TCR-$\alpha\beta^+$ AND TCR-$\gamma\delta^+$ T LYMPHOCYTES IN GRAFT AND PERIPHERAL BLOOD AFTER HEART TRANSPLANTATION

ISBN 90-9011091-7

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TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T lymphocytes in graft
and peripheral blood after heart transplantation

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Lay out: De *Werk*salon: Vanoordt/Groot, amsterdam
Printed by: Hentenaar Boek bv, Nieuwegein

TCR-$\alpha\beta^+$ AND TCR-$\gamma\delta^+$ T LYMPHOCYTES IN GRAFT AND PERIPHERAL BLOOD AFTER HEART TRANSPLANTATION

T-cel receptor $\alpha\beta^+$ en T-cel receptor $\gamma\delta^+$ T lymfocyten in het transplantaat en perifere bloed na harttransplantatie

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE
ERASMUS UNIVERSITEIT ROTTERDAM OP GEZAG VAN DE
RECTOR MAGNIFICUS
PROF DR P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES
DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP
WOENSDAG 26 NOVEMBER 1997 OM 15 UUR 45

door
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geboren te VENLO

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It is interesting to consider T-cell subsets in tissue transplantation, as this is an immunological process for which no evolutionary adaptation has occurred.

Judith E Allen and Rick Maizels

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[2] *Transplantation 1994; 57:119-1126 and in: The Human T cell receptor repertoire and transplantation, pp 117-147; editor: Peter J van den Elsen, (Molecular Biology Intelligence Unit) 1995 R.G. Landes Company, Austin, Texas, US*

[3] *Clin Exp Immunol 1992; 88:213-219*

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CHAPTER 1

INTRODUCTION

1.1 The Immune System

In vertebrates a highly complicated system has evolved to respond to invading micro-organisms such as bacteria, viruses, fungi and protozoa, and protect the individual from lethal infections. The same system frustrates the outcome of organ transplantation in seeing the lifesaving new organ as an alien element, judges it as dangerous and tries to eliminate it. This system is called the Immune System and can be divided into two main sections A: the innate immune system and B: the adaptive immune system. The innate immunity is considered to act as a fast, aspecific first line of defence. The adaptive immune response is more specific and becomes active when the first line of defence is not effective enough in eradicating the alien invasion. Recent insights suggest that the innate immunity may have an additional role in determining to which antigens the adaptive immune system will respond and in the nature of that response (*reviewed in ref 1*). Important parts of the innate immune system are skin, mucosal tissues, the complement system and phagocytes such as macrophages and granulocytes.

The specific immune system has two compartments, the humoral immune system, involved in the production of antibodies (immunoglobulins) by B lymphocytes, and the cellular immune system, involved in killing virus infected host cells and foreign cells by cytotoxic T lymphocytes (CTL) and the activation of B cells and macrophages by soluble mediators, called cytokines, produced by helper T lymphocytes (HTL).

The ability of B and T lymphocytes to recognize foreign structures (antigens) specifically, is mediated by antigen-specific receptors on the surface of these cells. Immunoglobulin (Ig) molecules are the antigen specific receptors on B lymphocytes, while the T cell receptor (TCR) has this function on T lymphocytes.

1.2 The T Cell Receptor

The TCR is a heterodimer composed of two chains, closely linked to the CD3 complex, which plays an important role in signal transduction from TCR to cytoplasm[2]. Two types of

