes which differ between patient and donor. In order to identify polymorphisms in the antigens, the respective ORFs were amplified by PCR.

Non-polymorphic genes	
Clone	**GenBank entry**
2-1	Hematopoietic protein 1 (HEM-1), mRNA
2-2	Ribosomal protein L31 (RPL31), mRNA Ribosomal protein S14 (RPS14), mRNA
20-1	Mitochondrion, complete genome
22-1	cDNA FLJ32372 , cDNA
22-2	cDNA FLJ32372 , cDNA
22-3	CDABP0163 (ICAP1), mRNA
31	Vacuolar protein sorting 28 (VPS28), mRNA
64	Ribosomal protein S11 (RPS11), mRNA
65-2	Similar to ribosomal protein L34, mRNA
69	Similar to KIAA1523 protein , mRNA
120-1	RP11-69E11, genomic DNA
120-2	Ribosomal DNA
133-2	
209	Myosin-IF, partial mRNA
233	Similar to eukaryotic translation elongation factor 1 alpha (EEF1A1), mRNA

Polymorphic genes in the population but not in the donor/recipient pair					
Clone	**ORF**	**GenBank entry**	**Pos.**	**Polymorphism**	
				Data base	**Donor/patient**
8	1	Chromodomain helicase DNA binding protein 2 (CHD2), mRNA	4173	TTA – L (Leu)	ATA – I (Ile)
116	1	FLJ23439, cDNA	673	GTC – V (Val)	GCC – A (Ala)

Polymorphic genes					
Clone	**ORF**	**GenBank entry**	**Pos.**	**Polymorphism**	
				Donor	**Patient**
21	1	FLJ23270, mRNA FLJ00159, mRNA	636	GTG – V (Val)	GTG – V (Val) CTG – L (Leu)
10	2	FLJ00087, mRNA FLJ21438, mRNA, and splice variant	3001	CGT – R (Arg)	CGT- R (Arg) TGT- C (Cys)
		FLJ00087, mRNA	792	CAC – H (His)	GAC – D (Asp)

Table 2

Three groups of antigens identified by serological screening. The SEREX-defined antigens were divided into three groups, according to sequence homologies with GenBank entries and donor/patient PCR products. The first group represents non-polymorphic antigens. In the second group contains antigens that are identical between patient and donor, but are polymorphic compared to sequences in the GenBank. The third group contains antigens that are polymorphic between patient and donor. Patient and donor-specific sequences were amplified from ORFs predicted in the indicated genes by PCR. In FLJ00087 mRNA two ORFs 10-1 and 10-2 were predicted.

Therefore, total RNA was isolated from PHA-stimulated T cells obtained before and after BMT, and subjected to RT-PCR. cDNAs were sequenced, aligned to sequences in GenBank and compared to one another. Based on these results, the identified antigens could be divided into three groups. The first group represents non-polymorphic genes. These genes are identical between patient and donor and GanBank entries. Group two consist of genes which are identical between patient and donor, but are polymorphic in

comparison to sequences in GenBank. In the third group are genes that differ between patient and donor (Table 2). This group contains candidate mHAs.

The sequences of the genes in the first and largest group are identical between patient and donor and also with sequences in the GenBank. Antigen 2-1, for example, is identical to the hematopoietic protein 1 (HEM-1). This gene is expressed in hematopoietic cells only (Hromas et al., 1991). The ORF is 1842 bp long and, as reported in the GenBank entry, contains a polymorphism ("A"/"C") at position 3366 (XM_028710). However, patient and donor both are homozygous for allele "C" at this position. The protein is 211 amino acids long and its function is not known. Antigen 22-3 has high similarity with integrin cytoplasmic domain-associated protein 1 (ICAP1) encoded by the gene CDABP0163. Two isoforms of ICAP1, a 200-amino acid protein (ICAP1-alpha), and a shorter 150-amino acid protein (ICAP1-beta), derived from alternatively spliced mRNA, are expressed in most cells. ICAP-1 is a phosphoprotein, binds beta-1 integrin, and plays an important role in integrin-dependent cell adhesion (Chang et al., 1997). The sequences of this gene in patient and donor are identical. Antigen 31 is a gene with high homology to yeast VPS 28 (vacuolar sorting protein). Human VPS 28 recognizes ubiquitin on ubiquitinated internalized EGF receptor and is involved in receptor sorting for endosomal/lysosomal degradation (Bishop et al., 2002). Antigen 69 is expressed in at least three different splicing variants. All three are similar with protein KIAA1523. This protein is ubiquitously expressed, represents a PHD zinc finger transcription factor and contains a region homologous to the human autoimmune regulator (AIRE) (Pitkanen and Peterson, 2003). The gene is not polymorphic between patient and donor. Antigen 209 is homologous to myosin-IF, which belongs to the family of unconventional myosins. Antigen 233 is homologous to eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) which is widely expressed. Autoantibodies against EEF1A1 have been described in an autoimmune disease known as Felty's syndrome (Ditzel et al., 2000). This factor is polymorphic in the population, but patient and donor are identical. Several of the identified antigens code for ribosomal proteins. Ribosomal proteins are polymorphic in the population but donor and patient express identical alleles.

In the second group are two antigens that have one amino acid difference in comparison with the genes in GenBank. The cDNA derived from clone 8 is homologous to chromodomain helicase DNA binding protein 2 (CHD2). Four novel polymorphisms have been detected within the 5220 bp long ORF, but patient and donor are identical at these positions. Another novel polymorphism has been detected at position 4271

(NM_001271), but it represents a conservative exchange. The patient is homozygous, contains allele “T” whereas the donor is heterozygous and contains alleles “T” and “C”. An additional, novel polymorphism was detected at position 4173 (NM_001271) located within the ORF. Patient and donor are identical at this position, boths carrying allele “A”, while in the GenBank a sequence expressed from allele “T” is reported. This exchange leads to substitution of leucine to isoleucine on the protein level. Clone 116 codes for an unknown protein with homology to the hypothetical proteins FLJ23439. A polymorphism has been reported at position 987 (AK027092), but patient and donor express identical alleles. A novel, nonsynonymous exchange was detected at position 673 (AK027092). While patient and donor are identical at this position, expressing allele “C”, the Gene Bank entry derived from allele “T”. This exchange results in a valine to alanine amino acid substitution.

3.3.3. Identification of polymorphic genes between patient and donor

The third and most important group contains antigens in which patient and donor differ. When interpreting the results of sequence analyses of donor and recipient RT-PCR products, one has to take into account that all genes located on autosomes are present in two copies, and, thus, the isolated mRNAs are a mixture of the transcription products from both alleles. Accordingly, the cDNA sequences obtained from donor and patient tissue after RT-PCR amplification of the ORFs, represent a mixture of both alleles, as well. cDNA sequence analyses reveal homogenous sequences when both alleles are identical, but mixed sequences when polymorphic sites are present. Such mixed sequences are recognized as two overlapping adsorption peaks in the electropherogram, as depicted for clone 21-1 (Fig. 13). Clone 21-1 represents one of the two antigens belonging to the third group of antigens, characterized by polymorphisms between patient and donor. The insert of clone 21-1 was about 1 kb in length and homologous to mRNA encoding the hypothetical protein FLJ23270. A single ORF, 783 amino acids in length, was predicted in the homologous mRNA. Within this ORF, two nonsynonymous exchanges were found. A novel polymorphism is located at position 184 (AK026923). At this site FLJ23270 mRNA represents allele “C” which codes for alanine (GCG), while the patient was homozygous for allele “T”, encoding valine (GTG). The donor is heterozygous at this position, carrying alleles “T/C” (not shown). However, an immune response relevant to GvL cannot be generated against this polymorphism, because the patient is homozygous and the donor

heterozygous and, thus, T cells recognizing mHA expressed from the "T" allele are negatively selected in the donor's thymus.

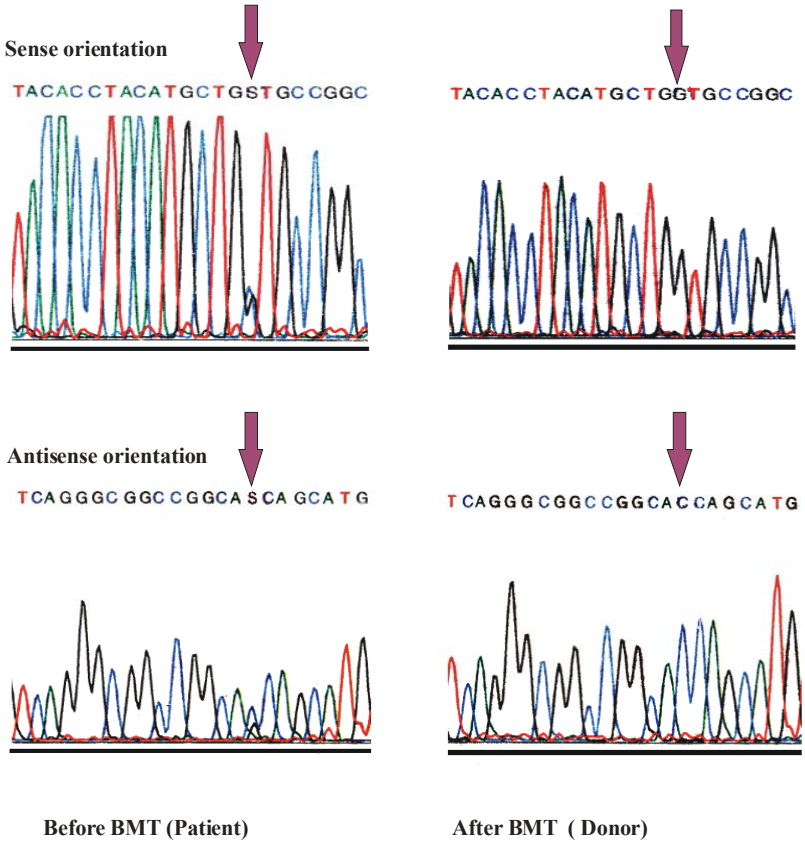


Fig. 13

Identification of a polymorphism in antigen 21-1 (pos. 636, AK0236923). Electropherograms in both sense and antisense orientation are shown. On the left hand side of the figure, the electropherogram obtained by sequencing the gene from the patient are shown in sense and antisense orientation. On the right hand side, the same part of the gene from the donor is shown in both orientations. The sequence obtained from the patient contains single letter code "S" (G or C) and, therefore, the patient is heterozygous and carries alleles "G" and "C". At the same position, the donor carries allele "G" and therefore, is homozygous. This polymorphism results in an amino acid substitution of valine by leucine.

A second novel nonsynonymous exchange, which was more important with respect to GvL, was found at position 636 (AK0236923) (Fig. 13). While FLJ23270 cDNA represents allele "G" which codes for valine (GTG) and the donor is homozygous, expressing the corresponding allele "G" (GTG), the patient is heterozygous, carrying the alleles "C/G" which encode leucine/valine (CTG/GTG). After BMT, GvH and GvL reactions due to recognition of patient type mHA derived from allele "C" may be elicited,

because this allele is not present in the donor, and donor T cells reactive against this allele are not deleted in the donor's thymus.

Gene FLJ23270		
cDNA	**Nonsynonymous exchange (pos. 184)**	**Nonsynonymous exchange (pos. 636)**
LCL #4	CCCCGGCAGG**Y**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL 1.20	CCCCGGCAGG**C**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL 1.23	CCCCGGCAGG**C**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL 1.24	CCCCGGCAGG**C**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL 1.26	CCCCGGCAGG**T**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL Kun	CCCCGGCAGG**Y**GCGGCAGTC	CTACATGCTG**G**TGCCGGCCGC
LCL #1		CTACATGCTG**G**TGCCGGCCGC
LCL 3.3		CTACATGCTG**G**TGCCGGCCGC
LCL GB		CTACATGCTG**G**TGCCGGCCGC
LCL JM		CTACATGCTG**G**TGCCGGCCGC
LCL #6		CTACATGCTG**S**TGCCGGCCGC
LCL #7		CTACATGCTG**S**TGCCGGCCGC
LCL UB		CTACATGCTG**G**TGCCGGCCGC

Table 3

Detection of nonsynonymous polymorphisms in FLJ23270 mRNA of unrelated individuals. The parts of the gene containing the polymorphisms are shown. The whole ORF of antigen 21-1 was sequenced in six individuals. In seven additional individuals, only the region encompassing the polymorphism at position 636 (AK026923) was sequenced. Both alleles of the polymorphic at pos. 184 are highly represented within the population. Two individuals contain single letter code "Y" (C or T) and therefore, are heterozygous; three are homozygous for allele "C" and one is homozygous for allele "T". Only two LCLs were heterozygous for the second polymorphism detected in this gene. This polymorphism is labeled with single letter code "S" (G or C). Allele "C" is present only in heterozygous state.

To show that the identified polymorphism is not limited to the patient/donor pair, expression of the different allelic variants of the gene in the population was examined. cDNAs from 13 unrelated donors were obtained and sequenced (Table 3). In six cases the whole gene was sequenced, while in seven cases only the part spanning the polymorphism at position 636 (AK026923) was analyzed. Both nonsynonymous exchanges have been

detected in the cDNAs when the whole ORF was sequenced, whereas no additional polymorphisms were detected. Therefore, this method is capable to detect single nucleotide differences. At the first polymorphic position (184) (AK026923), three of the cDNAs are homozygous and carry allele "C", one is homozygous and carries allele "T", and two are heterozygous and carry both alleles. Therefore, both alleles are present with high frequency in the population and no additional alleles appear to exist. At position 636, eleven cDNAs are homozygous, and carry allele "G" whereas two are heterozygous and carry both alleles. No homozygous carrier of allele "C" was found. For precise determination of the allele frequency within the population, a larger number of individuals has to be examined. The data obtained so far indicates that allele "C" is present at low frequency in the population.