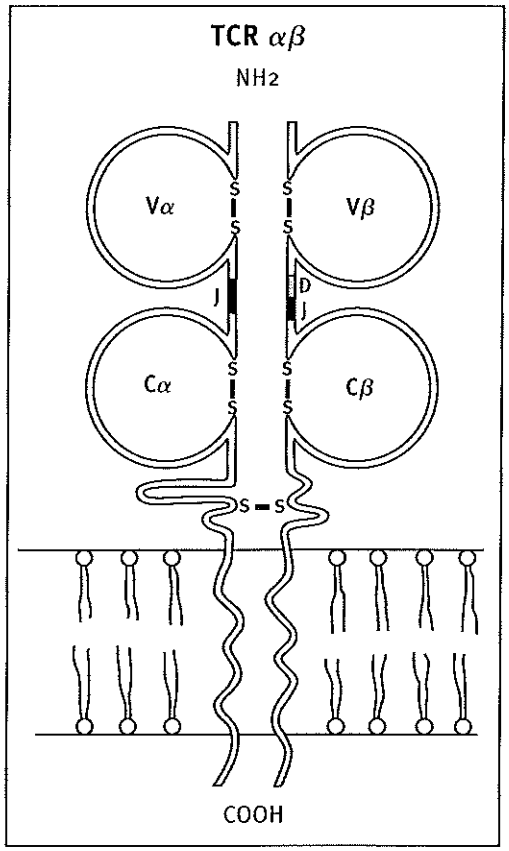


Figure 1.1 *Schematic presentation of the T-cell receptor αß*

TCR have been identified, each associated with a distinct T cell lineage. The majority of the mature T cells in peripheral blood and peripheral lymphoid tissues bear the TCR-α ß which consists of an α and ß chain, each with variable (V), diversity (D), joining (J) and constant (C) regions[3] (*Figure 1.1*). This is the main T cell population that mediates the specific immune response when antigen is encountered. For proper antigen recognition both, α and ß chains are required. Diversity in the TCR repertoire is achieved by gene rearrangements during T cell differentiation. Multiple germ line V, D, and J genes undergo ad random combinatorial rearrangements, after splicing mRNA is formed for TCR polypeptides. In the V domain of both α and ß chain of the TCR, 3 hypervariable regions

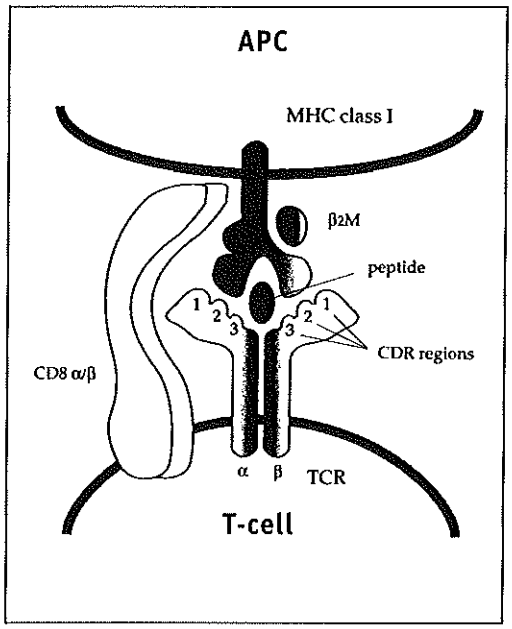
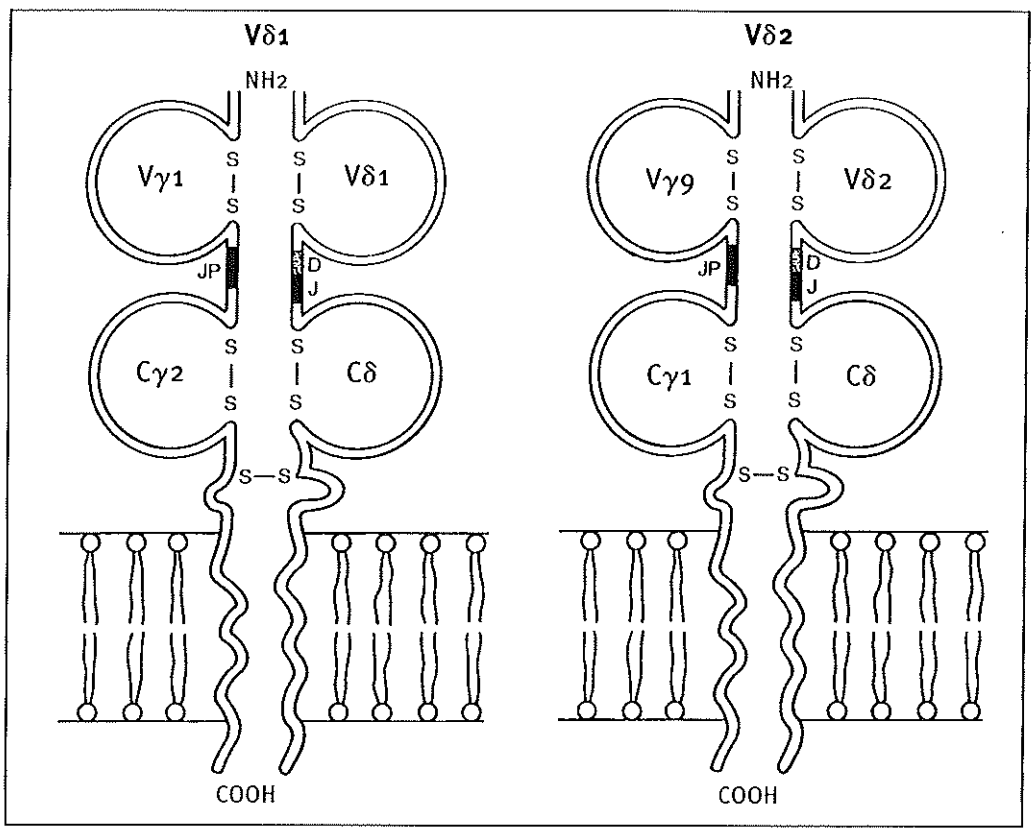