N terminal position	transmembrane region	C terminal Position	length
193	WLGWYTYML V/L PAALTGLLVFLSG	215	23

Table 4

Predicted transmembrane region in antigen 21-1. The amino acid sequence of the predicted transmembrane domain is shown. The identified polymorphic amino acids are shown in colors. Valine (V) is present in the protein sequence of the donor, whereas the patient expresses two forms with either valine or leucine (L) at this position.

In sequences obtained from LCLs #1, 3.3, GB, JM, #6, #7 and UB, a TCTC deletion was observed at position 383 (AK026923) in approximately 50% of the RT-PCR products. This deletion introduces a frameshift and a new premature stop codon, that probably represents a loss of function mutation. Because this deletion was not detected in the patient/donor pair, both alleles differing at position 636 (AK026923) in the ORF are expressed in frame, and therefore can induce immune responses after BMT. The SUSOI (http://sosui.proteome.bio.tuat.ac.j/about-sosui.html) program has been used for secondary structure prediction of the protein. This computational analysis predicted a transmembrane protein with seven transmembrane domains. InterPro (http://www.ebi.ac.uk /interpro/scan.html) database of protein domains and functional sites, which allows the

prediction of protein functions, did not give any results. The polymorphism detected between patient and donor resides within the first transmembrane domain (Table 4). Recently, a new gene has been deposited into GenBank with homology to antigen 21. This new gene codes for protein FLJ00159 (AK074088), and was cloned from human spleen (Fig. 14). The ORF is 5484 bp long and codes for a protein of 567 amino acids. This protein shares 102 amino acid with FLJ23270, and is predicted to contain three transmembrane domains. The first two transmembrane domains are identical in both proteins and encompass the nonsynonymous exchange detected in the patient/donor pair. The function of the protein is not known.

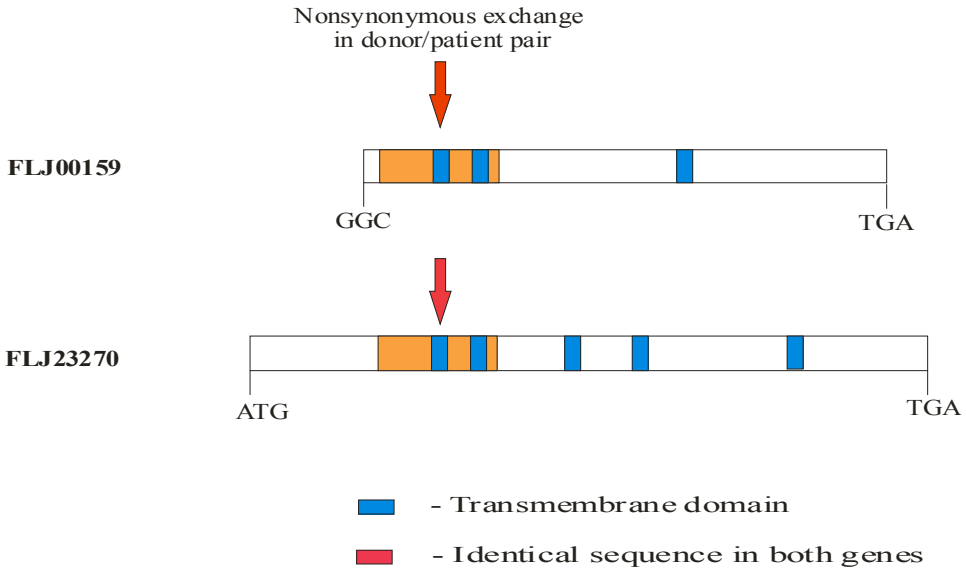


Fig. 14

Nonsynonymous polymorphism between patient and donor in antigen 21-1. The polymorphism detected in antigen 21-1 is present in two different hypothetical proteins, designated FLJ00159 and FLJ23270 in the GenBank database (pos. 636, AK026923). The two different corresponding ORFs share the sequence shown in red, where the nonsynonymous exchange, relevant to GvL responses, was detected in the donor/recipient pair. Another nonsynonymous exchange at pos. 184 (AK026923) is not shown. Predicted transmembrane domains are depicted in both genes. The first gene codes for a protein containing three transmembrane domains, whereas the second gene codes for a protein containing six transmembrane domains.

The second antigen of this group is encoded by clone 10 and designated 10-1. The 1.1kb cDNA insert is homologous to three mRNA GenBank entries (Acc.No. AK024488, XM_029084, and BC030281) form which three hypothetical proteins had been predicted (FLJ00087, FLJ21438 and alternatively spliced FLJ21438) (Table 2). As shown in Fig. 15, clone 10 contains one of two ATG-defined ORFs predicted in FLJ00087 cDNA.

Whitin this ORF (10-1), a novel nonsynonymous exchange was detected by sequencing of patient (C/T) and donor (C) PCR products (pos.3001, AK024488), resulting in a corresponding exchange of arginine to cysteine (Table 2). All homologous GenBank entries (AK024488, XM_029084, and BC030281) demonstrate C at this position, corresponding to arginine in the protein sequence (Table 2). Thus, T cells, recognizing the mHA expressed from the patient allele "T", are not negatively selected in the donor' thymus and may cause GvH and/or GvL reactions in the BMT patient. In order to determine the allele frequency within the population, the ORF 10-1 was sequenced from ten unrelated individuals. Nine out of ten are homozygous and carry allele "C" whereas one is heterozygous and carries both alleles "C/T", indicating that the BMT patient is heterozygous for an allele which is found at low frequency within the population. Automated computational analysis demonstrated that antigen 10-1 is possibly soluble (SOSUI prediction method) and might represent a downregulator of ras-like proteins (GTP-ase acticator, RasGAP) (InterPro-based proteome analysis). RT-PCR analysis of the expression profile of antigen in the NEDO database indicates that the antigen might preferentially be expressed in the spleen (http://www.kazusa.or.jp/NEDO/gfpage/FLJ00087/).

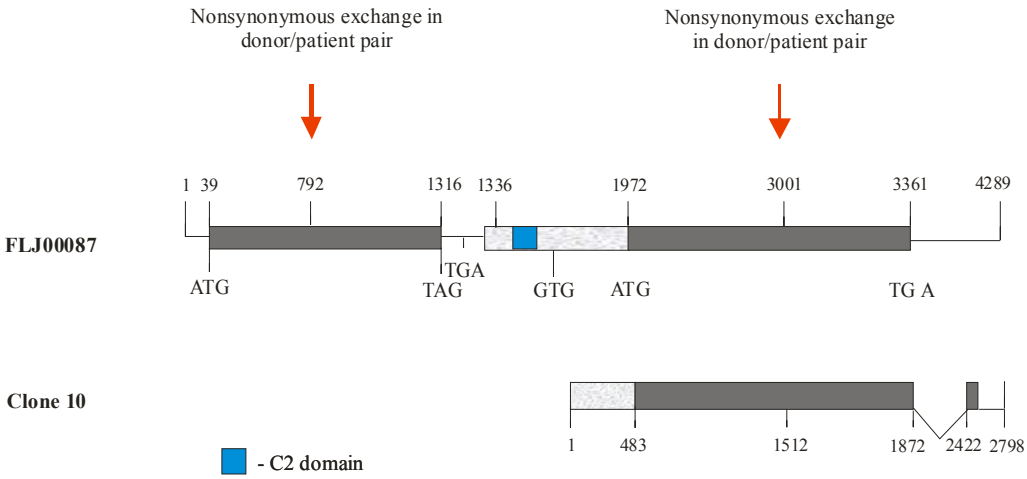


Fig. 15

Nonsynonymous exchanges between patient and donor in antigen 10-1 and 10-2. The cDNA insert of clone 10 was homologous to three mRNA GenBank entries (Acc.No. AK024488, XM_029084, and BC030281) from which three hypothetical proteins had been predicted (FLJ00087, FLJ21438 and alternatively spliced FLJ21438, respectively). The largest of all homologous mRNAs, FLJ00087, is depicted. This mRNA was predicted to contain two ATG-defined ORFs, designated here antigen ORF 10-1 and ORF 10-2, while the shorter, FLJ21438-related cDNAs contain ORF 10-1, only. When amplification of ORF-specific sequences from patient and donor tissue was performed using PCR, nonsynonymous polymorphisms were detected in both, ORF 10-1 (pos. 792, AK024488) and ORF 10-2 (pos. 3001, AK024488 and XM_029084, pos. 306, BC030281). ORF 10-1 (pos. 39-1316) and ORF 10-2 (pos. 1927-3363) are likely to be efficiently translated, since the indicated ATG start codons are integrated in ideal (10-1) or nearly ideal (10-2) Koszak's sequence (accATGg and gccATGg, respectively). The predicted ATG start codon of ORF-1, however, was not flanked by an upstream stop codon in the region up to pos. 1336 and, thus, an alternative upstream translation start site may be discussed, such as GTG at pos. 1459 or potential start sites located in upstream sequences spliced to ORF-1. Alternative translation from an upstream start codon would result in an extended N terminus of antigen 10-1 as indicated by dotted boxes. The potential alternative N terminus might contain a C2 domain (blue) involved e.g. in calcium-lipid binding as indicated by InterPro-based proteome analysis. Evidence for differential mRNA splicing of antigen-10-1, was provided by deleted 3'sequences in clone 10-1 compared to FLJ00087 as well as to both FLJ21438 mRNA variants. Accordingly, a very short (4aa) alternative COOH terminus was predicted. All positions indicated refer to Acc.No. AK024488 (FLJ00087) if not stated otherwise.

A second, upstream ORF (10-2) was predicted in FLJ00087 mRNA and might thus encode a second antigen, designated here antigen 10-2. Sequence comparison between patient and donor identified a nonsynonymous exchange at position 792 (AK024488), resulting in an exchange of histidine to asparagine. Patient and donor are homozygous for different alles, with allele "G" in the patient and allele "C" in the donor. Automated computational analyses indicated that the antigen 10-2 might be soluble as well (SOSUI prediction method) and revealed two proline rich regions (InterPro-based proteome analsis) between which the nonsynonymous exchange is located (Fig.16). ORF 10-2 was sequenced from eleven unrelated individuals who all turned out to be homozygous for "C", like the BM donor.

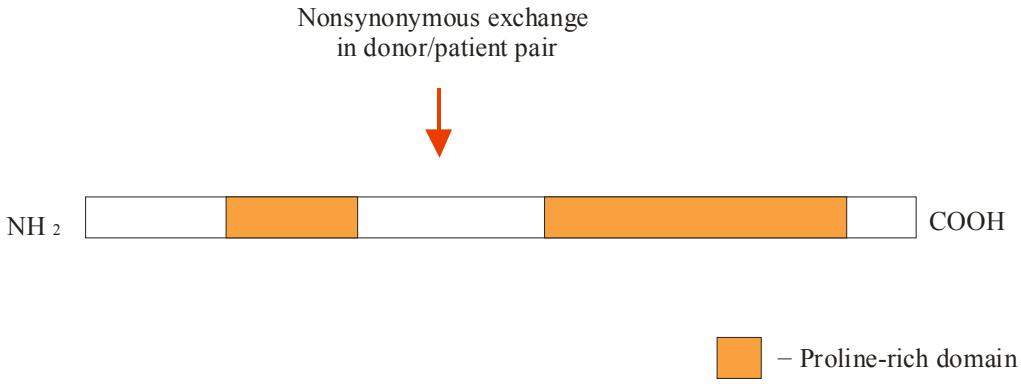


Fig. 16

Nonsynonymous polymorphism between patient and donor in antigen 10-2. A single nonsynonymous exchange was detected and located between two proline reach regions.

3.3.4. MHC class II epitope prediction

The mHA-specific T cell clones recognize four different antigens presented on three different HLA molecules. The binding cleft of MHC class II molecules contain nine so called pockets (minute indentations at the bottom of the cleft), each of which holds side chains of the amino acids in the peptide. Different side chains may have different effects on the binding affinity of the peptide; some may have positive (i.e. increase binding affinity), or negative (i.e. decrease binding affinity) while others may be neutral. Although the important binding positions are not well defined and appear to be different for different alleles, positions 3, 6 and 9 seem to be important for some MHC alleles. The minimal peptide length required for binding to different MHC class II molecules can vary from 9 to 15 amino acids. One of the best-characterized HLA-DR alleles for peptide binding is DR11, which is also the restriction element of two of the T cell clones. The complete protein sequences of all three antigens were submitted for epitope prediction. In all three proteins, the identified polymorphisms are located inside epitopes predicted to bind to HLA-DR11 molecules (Fig 17). Moreover, the polymorphisms are located inside of so-called “promiscuous binder” epitopes. These epitopes represent antigens or regions of an antigen that can bind to several HLA alleles. These regions are most suitable for vaccine development, because the immune response can be generated against a single epitope in a large population expressing different HLA molecules.