Figure (left) 1.2 *Schematic presentation of the key elements of the T-cell receptor (TCR) for recognition of peptides presented by HLA molecules. These elements are named CDR1, CDR2, CDR3, here marked as 1, 2 and 3. Depicted is a CD8 T-cell which recognizes an HLA Class I molecule on an antigen presenting cell (APC).*
(adapted from: PJ van der Elsen (Ed), Molecular Biol Intelligence Unit, RG Landes Company 1995, modified)

Figure 1.3 *Schematic presentation of the two main TCR-γδ T-cell subsets Vδ2 (A) and Vδ1 (B).*
(adapted from Lefranc and Rabbits, Trends Biochem Sci 1989; 14:214, modified)



are present, two are encoded by the V genes alone, and are called the complementary determining regions (CDR) 1 and 2, and the third one, the CDR3, is encoded by V(D)J genes. CDRs are arranged in such a way that the CDR3 regions of both α and ß chain form the central part of the TCR molecule whereas the CDRs 1 and 2 of both chains form the outer regions of the molecule in a symmetrical way[3] fig 1. It is thought that the CDR3 region binds to the peptide (antigen) in the groove of the Major Histocompatibility Complex (MHC) molecule (See §1.3.1) and the CDR1 and CDR2 are responsible for the interaction with the MHC molecule itself (*Figure 1.2*).
The other T cell lineage forms a minor T cell population in the adult peripheral blood and peripheral lymphoid organs and expresses a TCR consisting of a γ and δ chain (*Figure 1.3*).[4,5] The ligands and the exact function of these $\gamma\delta$ T lymphocytes are despite great effort in research still elusive.

1.3 The Major Histocompatibility Complex (MHC)

The evolution of the immune system of vertebrates has without doubt been driven by the need to survive pathogens. Especially the development of extensive polymorphism within the MHC seems crucial.[6]
The MHC gene complex encodes two major classes of peptide receptors, the MHC class I and class II molecules, whose function is to present antigen to $CD8^+$ and $CD4^+$ T cells respectively.[7] The genes of both classes are thought to originate from a common ancestral gene.
These loci separated at an early stage during evolution, probably in the late Cambrium, more than 500 million ago.[8]
Immunologists became aware of the polymorphism of the MHC when they noticed vigorous rejection of tumour grafts exchanged between two different members of a species.[9]
Transplantation studies showed that numerous genes controlled graft rejection, some were highly polymorphic and had a major influence on the rejection process.[10,11] In the mid fifties, similar histocompatibility antigens were demonstrated on human leucocytes.[12,13,14]
These serologically detectable, polymorphic antigen structures are now known as MHC class I molecules. Using cellular methods as mixed lymphocyte cultures (MLC) and later also with serology a second group of MHC antigens, the class II antigens, were detected.[15,16]
The human MHC is called HLA (Human Leucocyte Antigens).[17] Typing at the DNA level revealed that the HLA system was even much more polymorphic than already thought based on classical typing techniques. For A2 22 different alleles are known and for DR4 26 alleles are described now.[18] The genes coding for these antigens are located on the short arm of chromosome 6.[19,20] The genetic elements controlling the immune response and determining the specificity of T lymphocyte recognition were found to lie in the same region as the MHC loci.[21,22] It is now generally accepted that the MHC encoded class I and class II membrane glycoproteins, are involved in all of these T cell dependent phenomena as a consequence of their role in presenting peptides for recognition by T Cell Receptors.

1.3.1 HLA class I molecules

HLA class I molecules are surface glycoproteins consisting of a 45 kD heavy chain, which is non-covalently associated with ß2-microglobulin (ß2m), a 12 kD non-MHC-encoded soluble protein.[23] The heavy chain is encoded by the HLA-A,-B and -C genes of the HLA complex. The largest (extracellular) part of the heavy chain is organized into three globular domains, $\alpha1,\alpha2,\alpha3$.[24] The $\alpha3$ domain is connected to the cytoplasmic tail via a transmembrane part. The ß2m is associated with the $\alpha3$ domain.
Bjorkman and colleagues[25,26,27,28] elucidated the three-dimensional structure of the class I molecule, which revealed a peptide binding grove, formed by the $\alpha1$ and $\alpha2$ domains, required for presenting antigens to T cells (*Figure 1.4*). Each of the $\alpha1$ and $\alpha2$ subunits consists of four anti-parallel ß-strands followed by an α helix. The $\alpha1$ domain forms together with the $\alpha2$ domain a platform of a single eight-

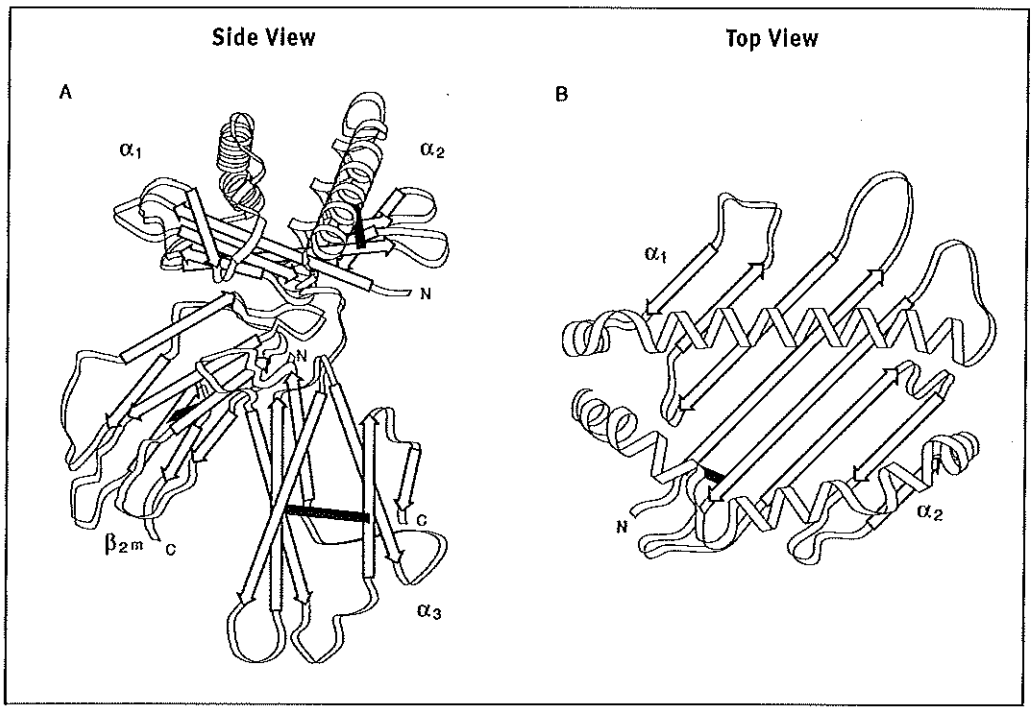


Figure 1.4 *Schematic presentation of the crystallographic structure of an HLA class I molecule. Panel A shows a side vieuw and panel B depicts a top view. The white arrows represent polypeptide folded as ß-pleated sheet. The coils represent polypeptide folded as α-helix.*
In panel A, the molecule is shown with the α3 domain and the ß2-Microglobulin at the bottom, and the polymorphic α1 and α2 domains at the top. The peptide binding groove is formed by the a1- and a2-helices.
Adapted from Bjorkman et al, Nature 1987; 329:506).