Antigen 10-1

```
                                                                  ▼
DRB1_1101:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1102:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1104:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1106:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1107:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1114:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1120:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1121:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
DRB1_1128:TPLISKSQSLRSVRRSESWARPRPDEERPLRRPRPVQRTQSVPVRRPARRRQS
```

Antigen 10-2

```
                                                ▼
DRB1_1101:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1102:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1104:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1106:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1107:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1114:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1120:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1121:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
DRB1_1128:RDGPPSALGSRESLATLSELDLGAERDVRIWPLHPSLLGEPHCFQVTWTGGS
```

Antigen 21-1

```
                ▼
DRB1_1101:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1102:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1104:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1106:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1107:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1114:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1120:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1121:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
DRB1_1128:WLGWYTYMLVPAALTGLLVFLSGFSLFEASQISKEICEAHDILMCPLGDHSR
```

Fig. 17

MHC class II binding peptide (http://www.imtech.res.in/raghava/propred/) prediction of the antigens 10-1, 10-2 and 21-1. The amino acid sequences have been submitted and the predicted peptides that bind to the HLA-DR 11 are shown in color. Red colored letters represent predicted amino acids that bind at position one in the binding groove of the HLA molecule (P1 binding amino acid). Binding prediction for all three antigens was done for nine different suballeles of HLA-DR11. Identical binding predictions were obtained when the alleles of the donor were submitted. Arrowheads indicate the position of the polymorphic amino acids.

3.3.5. Recognition assay

In order to directly test whether the polymorphic antigens 10-1, 10-2, and 21-1 identified by the SEREX method, are recognized by the $CD4^+$ mHA-specific T cell clones, peptides spanning the polymorphic region were synthesized. Because the restriction molecules presenting the polymorphic peptides are not known, 15-mer peptides were synthesized with the polymorphic amino acid in the middle of the peptide. Peptides with this length should contain important binding amino acids for most of the HLA alleles. Six peptides have been synthesized (Fig. 18), covering the polymorphic region present in the patient and the donor.

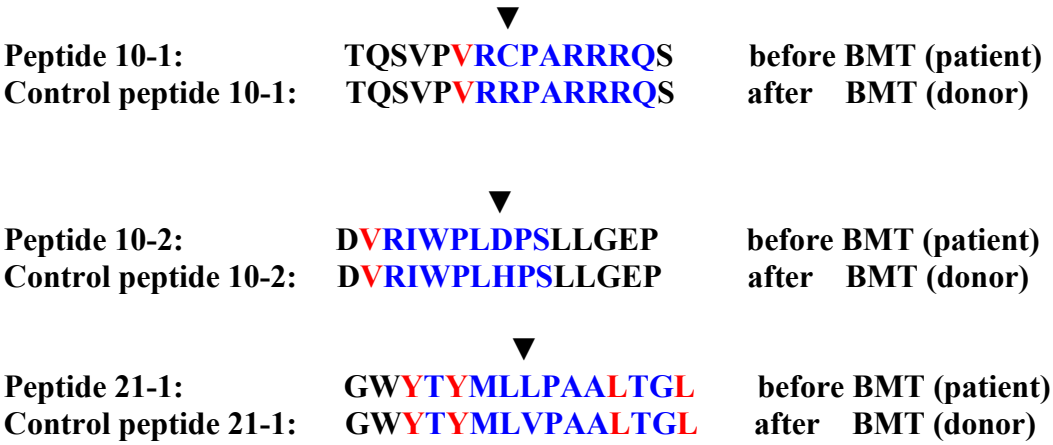


Fig. 18

15-mer peptides spanning the polymorphisms in the patient and the corresponding control peptides with the sequence from the donor were synthesized. Predicted binding motifs for DR11 are shown in blue. Amino acids shown in red are predicted P1 (amino acid that binds at position one in the binding groove of HLA molecule) binding amino acids. In the case of antigen 21, more predicted epitopes exists in the polymorphic region. Arrowheads indicate the polymorphic positions.

Specific recognition assays were performed with the mHA-specific clones as effector cells and LCL donor, incubated with the peptides as target cells. None of the T cell clones recognized LCL donor incubated with any of these peptides (Fig. 19).

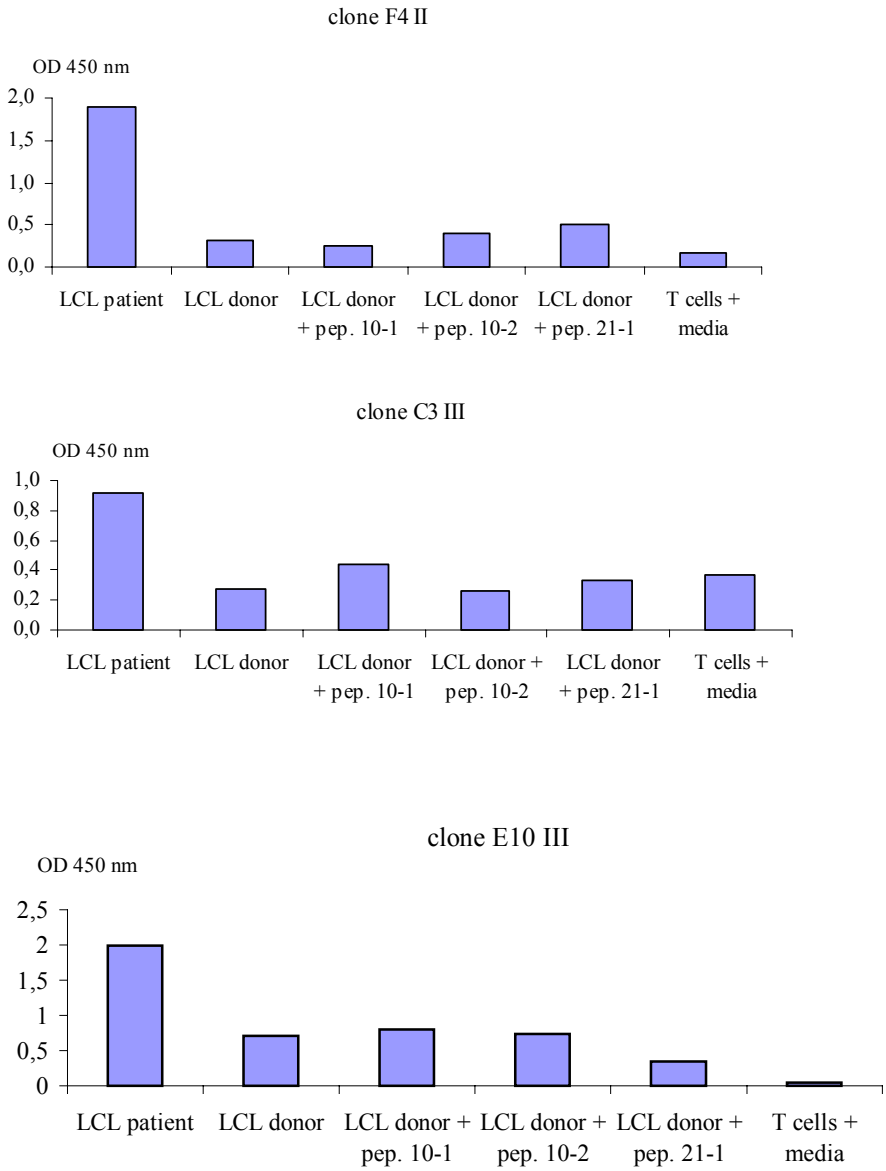


Fig. 19

Peptide recognition assay. LCL patient incubated with peptides were used as a target cells for the mHA-specific T cell clones. Secretion of GM-CSF was determined by ELISA.

The T cell clones isolated after BMT failed to recognize the polymorphic peptides. One possible explanation for this non-recognition could be that not all antigens were detected with the SEREX method, and that T cells specific for only a limited set of different

antigens have been generated. Another explanation could be that the synthetic peptides are not long enough and lack amino acids that are important for binding to MHC class II

molecules or T cell recognition. An alternative explanation has recently been described for the mHA HA-8 (Brickner et al., 2001). The two allelic variants of HA-8, which differ in one amino acid, are differentially processed by the proteasome and the resulting peptides are transported with different efficiency by TAP into the endoplasmatic reticulum (ER). This leads to an inefficient presentation of one allelic variant on MHC class I. These results could offer an explanation why the peptides were not recognized by the mHA-specific T cell clones. The polymorphisms may affect the processing of the proteins and thereby lead to the generation of different sets of peptides, but may not be directly recognized by the T cells. In order to test these possibilities, the specific recognition pattern of the mHA-specific T cell clones was compared with the allele transmission of the polymorphic antigens through the family (Fig. 8). Therefore, the ORFs of antigens 10, 10-2 and 21 from twelve LCLs were amplified by RT-PCR and the polymorphic parts of all three genes sequenced. LCLs 1, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 17 are homozygous for the identified polymorphisms in antigens 10, 10-2 and 21 and carry the same the alleles as the donor. Thus, the patterns of recognition and allele expression were not in accordance, indicating that the T cell clones probably recognize different antigens.

3.3.6. Frequency of mHA-specific T cells

The identification of a polymorphism between patient and donor does not necessarily imply an immunological importance of this antigen. Evidence for such immunological importance would be an increase in the frequency of T cells specific for this antigen after BMT in comparison to before BMT. In order to determine the frequency of T cells specific for these antigens after BMT, IFN-γ ELISPOT assays were performed. Due to limitations in the amount of PBL available from the patient, T cell lines stimulated 3, 4 and 17 times with LCL patient were used. The T cell lines were tested against LCL patient and LCL donor. As expected, T cells restimulated 3 or 4 times contained a higher number of T cells specific for antigens presented on LCL patient than on LCL donor. These differences are lost after more passages because the T cells were restimulated with LCL that also very efficiently present EBV antigen. Consequently, EBV-specific or autoantigen-specific T cells dominate the T cell line after seventeen restimulations (Fig 20).

These data show that the T cell lines generated from PBL after BMT contain T cells specific for mHA, and T cells specific for EBV antigens.

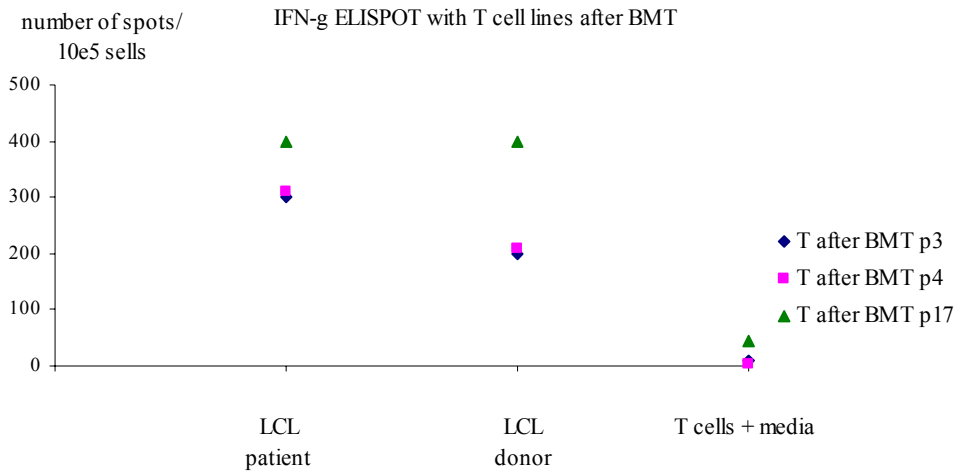


Fig 20

IFN-γ ELISPOT assay with a representative T cells line (I) at passages p3, p4 and p17. The T cell line at p3 and p4 contains a higher frequency of T cells specific for antigens presented on LCL patient in comparison with LCL donor. These differences are lost after seventeen restimulations when EBV-specific T cells dominate the T cell line.

In order to determine the frequency of T cells specific for the polymorphic peptides in these lines, ELISPOT assays were performed. To reduce the EBV background, PHA blasts obtained from the patient PBL after BMT were used as APCs instead of LCL. A transient increase in the frequency of T cells specific for all three polymorphic peptides was seen. This increase was not seen when a non-polymorphic control peptide were used in the same assay. As expected, this increase was lost after repeated T cell stimulations due to the domination of EBV-specific or autoantigen-specific T cells (Fig. 21). These experiments were repeted three times and indicated that T cells specific for the SEREX defined antigens are present after BMT, but they decrease in frequency after repeated stimulation in vitro.

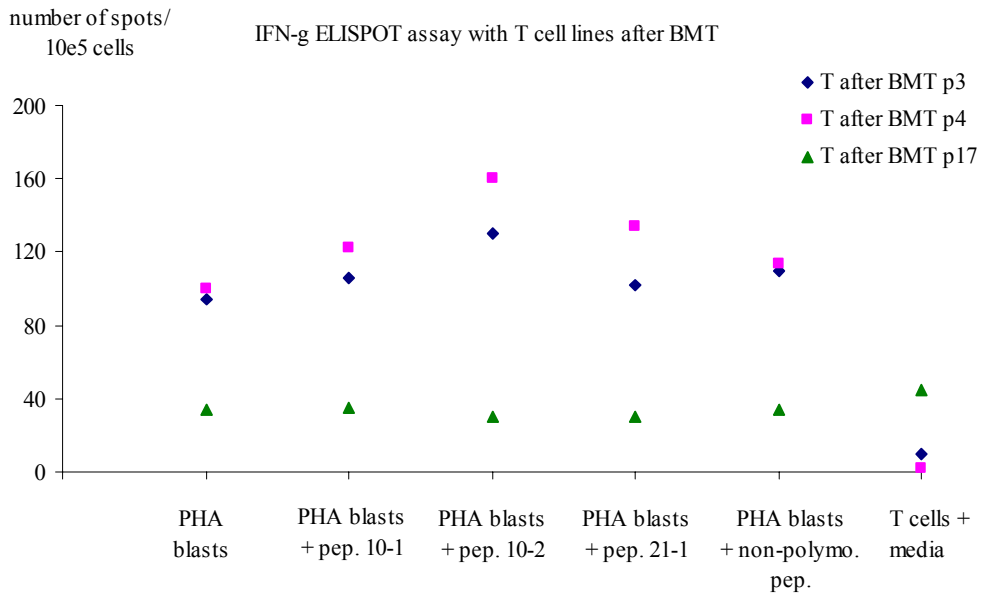


Fig. 21

IFN-γ ELISPOT assay performed with a representative T cell line (I) generated by stimulating PBL obtained after BMT with LCL patient taken at the indicated passage (p3, p4, and p17) and exposed to PHA blasts with or without peptides. A small but reproducible increase in the frequency of T cells specific for the three identified antigens was seen when the line was 3 and 4 times stimulated with LCL patient but not when stimulated 17 times. This increase was not seen with a non-polymorphic peptide.