stranded ß-sheet covered by two helices. The large groove between the α-helices provides a binding site for, both foreign as well as self peptides. The polymorphism of the class I molecules is mainly caused by the differences in residues in the α1- and α2-domains which are located along the groove.[27,29] Functional residues pointing away from the peptide-binding site, or located out-side the groove may also be involved in allorecognition by the TCR of T cells. Crystallographic studies at high resolution revealed six subsites, called pockets A-F, located within the groove.[26,28,30,31,32] The ability of MHC molecules to bind to a diverse array of peptides is based on specific interactions with these pockets. The predominant length, nine residues, of the peptides associated with most class I molecules seems to be determined by the interaction of the N and C terminal ends of the peptide with conserved residues in the pockets A and F located at the opposite ends of the groove.[30,32,33] However between 20 and 40% of the peptides derived from class I molecules is found to be longer (up to 13 residues) than nine residues. In contrast to earlier data[34,35] a more recent study has shown that longer peptides can bind with affinities comparable to those of 9-mer sequences.[36] Polymorphic residues located in pocket F and four more centrally located pockets (B,C,D,E) influence the specificity of peptide binding. Strong selectivity of these pockets for particular amino acid side chains can give

rise to a motif of common peptide residues that are important for binding to different MHC alleles (*reviewed in 37*). Peptide binding to the class I heavy chain facilitates association with ß2m and stabilize the complex, allowing it to migrate to the cell surface[38] where it can be recognized by CD8 positive T cells. Class I molecules are present on most cells of the body, although the level of expression varies and is highest on haematopoietic cells. In most cells, the great majority of class I molecules are stabilized by peptides derived from cytosolic degradated, endogenously synthesized proteins.[39] Antigen presenting cells (APC) are also able to feed exogenous (foreign) proteins into the proteolytic machinery of the cytosol.[40,41] The peptides are generated in the cytosol by proteasomes and are translocated into the endoplasmic reticulum (ER) by the peptide transporters TAP (TAP stands for transporter associated with antigen processing). This TAP mediated transport is ATP dependent and peptide specific.[42,43] The genes for these peptide transporters map in the class II region of the MHC complex[44] and the proteins are localized largely to ER and cis-Golgi membranes.[45] The location and the critical role for TAP in providing ligands for class I molecules makes it clear that antigen processing for class I presentation occurs primarily in the cytoplasm.

1.3.2 HLA Class II molecules

The Class II molecules have a more restricted distribution, and are expressed primarily on B cells, macrophages, monocytes, dendritic cells. T cells, endothelial cells and many epithelial cells will express Class II antigens when activated. The HLA class II molecules are the products of the HLA-DP, -DQ and -DR genes. In contrast to Class I molecules, Class II molecules are composed of two membrane anchored glycoproteins, a 33 kD α-chain and a 29 kD ß-chain. Both chains consist each of two extra cellular domains, α1, α2 and ß1, ß2. Like the α3 and ß2m domain of class I molecules, the membrane-proximal α2 and ß2 domains resemble constant region domains of immunoglobulins.[46] With exception of the DR α-chain and DQ ß-chain, all other MHC class II α and ß chains are polymorphic. Similar to class I molecules the membrane-distal domains α1 and ß1 are most polymorphic ones.[47]

X-ray analysis of HLA-DR crystals revealed a peptide binding side formed by the α1 and ß1 domains, creating a floor of ß pleated sheets covered by two α helices, analogous to class I molecules.[48] Further analysis of a DR1 molecule presenting a peptide from influenza haemagglutonin showed five pockets in the groove P1, P4, P6, P7 and P9[49] determining specific peptide-binding. Synthetic peptides of nine, or even fewer amino acids can bind very well to Class II molecules but natural ligands are usually longer, ranging from 12 to 25 amino acids.[50] These 12- to 25-mer peptides extend out of the cleft, since the peptide binding groove of class II molecules is open at both ends,[48] this in contrast to the binding groove of class I molecules, which is closed on both sides.

Initially it was postulated that a class I molecule is stabilized by peptides processed in the endogenous pathway and therefore present only peptides processed by this pathway, while class II molecules preferentially bind peptides obtained through proteolytic processing of antigens internalized by endocytosis or phagocytosis, the exogenous pathway. However evidence is accumulating that a fraction of the class II stabilising ligands may be derived from endogenously processed proteins.[51] For MHC class I molecules convincing evidence demonstrates that APC, in addition to presenting endogenous peptides, can also acquire exogenous antigens. The processing pathway of these antigens, which results in peptides that bind to and stabilize the MHC class I molecules, starts in the cytosol with proteasome cleavages, and probably continues in the endoplasmic reticulum (ER) were peptides are trimmed to the right size for stabilizing class I molecules.[41]

1.4 Antigen presentation pathways and allograft rejection

Tissue transplantation provides the unique setting in which there are two sets of APC available to stimulate the immune response. This provides two distinct recognition pathways by which T cells can be stimulated by allo-MHC antigens. The direct route where T cells recognize allo-antigens as intact molecules on donor APCs and the indirect route where T cells recognize allo-antigens as peptides in the groove of host MHC molecules on host APCs after antigen processing.