To enrich for these antigen-specific T cells, cytokine secretion and capture assays were performed. The T cell line p4 was stimulated with the three peptides for 16 hours. The cells were then labeled and separated using MACS MicroBeads. Positively selected cells were restimulated twice in order to obtain sufficient cell numbers and then tested in recognition assays. The same isolation procedure has been repeated four times independently. In all cases the isolated T cells recognized the peptides from the patient, but not the control peptides. Interestingly, neither LCL patient nor LCL donor were recognized (Fig. 21).

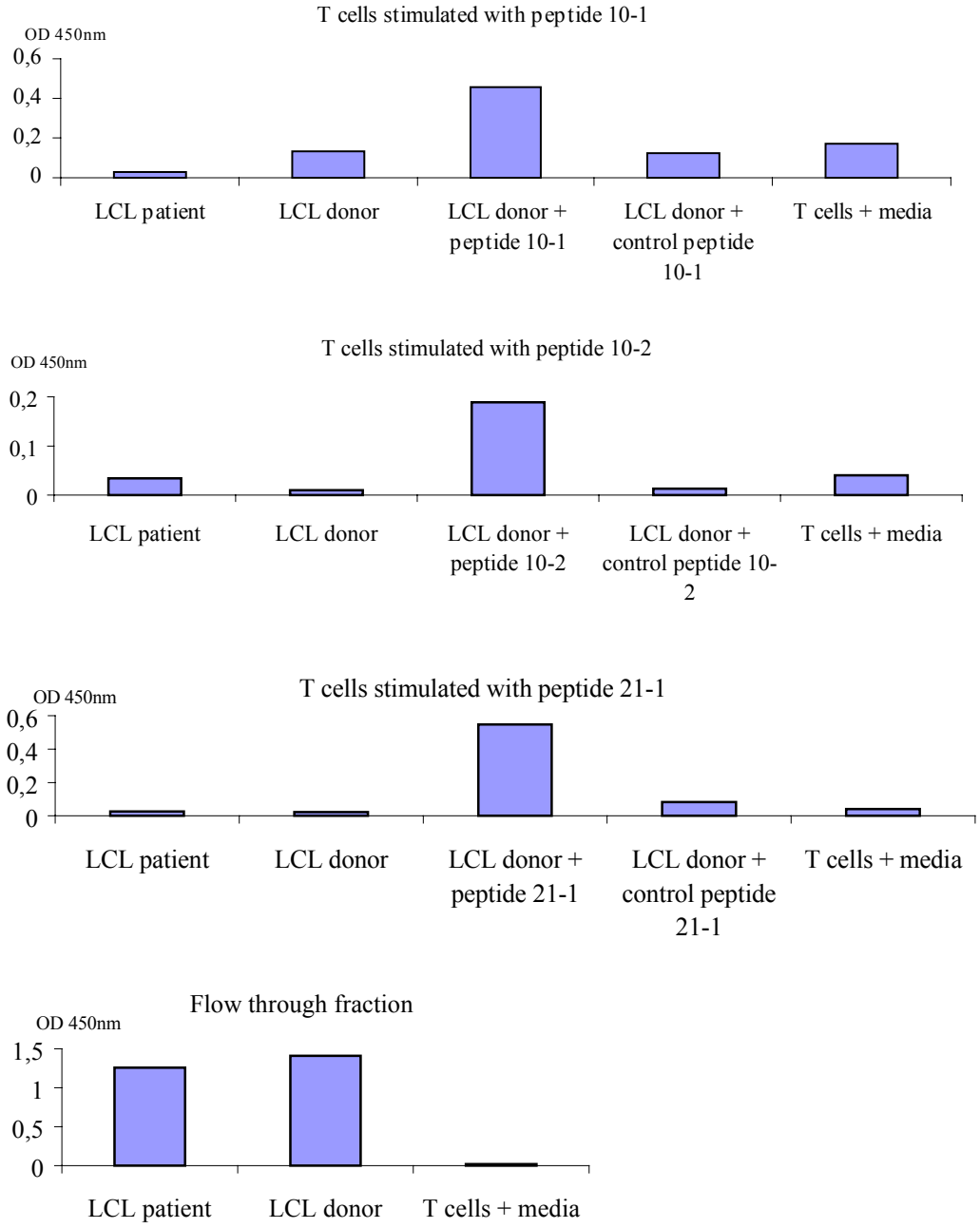


Fig. 22

T cells specific for polymorphic antigens identified by SEREX method were successfully isolated from T cell line I p4 of the patient after BMT using cytokine secretion and capture assay. The isolated T cells specifically recognize peptides containing polymorphic amino acid sequence expressed in the patient but not control peptide containing amino acid sequence expressed in the donor. Non-selected fraction was tested against LCL patient and LCL donor and represents the control.

These experiments clearly demonstrated that T cells specific for polymorphic antigens identified with the SEREX method exist in the blood of the patient after BMT. Further characterisation of these T cells is underway. However, the T cell clones generated by repeated stimulation with LCL patient recognize different antigens than those defined by SEREX. Taken together, the SEREX method does not facilitate the molecular identification of the antigens recognized by mHA-specific T cell clones, but leads to the identification of a different set of mHA recognized by T helper cells as shown by early passage Th cell lines.

4. Discussion

4.1. mHAs are recognize by T cells

mHA are the protein products of polymorphic genes, and peptides derived from mHA are presented on MHC class I and II for recognition by donor-derived mHA-specific $CD4^+$ and $CD8^+$ T cells. The development of techniques for cultivating T cells in vitro has allowed to isolate and expand mHA-specific T cells from the blood of patients after allogeneic BMT. These T cells have been used for the molecular identification of the genes that encode mHA. Current methods for the isolation of mHA-specific T cell usually rely on the stimulation of T cells taken from the patient after BMT with irradiated APC. For the generation of $CD8^+$ mHA-specific T cell clones, different APCs including leukemia cells, PBL, DC, and LCLs have been successfully used. With this approach, T cell clones specific for a large number of different mHAs have been generated, suggesting that an even larger number mHAs exists.

$CD8^+$ T cells and $CD4^+$ T cells play an important role in GvH and GvL responses after BMT (Jiang and Barrett J, 1997; Russell et al., 2001; Dodi et al., 2002; Zorn et al., 2002). However, evidences for a pivotal role of $CD4^+$ T cells in GvL induction without GvHD was obtained by the infusion of donor lymphocytes that had been depleted of $CD8^+$ T cells (Giralt et al., 1995; Alyea et al., 1998). In the large study of Alyea et al. (1998), 80% of the patients with relapsed hematological malignancies and subsequent $CD4^+$ DLI showed clinical responses. All patients in this trial who developed GvHD demonstrated tumor regression, but the incidence of GvHD in responding patients was lower than reported for patients with tumor remission after infractionated DLI (Collins et al., 1997).

Despite the importance of $CD4^+$ T cells in the immune response after allogeneic BMT, only few $CD4^+$ mHA-specific T cell clones have been isolated. Faber et al., generated $CD4^+$ mHA-specific T cell clones with specificity for two different antigens (Faber et al., 1995). The same group recently reported the isolation of additional six $CD4^+$ mHA-specific T cell clones (Eljaafari et al., 2001), demonstrating that mHA-specific $CD4^+$ T cells exist, and that their isolation in vitro is feasible. Because of their unsurpassed T cell stimulatory capability, ideal APCs for the stimulation and isolation of mHA-specific $CD4^+$ T cells are DC. DCs can be generated from precursor cells present in the blood of the patient and differentiated into DC in vitro.

4.2. Generation of $CD4^+$ mHA-specific T cells

In the present study it was not possible to collect enough material (PBL before BMT) for generating DC from the pediatric patient. Similar to DC, LCL express high levels of MHC class I and II, as well as costimulatory molecules. In contrast to DC, LCL can be generated from few milliliters of blood by EBV infection. LCLs represent immortalized B cells, which may proliferate infinitely in vitro, and thus provide enough cells of hematopoietic origin for all analyses performed in this work. In order to generate mHA-specific $CD4^+$ T cells, PBL obtained from the patient after BMT, therefore, were stimulated with LCL generated from PBL before BMT. A number of $CD4^+$ mHA-specific T cell clones were isolated using this protocol. LCL, however, not only present cellular antigens but also EBV antigens on MHC molecules. EBV-specific memory T cells are present in EBV-positive donors (Dolcetti et al., 1995; Hofmann et al., 2002), and EBV-specific T cells will expand when repeatedly stimulated with LCL. ELISPOT assays were performed to follow the frequency of mHA-specific T cells in T cell lines established from the patient 3,5 months after BMT. T cell lines stimulated 3-4 times with LCL patient showed a high frequency of mHA-specific T cells. However, seventeen times stimulated PBL contain predominantly T cells that are EBV- or autoantigen-specific. These experiments demonstrated that mHA-specific donor T cells could be stimulated using EBV-transformed patient B cells. However, reactivation of EBV-specific T cell responses may occur and even overgrow mHA-specific T cell responses. Therefore, PBL from the patient obtained after BMT were stimulated only few times with irradiated LCLs. From these mHA-specific T cell lines, a large number of $CD4^+$ mHA-specific T cell clones were isolated. These $CD4^+$ mHA-specific T cell clones recognize four different antigens. Clone E10 III recognizes an antigen that is presented on HLA-DR11. Three LCLs positive for HLA-DR11 were not recognized by this T cell clone, suggesting that these three LCLs do not express the mHA. Most of the generated T cell clones showed this pattern of recognition, indicating that the antigen recognized represents an immunodominant mHA. Clone C3 III is also HLA-DR11 restricted, but all DR11 positive LCLs were recognized. This pattern of recognition shows that the C3 III clone recognizes a different antigen than clone E10 III, and that all family members express the mHA. Clone D5-3 II is HLA-DR52 restricted, while clone F4 II is HLA-DQ6 restricted. Besides using different restriction elements, these T cell clones recognize a non-overlapping set of LCLs, and therefore recognize different antigens. FACS analysis and cytokine secretion assays showed that all

generated clones are of Th-1, and of memory phenotype. Two recent reports have suggested that Th-1 donor derived $CD4^+$ T cells are responsible for the development of GvHD, whereas Th-2 cells are important for GvL responses and the prevention of GvHD (Fowler et al., 2000; Zeis et al., 2001). It remains to be seen, whether the exclusive generation of Th-1 type T cell clones in this study is due to a higher frequency of Th-1 versus Th-2 cells, or whether the in vitro T cell culture conditions selectively favor Th-1 cell expansion.

Because generic methods for the identification of antigens presented on MHC class II are currently not available, only few antigens recognized by $CD4^+$ T cells have been identified molecularly. By testing candidate mHAs identified previously as targets of $CD8^+$ T cells, an epitope derived from DBY has recently been shown to be recognized by a mHA-specific $CD4^+$ T cell clone (Vogt et al., 2002). No other MHC class II-restricted mHA has been identified. Therefore, an indirect approach was taken to identify the antigens recognized by the $CD4^+$ T cell clones. In tumor patients, a close correlation between humoral and cellular immune responses has been observed (Jäger et al., 2000). In patients undergoing allogeneic BMT, little is known about humoral immune responses against tissue antigens. Increased numbers of circulating B cells and plasma cells have been observed in patients treated with DLI. A recent study assessed the targets of the humoral immune response in three patients with recurrent disease showing clinical response to DLI treatment (Wu et al., 2000). Interestingly, humoral immune responses against CHD-2 were observed in all three patients. CHD-2 was also identified as target of the antibody response in this study (clone # 8). No sequence comparison between patient and donor was performed in that study, therefore, it is not clear if any of the 13 identified antigens was polymorphic. Furthermore, since the expression library was established from tumor cells (CML), the observed sequence differences between the identified antigens and the GenBank database could be the results of tumor-specific alterations.

4.3. SEREX method could be applied for identification of polymorphic antigens

To examine the MHC class II-restricted T helper cell response after allogeneic BMT, the SEREX method was used to indirectly define the antigens involved in T helper cell response. Twenty antigens were identified in the patient/donor pair examined. Sequence analysis of the ORFs identified three antigens that are polymorphic between

donor and patient. While these antigens represent candidate mHAs, no polymorphism has been detected in the majority of antigens identified, and thus the remaining antigens could be autoantigens. In fact, many of the SEREX-defined antigens cloned from tumors were demonstrated to be autoantigens, since they were not only recognized by sera of tumor patients but by sera of healthy controls as well, as demonstrated by our group (Behrends et al., 2002; Behrends et al., 2003) and numerous others. After allogeneic BMT, a similarly high percentage of autoantigens might be identified since toxic conditioning regimes can cause significant tissue damage and possibly thereby lead to a secondary break down of immunotolerance. A simple method to directly detect and exclude autoantigens identified by SEREX after allogeneic BMT would be to test all antigens with serum collected from the patient before BMT and serum from the healthy donor. Unfortunately, such serum samples were not available in this study. However, immune responses against such "BMT-associated autoantigens" might be transient and antibodies against these antigens might diminish over time. It will, therefore, be challenging, to test the identified antigens with serum collected at different time points after BMT and investigate any changes of the SEREX-antigen-specific humoral immune responses. Furthermore, by comparing antigens identified in a larger number of patient/donor pairs, a group of common BMT-associated autoantigens may be defined. Preliminary data obtained from one additional HLA-identical BMT donor/patient pair suggests that ribosomal proteins may be such BMT-associated autoantigens, because antibody responses against several ribosomal proteins were detected in both cases. Although ribosomal proteins are polymorphic in the population, they were identical in both patient and donor pairs examined. Another possible explanation for the apparent identification of many non-polymorphic antigens might be that these antigens are indeed polymorphic, but the polymorphisms were not detected.

4.4. Identification of polymorphisms

Polymorphisms between patient and donor were identified by comparing the cDNA sequences of the genes identified with the SEREX method. For this purpose, RNA was purified from T cells derived from patient and donor, reverse-transcribed into cDNA and then PCR amplified to obtain enough DNA for the sequencing reactions. Essential for this analysis, therefore, is precise RNA purification, cDNA preparation, and PCR amplification. By sequencing antigen 21-1 (FLJ23270) from several unrelated donors, a

four-nucleotide deletion was observed in 7 out of 10 cases, but not in the patient/donor pair. This deletion leads to a frameshift in the open reading frame and the appearance of a premature stop codon. This deletion was detected in about 50% of the cDNAs, and therefore, may be derived from one allele. These results indicated that both alleles are expressed equally, and this ratio was not affected by the preparation of cDNA.

Polymorphisms in regulatory sequences of genes, however, may influence the level of transcription (Yamaguchi et al., 2002). Because PCR amplified mixes of cDNAs are sequenced, polymorphisms will only be detected if both alleles are transcribed at a similar level. Therefore, it cannot be excluded that polymorphisms were missed because the polymorphic allele is expressed at a lower level. Polymorphism analysis would not be affected by such differential gene expression, if the genes were analyzed at the DNA level. The copy numbers of both alleles would be the same and should be amplified by PCR in an equimolar ratio. However, for this kind of analysis, the genomic structure of the gene has to be known.

Another possible explanation for the failure to detect polymorphisms could be that the polymorphisms are located in variants of the antigens created by alternative splicing. Because epitopes recognized by antibodies and T cells are not necessarily identical, it could be that a polymorphism is located in a splicing variant of the antigen, which has not been analyzed. For example, three different splicing variants of hematopoietic protein 1 (HEM-1) were identified and additional variants may exist. Another example is the protein product derived from the sequence XM_031425 (clone 69), which shares 270 amino acids with the autoimmune regulator (AIRE) and therefore, may represent a splicing variant. AIRE plays an important role in central tolerance induction, probably by promoting the ectopic expression of peripheral tissue-restricted antigens in medullary epithelial cells of the thymus. A mutation in the transcription factor AIRE is responsible for the development of multiorgan autoimmune disease (Anderson et al., 2002). AIRE is exclusively expressed in thymic epithelial cells. The protein product from XM_031425 is also a transcription factor with unknown function and, because discovered in a cDNA library derived from T cells, with a different expression pattern. Further analysis of XM_031425 has to reveal whether this antigen plays any role in the immunological response after BMT. Finishing the human genome project will facilitate the identification of more splicing variants and will hopefully aid at answering this question.

4.5. T cells specific for polymorphic antigens are present in the blood of the patient

Single nucleotide polymorphisms (SNPs) are common DNA sequence variations among individuals and in the last decade public databases containing several hundred thousands of SNPs have been established. Therefore, it is expected that SNP can be detected just by random comparison of 20 genes between two individuals. What are then the evidences that the polymorphic differences between the patient and the donor are not just polymorphisms detected by chance? After identification of the polymorphic antigens in the patient/donor pair, peptides carrying the allelic variants from patient and donor were synthesized. Evidence for an immunological importance of the identified polymorphic antigens would be the detection of T cells in transplanted patient which are specific for peptides derived from patient alleles compared control peptides derived from donor alleles. By using cytokine secretion and purification assays, T cells specific for all three polymorphic antigens were isolated. These T cells recognized the peptides carrying the allelic variant of the patient, but not the control peptides. These results demonstrated that T cells specific for the polymorphic antigens are present in the blood of the patient after BMT. Thus, the antigens identified by the SEREX method caused both humoral and cellular immune responses. Surprisingly, neither LCL obtained from the patient nor LCL obtained from the donor were recognized by these T cells. This unresponsiveness of the T cells to the LCLs may indicate that these antigens are inefficiently presented on MHC class II, and thereby may offer an explanation why the mHA-specific T cell clones generated by repeated stimulation with LCL patient did not recognize the polymorphic antigens identified with the SEREX method. Antigens presented on MHC class II are usually derived from exogenous proteins that have been internalized and degraded in the endosomal/lysosomal system. Besides exogenous antigens, endogenous antigens synthesized within the APC are also presented on MHC class II (Nuchtern et al., 1990; Chen et al., 1990). Recently, our group demonstrated that LCL can efficiently present a cytosolic protein on MHC class II (Nimmerjahn et al., 2003). The efficiency, however, by which endogenous antigens are presented on MHC class II, is dependent on the subcellular localisation as well as expression level and turnover rate of the protein. Intracellular antigens, therefore, are expected to be presented with varying efficiencies on MHC class II molecules. Activation and proliferation of T cells is dependent on antigen density, i.e. the number of MHC/antigen complexes presented on the cell surface. Consequently, T cells

specific for antigens which are efficiently presented on MHC class II will expand, while other T cell specificities may be lost under these in vitro culture conditions. These differences in antigen presentation may offer an explanation why no T cells specific for the SEREX-defined antigens have been generated, but do not explain why the antigens recognized by the T cell clones have not been identified by the serological approach. The SEREX method entails the expression of a protein library in E. coli. Bacterial expression of proteins is usually very efficient, but may be problematic in certain cases, e.g. membrane proteins which tend to aggregate, or proteins toxic to E. coli. Therefore, the expression level of some proteins may be too low for detection by serum antibodies. While expression of membrane proteins in E. coli appears to be difficult, proteins localized in the cell membrane very efficiently enter the MHC class II presentation pathway. Thus, it seems possible that the antigens recognized by the T cell clones are derived from membrane proteins, which are difficult to express in bacteria. Alternatively, the differences in the antigens identified with the serological and the cellular approach could be a reflection of the different methods used. The expression library used for screening with serum of the patient after BMT was prepared from T cells, whereas stimulation of the T cells was performed with LCL. Therefore, it is possible that the antigens identified by the SEREX method are not expressed in EBV-immortalized B cells used for T cell stimulation and vice versa. To address these possibilities, it will be important to examine protein expression level, protein turnover and localisation of the identified SEREX antigens in T and B cells.

4.6. Frequency of polymorphic antigens in the population

An immune response against mHAs after BMT is generated because they differ between patient and donor. Three antigens that are polymorphic between patient and donor were identified. Clone 10 contains two ORFs 10-1 and 10-2 and both are polymorphic. The protein product from ORF 10 has been described as FLJ00087 whereas the ORF 10-2 has been identified in this work. The cDNA encoding antigen 10-2 was homologous to mRNA FLJ00087 and two different splice variants. All three are found in hematopoietic tissues (blood, adult leukocytes and spleen), but the functions of the proteins they encode are not known. The sequence of clone 21-1 was found in two different genes (FLJ23270 and FLJ00159). Both are potential transmembrane proteins

with unknown functions, expressed in hematopoietic tissues, and both share the first transmembrane domain where the polymorphism is located. The clinical relevance of mHA is dependent on allele frequency within the population. For enhancement of immune responses against residual leukemia cells, patient and donor should differ in hematopoiesis-specific mHA. For example, two alleles of the hematopoiesis-specific mHA HA-1 have been identified in the human population. These two alleles, HA-1^H (histidine) and HA-1^R (arginine), are present within the population in 55.9% and 44.1%, respectively (Tseng et al., 1998). The HA-1^H allele gives rise to an epitope that can be present on HLA-A2. Thus, a significant number of HLA-A2 positive patients would have an appropriately discordant donor for HA-1. The frequency for the two alleles of HA-2 is 95% and 5%. Therefore, this mHA is suitable for enhancing GvL responses after BMT in only a small percentage of patients. HB-1 is presented on HLA-B44 and HB-1^H (histidine) and HB-1^Y (tyrosine) are present in 79% and 21% of the population, respectively. The allele frequencies for the three identified polymorphic antigens have been determined in this work by sequencing the polymorphic part of the genes in 13 unrelated volunteers. These experiments indicated, that the alleles identified are present at varying frequencies within the population. For example, antigen 21-1^L (leucine, allele in patient) is present in 15% of all individuals analyzed and is always found in a heterozygous state, whereas 21-1^V is present in 100% of the cases. Thus, in about 15% of the patient/donor pairs, 21-1^L may play a role in the immune response after BMT. However, for statistical reasons, more individuals have to be examined. Sequence analysis of the ORFs, however, is time- and cost-consuming. Therefore, primers specific for the polymorphic part of the alleles will be generated, and the frequency of the various alleles analyzed by PCR in a larger number of cases.

Although allogeneic BMT has proven very effective for the treatment of certain malignancies, the treatment of tumor relapse is still difficult. DLI is used with great clinical success in patients with recurrent leukemia after BMT (Kolb et al., 1990; Kolb et al., 1995; Slavin et al., 1996; Kolb et al., 1997). However, the infusion of non-selected donor lymphocytes is very often associated with the development of GvHD. Different strategies have been used to prevent the development of GvHD but to preserve GvL reactivity in the infused lymphocytes. These strategies include: (i) titration of the infused T cell numbers, (ii) depletion of $CD4^+$ or $CD8^+$ T cell subsets, (iii) selective removal of alloreactive donor T cells, (iv) induction of T cell anergy specifically to alloantigens and,

(v) the introduction of suicide genes into the donor T cells. The thymidin kinase gene of herpes simplex virus (HSV-TK) has been transduced into T lymphocytes, which were then used in DLI. The development of GvHD could be efficiently reversed by the treatment of the patient with ganciclovir (Bonini et al., 1997). Tumor regression following DLI provides further evidence that T cells reactive against leukemia cells exist in the blood of the donor. Instead of using unselected lymphocytes, the infusion of T cells specific to hematopoiesis-specific mHA expanded in vitro may lead to the eradication of residual leukemia cells after BMT without inducing GvHD. Such adoptive transfer of T cell clones has been successfully applied to reconstitute CMV and EBV immunity in immunocompromised patients (Walter et al., 1995; Gahn et al., 2002). Another possibility to specifically prime immune responses against antigens expressed on leukemia cells is by active vaccination. For this purpose, well defined, sufficiently immunogenic, hematopoiesis-specific mHA have to be identified. Whether the mHA identified in this work recognize ubiquitously expressed, or hematopoiesis-specific mHA, still has to be determined.

4.7. Conclusions

It has been shown in this work that minor histocompatibility antigens can be identified by serological screening of a cDNA expression library generated from normal tissue of the patient. Furthermore, T cell culture conditions have been established and optimized to efficiently generate $CD4^+$ mHA-specific T cell lines and clones. The antigens identified with the modified SEREX approach were not recognized by the mHA-specific T cell clones. Thus, these two approaches have led to the definition of a complementary rather than an overlapping set of antigens. Further analyses are required to see whether this holds true in a larger number of patient/donor pairs, and whether the antigens identified are unique to the patient/donor pair examined, or shared among recipients of allogeneic BMT. In summary, immunotherapeutic approaches to enhance GvL and reduce GvH responses after allogeneic BMT are greatly dependent on the molecular definition of mHA. Methods for the definition of mHA recognized by $CD4^+$ T cells, as described in this work, may help to achieve this goal.

Summary

After allogeneic HLA-identical BMT, the cells of the new immune system recognize peptides derived from mHAs in which patient and donor differ. The identification of mHAs therefore, is important for the development of immunotherapeutic approaches, which aim at enhancing GvL and decreasing GvH responses. Since T helper cells play a central role in various immune responses, mHA recognized by Th cells are of special interest.

In order to identify mHAs recognized by T helper cells, a dual strategy was designed to define the targets of the T helper cell response after BMT. In a serological approach to define the targets of the humoral immune response, 20 different antigens were identified. By comparing patient and donor mHA sequences, three antigens were found to have interindividual differences. Furthermore, analysis of unrelated individuals confirmed that these antigens are polymorphic in the population. To assess the targets of the cellular immune response, a number of mHA-specific T cell lines and clones were generated. These T cell clones were all $CD4^+$ and of Th-1 phenotype, and recognized four different mHAs presented on three different HLA molecules. Specific recognition assays showed that these clones do not recognize mHAs identified as the targets of humoral immune response. The recognition pattern of the mHA-specific T cell clones did not correlate with the distribution of polymorphic alleles within unrelated donors, confirming that antibodies and T cells recognize different mHAs. However, increased numbers of T cells specific for these antigens were found in the blood of the patient after BMT. The detection and successful isolation of these T cells provides further evidence that these antigens are important in the immune response after BMT.

This work shows that mHAs recognized by T helper cells can be identified through serological methods. The method applied here, however, did not lead to the identification of mHAs recognized by independently generated mHA-specific T cell clones. It remains to be seen whether antigens identified by these two approaches are different or overlapping in a larger number of patient/donor pairs. Furthermore, analysis of a larger number of patient/donor pairs should reveal whether the antigens identified with these two approaches are unique to the analysed patient/donor pair, or shared among several patient/donor pairs. The latter set of antigens could be especially useful for increasing GvL and decreasing GvH responses.

References

Alyea, E., Weller, E., Schlossman, R., Canning, C., Webb, I., Doss, D., Mauch, P., Marcus, K., Fisher, D., Freeman, A., et al. (2001). T-cell--depleted allogeneic bone marrow transplantation followed by donor lymphocyte infusion in patients with multiple myeloma: induction of graft-versus-myeloma effect, Blood 98, 934-9.

Alyea, E. P., Soiffer, R. J., Canning, C., Neuberg, D., Schlossman, R., Pickett, C., Collins, H., Wang, Y., Anderson, K. C., and Ritz, J. (1998). Toxicity and efficacy of defined doses of CD4(+) donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant, Blood 91, 3671-80.

Anderson, M. S. (2002). Autoimmune endocrine disease, Curr Opin Immunol 14, 760-4.

Bach, F. H., Albertini, R. J., Joo, P., Anderson, J. L., and Bortin, M. M. (1968). Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome, Lancet 2, 1364-6.

Bay, J. O., Choufi, B., Pomel, C., Dauplat, J., Durando, X., Tournilhac, O., Travade, P., Plagne, R., and Blaise, D. (2000). Potential allogeneic graft-versus-tumor effect in a patient with ovarian cancer, Bone Marrow Transplant 25, 681-2.

Behrends, U., Jandl, T., Golbeck, A., Lechner, B., Muller-Weihrich, S., Schmid, I., Till, H., Berthold, F., Voltz, R., and Mautner, J. M. (2002). Novel products of the HUD, HUC, NNP-1 and alpha-internexin genes identified by autologous antibody screening of a pediatric neuroblastoma library, Int J Cancer 100, 669-77

Behrends, U., Schneider, I., Roessler, S, Frauenknecht, H., Golbeck, A., Lechner, B., Eigenstetter, G., Zobywalki, C., Müller-Weihrich, S., Graubner, U., Schmid, I., Sackerer, D, Späth, M, Götz, C., Prantl, F., Asmuss, H.P., Bise, K., and Mautner, J. M. (2003). Novel tumor antigens identified by autologous antibody screening of childhood medulloblastoma cDNA libraries. Int J Cancer, in press.

Berke, Z., Andersen, M. H., Pedersen, M., Fugger, L., Zeuthen, J., and Haurum, J. S. (2000). Peptides spanning the junctional region of both the abl/bcr and the bcr/abl fusion proteins bind common HLA class I molecules, Leukemia 14, 419-26.

Bernaudin, F. (1999). [Results and current indications of bone marrow allograft in sickle cell disease], Pathol Biol (Paris) 47, 59-64.

Bishop, N., Horman, A., and Woodman, P. (2002). Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates, J Cell Biol 157, 91-101.

Bocchia, M., Korontsvit, T., Xu, Q., Mackinnon, S., Yang, S. Y., Sette, A., and Scheinberg, D. A. (1996). Specific human cellular immunity to bcr-abl oncogene-derived peptides, Blood 87, 3587-92.

Bocchia, M., Wentworth, P. A., Southwood, S., Sidney, J., McGraw, K., Scheinberg, D. A., and Sette, A. (1995). Specific binding of leukemia oncogene fusion protein peptides to HLA class I molecules, Blood 85, 2680-4.

Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponzoni, M., Rossini, S., Mavilio, F., Traversari, C., and Bordignon, C. (1997). HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia, Science 276, 1719-24.

Bonini, C., Lee, S. P., Riddell, S. R., and Greenberg, P. D. (2001). Targeting antigen in mature dendritic cells for simultaneous stimulation of CD4+ and CD8+ T cells, J Immunol 166, 5250-7.

Bonnet, D., Warren, E. H., Greenberg, P. D., Dick, J. E., and Riddell, S. R. (1999). CD8(+) minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells, Proc Natl Acad Sci U S A 96, 8639-44.

Bortin, M. M., Horowitz, M. M., Mrsic, M., Rimm, A. A., and Sobocinski, K. A. (1991). Progress in bone marrow transplantation for leukemia: a preliminary report from the Advisory Committee of the International Bone Marrow Transplant Registry, Transplant Proc 23, 61-2.

Bosch, G. J., Joosten, A. M., Kessler, J. H., Melief, C. J., and Leeksma, O. C. (1996). Recognition of BCR-ABL positive leukemic blasts by human CD4+ T cells elicited by primary in vitro immunization with a BCR-ABL breakpoint peptide, Blood 88, 3522-7.

Brickner, A. G., Warren, E. H., Caldwell, J. A., Akatsuka, Y., Golovina, T. N., Zarling, A. L., Shabanowitz, J., Eisenlohr, L. C., Hunt, D. F., Engelhard, V. H., and Riddell, S. R. (2001). The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing, J Exp Med 193, 195-206.

Collins, R., Shpilberg, O., Drobyski, W., Porter, D., Giralt, S., Champlin, R., Goodman, S., Wolff, S., Verfaillie, C., List, A., Dalton, W., Ognoskie, N., Chetrit, A., Antin, J., Nemunaitis, J. (1997). Donor leukocyte infusion in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol 15:433

Champlin, R. (1992). Graft-versus-leukemia without graft-versus-host disease: an elusive goal of bone marrow transplantation, Semin Hematol 29, 46-52.

Chang DD, Wong C, Smith H, Liu J.(1997). ICAP-1, a novel beta1 integrin cytoplasmic domain-associated protein, binds to a conserved and functionally important NPXY sequence motif of beta1 integrin. J Cell Biol. 1997 Sep 8;138(5):1149-57.

Chen, B. P., Madrigal, A., and Parham, P. (1990). Cytotoxic T cell recognition of an endogenous class I HLA peptide presented by a class II HLA molecule, J Exp Med 172, 779-88.

Childs, R., Chernoff, A., Contentin, N., Bahceci, E., Schrump, D., Leitman, S., Read, E. J., Tisdale, J., Dunbar, C., Linehan, W. M., et al. (2000). Regression of metastatic renal-

cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation, N Engl J Med 343, 750-8.

Childs, R. W., Clave, E., Tisdale, J., Plante, M., Hensel, N., and Barrett, J. (1999). Successful treatment of metastatic renal cell carcinoma with a nonmyeloablative allogeneic peripheral-blood progenitor-cell transplant: evidence for a graft-versus-tumor effect, J Clin Oncol 17, 2044-9.

de Bueger, M., Bakker, A., Van Rood, J. J., Van der Woude, F., and Goulmy, E. (1992). Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens, J Immunol 149, 1788-94.

De Koning, J., Van Bekkum, D. W., Dicke, K. A., Dooren, L. J., Radl, J., and Van Rood, J. J. (1969). Transplantation of bone-marrow cells and fetal thymus in an infant with lymphopenic immunological deficiency, Lancet 1, 1223-7.

den Haan, J. M., Meadows, L. M., Wang, W., Pool, J., Blokland, E., Bishop, T. L., Reinhardus, C., Shabanowitz, J., Offringa, R., Hunt, D. F., et al. (1998). The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism, Science 279, 1054-7.

den Haan, J. M., Sherman, N. E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J. W., Skipper, J., Shabanowitz, J., Hunt, D. F., Engelhard, V. H., and et al. (1995). Identification of a graft versus host disease-associated human minor histocompatibility antigen, Science 268, 1476-80.

Ditzel, H. J., Masaki, Y., Nielsen, H., Farnaes, L., and Burton, D. R. (2000). Cloning and expression of a novel human antibody-antigen pair associated with Felty's syndrome, Proc Natl Acad Sci U S A 97, 9234-9.

Dodi, I. A., Van Rhee, F., Forde, H. C., Roura-Mir, C., Jaraquemada, D., Goldman, J. M., and Madrigal, J. A. (2002). CD4(+) bias in T cells cloned from a CML patient with active graft versus leukemia effect, Cytotherapy 4, 353-63.

Dolcetti, R., Frisan, T., Sjoberg, J., De Campos-Lima, P. O., Pisa, P., De Re, V., Gloghini, A., Rizzo, S., Masucci, M. G., and Boiocchi, M. (1995). Identification and characterization of an Epstein-Barr virus-specific T-cell response in the pathologic tissue of a patient with Hodgkin's disease, Cancer Res 55, 3675-81.

Dolstra, H., Fredrix, H., Maas, F., Coulie, P. G., Brasseur, F., Mensink, E., Adema, G. J., de Witte, T. M., Figdor, C. G., and van de Wiel-van Kemenade, E. (1999). A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia, J Exp Med 189, 301-8.

Dolstra, H., Fredrix, H., Preijers, F., Goulmy, E., Figdor, C. G., de Witte, T. M., and van de Wiel-van Kemenade, E. (1997). Recognition of a B cell leukemia-associated minor histocompatibility antigen by CTL, J Immunol 158, 560-5.

Eljaafari, A., Farre, A., Duperrier, K., Even, J., Vie, H., Michallet, M., Souillet, G., Catherine Freidel, A., Gebuhrer, L., and Rigal, D. (2001). Generation of helper and cytotoxic CD4+T cell clones specific for the minor histocompatibility antigen H-Y, after in vitro priming of human T cells by HLA-identical monocyte-derived dendritic cells, Transplantation 71, 1449-55.

Epstein, R. B., Storb, R., Ragde, H., and Thomas, E. D. (1968). Cytotoxic typing antisera for marrow grafting in littermate dogs, Transplantation 6, 45-58.

Faber, L. M., van der Hoeven, J., Goulmy, E., Hooftman-den Otter, A. L., van Luxemburg-Heijs, S. A., Willemze, R., and Falkenburg, J. H. (1995). Recognition of clonogenic leukemic cells, remission bone marrow and HLA-identical donor bone marrow by CD8+ or CD4+ minor histocompatibility antigen-specific cytotoxic T lymphocytes, J Clin Invest 96, 877-83.

Faber, L. M., van Luxemburg-Heijs, S. A., Veenhof, W. F., Willemze, R., and Falkenburg, J. H. (1995). Generation of CD4+ cytotoxic T-lymphocyte clones from a patient with severe graft-versus-host disease after allogeneic bone marrow transplantation: implications for graft-versus-leukemia reactivity, Blood 86, 2821-8.

Falkenburg, J. H., Faber, L. M., van den Elshout, M., van Luxemburg-Heijs, S. A., Hooftman-den Otter, A., Smit, W. M., Voogt, P. J., and Willemze, R. (1993). Generation of donor-derived antileukemic cytotoxic T-lymphocyte responses for treatment of relapsed leukemia after allogeneic HLA-identical bone marrow transplantation, J Immunother 14, 305-9.

Fassas, A., Anagnostopoulos, A., Kazis, A., Kapinas, K., Sakellari, I., Kimiskidis, V., Smias, C., Eleftheriadis, N., and Tsimourtou, V. (2000). Autologous stem cell transplantation in progressive multiple sclerosis--an interim analysis of efficacy, J Clin Immunol 20, 24-30.

Fournier HN., Dupe-Manet S., Bpuvard D., Lacombe ML., Marie C., Block MR., Albigez-Rizo C. (2002). Integrin cytoplasmatic domain-associated protein 1alpha (ICAP-1 alpha) interact directli with the metastasis supressor nm23-H2, and both proteins are targeted to newly formed cell adhesion sites upon integrin engagement, J Biol Chem Jun 7;277 (23):20895-902

Fowler, D. H., and Gress, R. E. (2000). Th2 and Tc2 cells in the regulation of GVHD, GVL, and graft rejection: considerations for the allogeneic transplantation therapy of leukemia and lymphoma, Leuk Lymphoma 38, 221-34.

Fujii, S., Senju, S., Chen, Y. Z., Ando, M., Matsushita, S., and Nishimura, Y. (1998). The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells, Hum Immunol 59, 607-14.

Gahn, B., Hunt, G., Rooney, C. M., and Heslop, H. E. (2002). Immunotherapy to reconstitute immunity to DNA viruses, Semin Hematol 39, 41-7.

Gao, L., Bellantuono, I., Elsasser, A., Marley, S. B., Gordon, M. Y., Goldman, J. M., and Stauss, H. J. (2000). Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1, Blood 95, 2198-203.

Gatti, R. A., Meuwissen, H. J., Allen, H. D., Hong, R., and Good, R. A. (1968). Immunological reconstitution of sex-linked lymphopenic immunological deficiency, Lancet 2, 1366-9.

Giardini, C., and Lucarelli, G. (1999). Bone marrow transplantation for beta-thalassemia, Hematol Oncol Clin North Am 13, 1059-64, viii.

Giralt, S., Hester, J., Huh, Y., Hirsch-Ginsberg, C., Rondon, G., Seong, D., Lee, M., Gajewski, J., Van Besien, K., Khouri, I., and et al. (1995). CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation, Blood 86, 4337-43.

Giralt, S. A., and Champlin, R. E. (1994). Leukemia relapse after allogeneic bone marrow transplantation: a review, Blood 84, 3603-12.

Goulmy, E. (1997). Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy, Immunol Rev 157, 125-40.

Goulmy, E. (1997). Minor histocompatibility antigens: from T cell recognition to peptide identification, Hum Immunol 54, 8-14.

Goulmy, E., Gratama, J. W., Blokland, E., Zwaan, F. E., and van Rood, J. J. (1983). A minor transplantation antigen detected by MHC-restricted cytotoxic T lymphocytes during graft-versus-host disease, Nature 302, 159-61.

Gratwohl, A., Brand, R., Apperley, J., Biezen Av, A., Bandini, G., Devergie, A., Schattenberg, A., Frassoni, F., Guglielmi, C., Iacobelli, S., et al. (2002). Graft-versus-host disease and outcome in HLA-identical sibling transplantations for chronic myeloid leukemia, Blood 100, 3877-86.

Hoffmann, T., Russell, C., and Vindelov, L. (2002). Generation of EBV-specific CTLs suitable for adoptive immunotherapy of EBV-associated lymphoproliferative disease following allogeneic transplantation, Apmis 110, 148-57.

Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H. J., Rimm, A. A., Ringden, O., Rozman, C., Speck, B., and et al. (1990). Graft-versus-leukemia reactions after bone marrow transplantation, Blood 75, 555-62.

Howe CWS, Radde-Stepaniak T: Hematopoietic cell donor registries, in Thomas ED, Blume KG, Forman SJ (eds): Hematopoietic cell transplantation(ed “). Boston, MA, Blackwell Science, 1999, pp 503-512

Hromas, R., Collins, S., Raskind, W., Deaven, L., and Kaushansky, K. (1991). Hem-1, a potential membrane protein, with expression restricted to blood cells, Biochim Biophys Acta 1090, 241-4.

Irle, C., Beatty, P. G., Mickelson, E., Thomas, E. D., and Hansen, J. A. (1985). Alloreactive T cell responses between HLA-identical siblings. Detection of anti-minor histocompatibility T cell clones induced in vivo, Transplantation 40, 329-33.

Irle, C., Deeg, H. J., Buckner, C. D., Kennedy, M., Clift, R., Storb, R., Appelbaum, F. R., Beatty, P., Bensinger, W., Doney, K., and et al. (1985). Marrow transplantation for leukemia following fractionated total body irradiation. A comparative trial of methotrexate and cyclosporine, Leuk Res 9, 1255-61.

Jacobson LO, Marks EK, Robson MJ, et al: Effect of spleen protection on mortality following x-irradiation. J Lab Clin Med 34: 1538-1543, 1949

Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., et al. (2000). Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers, Proc Natl Acad Sci U S A 97, 12198-203.

Jager, E., Jager, D., Karbach, J., Chen, Y. T., Ritter, G., Nagata, Y., Gnjatic, S., Stockert, E., Arand, M., Old, L. J., and Knuth, A. (2000). Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma, J Exp Med 191, 625-30.

Jager, E., Nagata, Y., Gnjatic, S., Wada, H., Stockert, E., Karbach, J., Dunbar, P. R., Lee, S. Y., Jungbluth, A., Jager, D., et al. (2000). Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses, Proc Natl Acad Sci U S A 97, 4760-5.

Jiang, Y. Z., and Barrett, J. (1997). The allogeneic CD4+ T-cell-mediated graft-versus-leukemia effect, Leuk Lymphoma 28, 33-42.

Kernan, N.A., Bordignon, C., Heller, G., Cunningham, I., Castro Malaspina, H., Shank, B., Flomenberg, N., Burns, J., Yang, S.Y., Black, P., et al. (1989). Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukaemia: I. Analysis of risk factors and results of secondary transplants. Blood 74, 2227-2236.

Kessler, J. H., Beekman, N. J., Bres-Vloemans, S. A., Verdijk, P., van Veelen, P. A., Kloosterman-Joosten, A. M., Vissers, D. C., ten Bosch, G. J., Kester, M. G., Sijts, A., et al. (2001). Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis, J Exp Med 193, 73-88.

Kolb, H. J., and Holler, E. (1997). Hematopoietic transplantation: state of the art, Stem Cells 15, 151-7; discussion 158.

Kolb, H. J., and Holler, E. (1997). Adoptive immunotherapy with donor lymphocyte transfusions, Curr Opin Oncol 9, 139-45.

Kolb, H. J., Mittermuller, J., Clemm, C., Holler, E., Ledderose, G., Brehm, G., Heim, M., and Wilmanns, W. (1990). Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients, Blood 76, 2462-5.

Kolb, H. J., Schattenberg, A., Goldman, J. M., Hertenstein, B., Jacobsen, N., Arcese, W., Ljungman, P., Ferrant, A., Verdonck, L., Niederwieser, D., and et al. (1995). Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia, Blood 86, 2041-50.

Lorenz E, Uphoff D, Reid TR, et al: Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst 12: 197-201, 1951

Lucarelli, G., Galimberti, M., Polchi, P., Angelucci, E., Baronciani, D., Giardini, C., Politi, P., Durazzi, S. M., Muretto, P., and Albertini, F. (1990). Bone marrow transplantation in patients with thalassemia, N Engl J Med 322, 417-21.

Malcherek, G., Wirblich, C., Willcox, N., Rammensee, H. G., Trowsdale, J., and Melms, A. (1998). MHC class II-associated invariant chain peptide replacement by T cell epitopes: engineered invariant chain as a vehicle for directed and enhanced MHC class II antigen processing and presentation, Eur J Immunol 28, 1524-33.

Mandalfino, P., Rice, G., Smith, A., Klein, J. L., Rystedt, L., and Ebers, G. C. (2000). Bone marrow transplantation in multiple sclerosis, J Neurol 247, 691-5.

Mannick JA, Lochte HL Jr, Ashley CA, et al: Autografs of bone marrow in dogs after lethal total-body radiation. Blood 15: 255-266, 1960

Marmont, A. M. (1991). Alloimmune effects of bone marrow transplantation for leukaemia on the leukaemic diseases, Bone Marrow Transplant 7, 2-3.

Marmont, A.M., Horowitz, M.M., Gale, R.P., Sobocinski, K., Ash, R.C., van Bekkum, D.W., Chaplin, R.E., Dicke, K.A., Goldman, J.M., Good, R.A., et al. (1991). T cell depletion of HLA-identical transplants in leukaemia. Blood 78, 2120-2130.

Mathe, G., Amiel, J. L., Schwarzenberg, L., Cattan, A., and Schneider, M. (1965). Adoptive immunotherapy of acute leukemia: experimental and clinical results, Cancer Res 25, 1525-31.

Meadows, L., Wang, W., den Haan, J. M., Blokland, E., Reinhardus, C., Drijfhout, J. W., Shabanowitz, J., Pierce, R., Agulnik, A. I., Bishop, C. E., et al. (1997). The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition, Immunity 6, 273-81.

Miller, S. T., Sleeper, L. A., Pegelow, C. H., Enos, L. E., Wang, W. C., Weiner, S. J., Wethers, D. L., Smith, J., and Kinney, T. R. (2000). Prediction of adverse outcomes in children with sickle cell disease, N Engl J Med 342, 83-9.

Minev, B., Hipp, J., Firat, H., Schmidt, J. D., Langlade-Demoyen, P., and Zanetti, M. (2000). Cytotoxic T cell immunity against telomerase reverse transcriptase in humans, Proc Natl Acad Sci U S A 97, 4796-801.

Molldrem, J., Dermime, S., Parker, K., Jiang, Y. Z., Mavroudis, D., Hensel, N., Fukushima, P., and Barrett, A. J. (1996). Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells, Blood 88, 2450-7.

Molldrem, J. J., Clave, E., Jiang, Y. Z., Mavroudis, D., Raptis, A., Hensel, N., Agarwala, V., and Barrett, A. J. (1997). Cytotoxic T lymphocytes specific for a nonpolymorphic proteinase 3 peptide preferentially inhibit chronic myeloid leukemia colony-forming units, Blood 90, 2529-34.

Molldrem, J. J., Lee, P. P., Wang, C., Felio, K., Kantarjian, H. M., Champlin, R. E., and Davis, M. M. (2000). Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia, Nat Med 6, 1018-23.

Monach, P. A., Meredith, S. C., Siegel, C. T., and Schreiber, H. (1995). A unique tumor antigen produced by a single amino acid substitution, Immunity 2, 45-59.

Nair, S. K., Heiser, A., Boczkowski, D., Majumdar, A., Naoe, M., Lebkowski, J. S., Vieweg, J., and Gilboa, E. (2000). Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells, Nat Med 6, 1011-7.

Nieda, M., Nicol, A., Kikuchi, A., Kashiwasa, K., Taylor, K., Suzuki, K., Tadokoro, K., Juji, T. (1998). Dendritic cells stimulate the expansion of bcr-abl specific CD8+ T cells with cytotoxic activity against leukemic cells from patiens with chronic myeloid leukaemia. Blood. 1998 Feb 1; 91(3):977-83.

Niederwieser, D., Grassegger, A., Aubock, J., Herold, M., Nachbaur, D., Rosenmayr, A., Gachter, A., Nussbaumer, W., Gaggl, S., Ritter, M., and et al. (1993). Correlation of minor histocompatibility antigen-specific cytotoxic T lymphocytes with graft-versus-host disease status and analyses of tissue distribution of their target antigens, Blood 81, 2200-8.

Nijmeijer, B. A., Willemze, R., and Falkenburg, J. H. (2002). An animal model for human cellular immunotherapy: specific eradication of human acute lymphoblastic leukemia by cytotoxic T lymphocytes in NOD/scid mice, Blood 100, 654-60.

Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E., Pardoll, D., Bornkamm, G., Mautner, J. (2003). Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy Eur J Immunol, in press.

Norbury, L. C., Clark, R. E., and Christmas, S. E. (2000). b3a2 BCR-ABL fusion peptides as targets for cytotoxic T cells in chronic myeloid leukaemia, Br J Haematol 109, 616-21.

Nuchtern, J. G., Biddison, W. E., and Klausner, R. D. (1990). Class II MHC molecules can use the endogenous pathway of antigen presentation, Nature 343, 74-6.

Ohminami, H., Yasukawa, M., Kaneko, S., Yakushijin, Y., Abe, Y., Kasahara, Y., Ishida, Y., and Fujita, S. (1999). Fas-independent and nonapoptotic cytotoxicity mediated by a human CD4(+) T-cell clone directed against an acute myelogenous leukemia-associated DEK-CAN fusion peptide, Blood 93, 925-35.

Ohminami, H., Yasukawa, M., and Fujita, S. (2000). HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide, Blood 95, 286-93.

Oka, Y., Elisseeva, O. A., Tsuboi, A., Ogawa, H., Tamaki, H., Li, H., Oji, Y., Kim, E. H., Soma, T., Asada, M., et al. (2000). Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product, Immunogenetics 51, 99-107.

Old, L. J. (1981). Cancer immunology: the search for specificity--G. H. A. Clowes Memorial lecture, Cancer Res 41, 361-75.

Orsini, E., Alyea, E. P., Schlossman, R., Canning, C., Soiffer, R. J., Chillemi, A., Neuberg, D., Anderson, K. C., and Ritz, J. (2000). Changes in T cell receptor repertoire associated with graft-versus-tumor effect and graft-versus-host disease in patients with relapsed multiple myeloma after donor lymphocyte infusion, Bone Marrow Transplant 25, 623-32.

Osman, Y., Takahashi, M., Zheng, Z., Koike, T., Toba, K., Liu, A., Furukawa, T., Aoki, S., and Aizawa, Y. (1999). Generation of bcr-abl specific cytotoxic T-lymphocytes by using dendritic cells pulsed with bcr-abl (b3a2) peptide: its applicability for donor leukocyte transfusions in marrow grafted CML patients, Leukemia 13, 166-74.

Pieper, R., Christian, R. E., Gonzales, M. I., Nishimura, M. I., Gupta, G., Settlage, R. E., Shabanowitz, J., Rosenberg, S. A., Hunt, D. F., and Topalian, S. L. (1999). Biochemical identification of a mutated human melanoma antigen recognized by CD4(+) T cells, J Exp Med 189, 757-66.

Pitkanen J., Peterson P. Autoimmune regulator: from loss of function to autoimmunity, Genes Immun 2003 Jan;4(1):12-21

Posthuma, E. F., Falkenburg, J. H., Apperley, J. F., Gratwohl, A., Roosnek, E., Hertenstein, B., Schipper, R. F., Schreuder, G. M., D'Amaro, J., Oudshoorn, M., et al. (1999). HLA-B8 and HLA-A3 coexpressed with HLA-B8 are associated with a reduced risk of the development of chronic myeloid leukemia. The Chronic Leukemia Working Party of the EBMT, Blood 93, 3863-5.

Posthuma, E.F., Falkenburg, J.H., Apperley, J.F., Gratwohl, a., Hertenstein, b., Schipper, R.F., Oudshoom M., Biezen, J.H., Harmans, j., Willemze R., Roosnek, E., Niederwieser, D. (2000). HLA-DR4 is associated with a diminish risk of the development of chronic myeloid leukaemia (CML). Chronic Leukemia Working Party of the European Blood and Marrow Transplant Registry. Leukemia. 2000 May; 14(5): 859-62.

Rini, B. I., Zimmerman, T. M., Gajewski, T. F., Stadler, W. M., and Vogelzang, N. J. (2001). Allogeneic peripheral blood stem cell transplantation for metastatic renal cell carcinoma, J Urol 165, 1208-9.

Russell, C. A., Heilmann, C., Madsen, H. O., and Vindelov, L. L. (2001). The predictive value of helper T lymphocyte precursor frequencies for graft-versus-host disease and graft-versus-leukaemia effects in allogeneic bone marrow transplantation, Bone Marrow Transplant 27, 949-58.

Sahara, H., and Shastri, N. (2003). Second Class Minors: Molecular Identification of the Autosomal H46 Histocompatibility Locus as a Peptide Presented by Major Histocompatibility Complex Class II Molecules, J Exp Med 197, 375-85.

Sahin, U., Tureci, O., and Pfreundschuh, M. (1997). Serological identification of human tumor antigens, Curr Opin Immunol 9, 709-16.

Sanderson, S., Frauwirth, K., and Shastri, N. (1995). Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins, Proc Natl Acad Sci U S A 92, 7217-21.

Scott, D., Addey, C., Ellis, P., James, E., Mitchell, M. J., Saut, N., Jurcevic, S., and Simpson, E. (2000). Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens, Immunity 12, 711-20.

Shenoy, S., Desch, K., Duffy, B., Thorson, P., and Mohanakumar, T. (1998). Analysis of graft-versus-host disease (GVHD) and graft rejection using MHC class I-deficient mice, Clin Exp Immunol 112, 188-95.

Sierra, J., Perez, W. S., Rozman, C., Carreras, E., Klein, J. P., Rizzo, J. D., Davies, S. M., Lazarus, H. M., Bredeson, C. N., Marks, D. I., et al. (2002). Bone marrow transplantation from HLA-identical siblings as treatment for myelodysplasia, Blood 100, 1997-2004.

Slavin, S., Ackerstein, A., Naparstek, E., Or, R., and Weiss, L. (1990). The graft-versus-leukemia (GVL) phenomenon: is GVL separable from GVHD?, Bone Marrow Transplant 6, 155-61.

Slavin, S., Naparstek, E., Nagler, A., Kapelushnik, Y., Ackerstein, A., and Or, R. (1996). Allogeneic cell therapy: the treatment of choice for all hematologic malignancies relapsing post BMT, Blood 87, 4011-3.

Storb, R., Deeg, H. J., Fisher, L., Appelbaum, F., Buckner, C. D., Bensinger, W., Clift, R., Doney, K., Irle, C., McGuffin, R., and et al. (1988). Cyclosporine v methotrexate for graft-v-host disease prevention in patients given marrow grafts for leukemia: long-term follow-up of three controlled trials, Blood 71, 293-8.

Storb, R., Deeg, H. J., Pepe, M., Doney, K., Appelbaum, F., Beatty, P., Bensinger, W., Buckner, C. D., Clift, R., Hansen, J., and et al. (1989). Graft-versus-host disease prevention by methotrexate combined with cyclosporin compared to methotrexate alone in patients given marrow grafts for severe aplastic anaemia: long-term follow-up of a controlled trial, Br J Haematol 72, 567-72.

Storb, R., Epstein, R. B., Rudolph, R. H., and Thomas, E. D. (1969). Allogeneic canine bone marrow transplantation following cyclophosphamide, Transplantation 7, 378-86.

Storb, R., Rudolph, R. H., and Thomas, E. D. (1971). Marrow grafts between canine siblings matched by serotyping and mixed leukocyte culture, J Clin Invest 50, 1272-5.

Thomas, E. D., Buckner, C. D., Sanders, J. E., Papayannopoulou, T., Borgna-Pignatti, C., De Stefano, P., Sullivan, K. M., Clift, R. A., and Storb, R. (1982). Marrow transplantation for thalassaemia, Lancet 2, 227-9.

Tseng, L. H., Lin, M. T., Martin, P. J., Pei, J., Smith, A. G., and Hansen, J. A. (1998). Definition of the gene encoding the minor histocompatibility antigen HA-1 and typing for HA-1 from genomic DNA, Tissue Antigens 52, 305-11.

Tsoi, M. S., Storb, R., Dobbs, S., Medill, L., and Thomas, E. D. (1980). Cell-mediated immunity to non-HLA antigens of the host by donor lymphocytes in patients with chronic graft-vs-host disease, J Immunol 125, 2258-62.

Tsoi, M. S., Warren, R. P., Storb, R., Witherspoon, R. P., Mickelson, E., Giblett, E. R., Schanfield, M. S., Weiden, P., and Thomas, E. D. (1983). Autologous marrow recovery and sensitization to non-HLA antigens after HLA-identical marrow transplantation for aplastic anemia, Exp Hematol 11, 73-81.

Tyndall, A., and Gratwohl, A. (2002). The use of high dose immunoablative therapy with hematopoietic stem cell support therapy in the treatment of severe autoimmune diseases, Int J Hematol 76, 218-22.

Uphoff DE: Genetic factors influencing irradiation protection by bone marrow. I. The F1 hybrid effect. J Natl Cancer Inst 19: 123-125, 1957

van Bekkum, D. W., de Vries, M. J., and van der Waay, D. (1967). Lesions characteristic of secondary disease in germfree heterologous radiation chimeras, J Natl Cancer Inst 38, 223-31.

van Bergen, J., Schoenberger, S. P., Verreck, F., Amons, R., Offringa, R., and Koning, F. (1997). Efficient loading of HLA-DR with a T helper epitope by genetic exchange of CLIP, Proc Natl Acad Sci U S A 94, 7499-502.

van der Harst, D., Goulmy, E., Falkenburg, J. H., Kooij-Winkelaar, Y. M., van Luxemburg-Heijs, S. A., Goselink, H. M., and Brand, A. (1994). Recognition of minor histocompatibility antigens on lymphocytic and myeloid leukemic cells by cytotoxic T-cell clones, Blood 83, 1060-6.

van Els, C., Bakker, A., Zwaan, F., van Rood, J. J., and Goulmy, E. (1989). Incidence of anti-host cytotoxic and proliferative T cell responses after HLA-identical bone marrow transplantation (BMT), Transplant Proc 21, 2987-8.

van Els, C. A., Bakker, A., Zwinderman, A. H., Zwaan, F. E., van Rood, J. J., and Goulmy, E. (1990). Effector mechanisms in graft-versus-host disease in response to minor histocompatibility antigens. I. Absence of correlation with cytotoxic effector cells, Transplantation 50, 62-6.

van Lochem, E., de Gast, B., and Goulmy, E. (1992). In vitro separation of host specific graft-versus-host and graft-versus-leukemia cytotoxic T cell activities, Bone Marrow Transplant 10, 181-3.

Vogt, M. H., van den Muijsenberg, J. W., Goulmy, E., Spierings, E., Kluck, P., Kester, M. G., van Soest, R. A., Drijfhout, J. W., Willemze, R., and Falkenburg, J. H. (2002). The DBY gene codes for an HLA-DQ5-restricted human male-specific minor histocompatibility antigen involved in graft-versus-host disease, Blood 99, 3027-32.

Walter, E. A., Greenberg, P. D., Gilbert, M. J., Finch, R. J., Watanabe, K. S., Thomas, E. D., and Riddell, S. R. (1995). Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor, N Engl J Med 333, 1038-44.

Wang, R. F., Wang, X., Atwood, A. C., Topalian, S. L., and Rosenberg, S. A. (1999). Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen, Science 284, 1351-4.

Wang, R. F., Wang, X., and Rosenberg, S. A. (1999). Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4(+) T cells, J Exp Med 189, 1659-68.

Warren, E. H., Gavin, M., Greenberg, P. D., and Riddell, S. R. (1998). Minor histocompatibility antigens as targets for T-cell therapy after bone marrow transplantation, Curr Opin Hematol 5, 429-33.

Warren, E. H., Gavin, M. A., Simpson, E., Chandler, P., Page, D. C., Disteche, C., Stankey, K. A., Greenberg, P. D., and Riddell, S. R. (2000). The human UTY gene encodes a novel HLA-B8-restricted H-Y antigen, J Immunol 164, 2807-14.

Warren, E. H., Greenberg, P. D., and Riddell, S. R. (1998). Cytotoxic T-lymphocyte-defined human minor histocompatibility antigens with a restricted tissue distribution, Blood 91, 2197-207.

Wu, C. J., Chillemi, A., Alyea, E. P., Orsini, E., Neuberg, D., Soiffer, R. J., and Ritz, J. (2000). Reconstitution of T-cell receptor repertoire diversity following T-cell depleted allogeneic bone marrow transplantation is related to hematopoietic chimerism, Blood 95, 352-9.

Wu, C. J., Yang, X. F., McLaughlin, S., Neuberg, D., Canning, C., Stein, B., Alyea, E. P., Soiffer, R. J., Dranoff, G., and Ritz, J. (2000). Detection of a potent humoral response associated with immune-induced remission of chronic myelogenous leukemia, J Clin Invest 106, 705-14.

Yamaguchi, S., Shinmura, K., Saitoh, T., Takenoshita, S., Kuwano, H., and Yokota, J. (2002). A single nucleotide polymorphism at the splice donor site of the human MYH base excision repair genes results in reduced translation efficiency of its transcripts, Genes Cells 7, 461-74.

Yotnda, P., Garcia, F., Peuchmaur, M., Grandchamp, B., Duval, M., Lemonnier, F., Vilmer, E., and Langlade-Demoyen, P. (1998). Cytotoxic T cell response against the

chimeric ETV6-AML1 protein in childhood acute lymphoblastic leukemia, J Clin Invest 102, 455-62.

Yotnda, P., Firat, H., Garcia-Pons, F., Garcia, Z., Gourru, G., Vernant, J. P., Lemonnier, F. A., Leblond, V., and Langlade-Demoyen, P. (1998). Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia, J Clin Invest 101, 2290-6.

Zeis, M., Uharek, L., Hartung, G., Glass, B., Steinmann, J., and Schmitz, N. Graft-vs-leukemia activity and graft-vs-host disease induced by allogeneic Th1- and Th2-type CD4(+) T cells in mice, 2001 2, 136-144.

Zeng, G., Wang, X., Robbins, P. F., Rosenberg, S. A., and Wang, R. F. (2001). CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production, Proc Natl Acad Sci U S A 98, 3964-9.

Zorn, E., Wang, K. S., Hochberg, E. P., Canning, C., Alyea, E. P., Soiffer, R. J., and Ritz, J. (2002). Infusion of CD4+ donor lymphocytes induces the expansion of CD8+ donor T cells with cytolytic activity directed against recipient hematopoietic cells, Clin Cancer Res 8, 2052-60.

Abbreviations

AIRE	autoimmune regulator
AP	alkaline phosphatase
APC	antigen-presenting cell
bp	base pair
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumine
cDNA	complementary DNA
CHD	chromodomain helicase
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CTL	cytotoxic T cells
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DLA	dog leukocyte antigens
DLI	donor lymphocytes infusion
DMSO	dimethylsulfoxyde
dNTP	desoxyribonucleotide
DTT	dithiothreitol
EBV	Epstein-Barr virus
E. coli	Escherichia coli
EEF1A1	eukaryotic translation elongation factor 1 alpha 1
EGF	epidermal growth factor
EDTA	(Titriplex III) ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assays
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
GvL	graft-versus-leukemia
GvHD	graft-versus-host disease
HEM	hematopoietic protein
HLA	human leukocyte antigens
HPLC	high performance liquid chromatography
HSV-TK	herpes simplex virus-thymidin kinase
ICAP	integrin cytoplasmic domain-associated protein

IFNγ	interferon gamma
Ig	immunoglobulin
IL-4	interleukin 4
INF	interferon
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobase pairs
LB-agar	Luria Bertani-agar
LCL	lymphoblastoid cell line
mHA	minor histocompatibility antigens
MHC	major histocompatibility complex
MOPS	3-[*N*-morpholino]propane-sulfonic acid
MuMLV	mouse moloney leukaemia virus
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
pAPC	professional antigen presenting cells
PE	phycoerythrin
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PR-3	proteinase-3
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEREX	serological analysis of recombinant cDNA expression libraries
SDS	sodium dodecylsulfate
SNP	single nucleotide polymorphisms
TAE	Tris-Acetate-EDTA
TAP	transporter associated with antigen processing
TBE	Tris-boric EDTA buffer
TBI	total body irradiation
TBS	Tris-Buffered Saline
TE	Tris-EDTA
TCR	T cell receptor
TEMED	N,N,N',N'-Tetramethylethylendiamin
TERT	telomerase reverse transcriptase
Th1, Th2	T helper cell type 1 or 2
Tween 20	polyoxyethylene sorbitane monolaurate
U	units

VPS	vacuolar sorting protein
WT-1	Wilm's tumor gene
x-gal	5-Bromo-4-Chloro-3-Indoyl-β-D Galactopyranosid

8. Curriculum vitae

Personal data:

Name:	Slavoljub Milosevic
Adress:	Maria-montessori str. 6
	81829 München
Date of birth:	13.08.1969
Birthplace:	Krusevac
Citizenship:	Serb
Family status:	married

Education:

1977-85	Primary school
1985-89	Gymnasium
1989-90	military service

College education:

1990-96	Study of molecular biology and physiology, University of Belgrad
1996	Diploma thesis, University of Belgrad, Military technical institute Belgrad

Doctoral thesis

1999-2003	GSF-Forschungszentrum für Umwelt und Gesundheit GmbH, München, Institute für Klinische Molekularbiologie und Tumorgenetik, Prof. Dr. G. W. Bornkamm: „Identification of minor histocompatibility antigens“
2003	Submission of the thesis to the Dekanat für Biology, LMU München

München, 27.2.2003