1.4.1 Direct antigen presentation.

The response of T cells to allogenic MHC molecules is more vigorous than to "normal" environmental antigens. This stronger response might be due to the extraordinarily powerful stimulation of T cell by allo-antigens expressed on donor APC (the direct recognition pathway). The vigorous response has also been assigned to the 100-1000 times higher precursor T cell frequency for a given allo-antigen compared to the precursor frequency for peptides of nominal antigens.[52,53] Two hypothesis have been put forward for this phenomenon. First Matzinger and Bevan[53] proposed their so called multiple binary complex hypothesis, in which a single allogenic MHC molecule can acquire multiple distinct specificities by forming binary complexes with an array of peptides carried by the MHC molecule. Most of the peptides presented by APCs are derived from self proteins giving rise to a large number of allo+Xs(X_1+X_2+X_3 ....X_n) each combination is able to simulate a T cell of different specificity. A few years later Bevan[54] launched the high determinant density hypothesis. In this theory, alloreactive T cells recognize all allo MHC molecules, expressed at high density on the foreign APC, irrespective of the peptide bound in the cleft. This means that also T cells with much lower avidity will be able to respond to the foreign MHC. When determinant density is high even low affinity TCR can bind, but when determinant density is low fewer receptors bind in a meaningful way.

Although for both hypothesis evidence has been brought up the precise nature of the molecular interactions is still not entirely clear.[55]
T cell responses that result in early, acute rejection seem primarily due to direct recognition of HLA allo antigens present on donor type dendritic cells (DC). Circumstantial evidence for this was provided by Batchelor and coworkers[57] who showed that long-surviving rat [(AS x AUG)F1] renal allografts derived from tolerant AS rats were not acutely rejected when they were retransplanted into normal (non-immunosuppressed) AS recipients. It was postulated that those grafts lacked donor type passenger leucocytes. This hypothesis was confirmed by staining those grafts with mAb recognizing donor type class II antigens specifically.[58] Administration of mature, donor type DC to the AS recipients, resulted in a rapid rejection of the DC depleted kidney graft.[59] indicating that these mature DC can induce acute rejection. Passive transfer experiments of Braun et al[58] showed that T cells which recognise donor MHC molecules by the direct route can initiate early acute rejection of rat renal allografts when DC are present, but not in the situation when DC had migrated from the graft.

1.4.2 Indirect antigen presentation

The common route for nominal antigen presentation is the indirect pathway. The hosts APC (Dendritic Cells (DC) and Macrophages (Mo) play a central role in this pathway. They capture an antigen at the peripheral sites and migrate to the secondary lymphoid organs where they initiate the specific immune response by triggering naive T cells.[60,61] DC and Mo internalize the foreign antigen, degraded it into peptides and present those in the cleft of their Class I and Class II molecules to the T cells,[56,62-64] *(See also §2.1 and §2.2).*
Since depletion of donor type DC from an allograft does not lead to indefinitive graft survival [59] the indirect pathway may also be important in allo-graft rejection.[65]
Soluble Allo-antigens shedded from the graft, or on cellular debris from the graft are most

likely picked up and processed by recipients' DC and Mo, in the same way as nominal antigens. There is *in vitro* and *in vivo* evidence that peptides derived from allo-antigens (Major and Minor) are indeed present in MHC class I and class II molecules on the surface of host APC and can be presented to responding CD8 and CD_4 T cells respectively[66-73,78-85] and induce graft rejection in mice.[72]

1.4.2.1 Antigen presentation by MHC class II molecules

In vitro evidence that MHC class II molecules can present peptides derived from class II allo-antigens was provided by De Koster et al[65] who showed that CD_4^+ T cell clones could recognize an HLA-DR peptide presented in an HLA-DP molecule. This was subsequently confirmed by others in different systems.[66,67] Indirect presentation of class I peptides by MHC class II molecules has also been reported by Chen and colleagues,[68] and Essaket et al.[69] They showed that CD_4^+ T cell clones and T cell lines were able to recognize peptides derived from HLA class I molecules when associated with class II molecules. That this presentation process might be a common *in vivo* event can be concluded from the experiments of Chicz et al[70] in humans, and Hunt et al[71] and Benichou et al[72] in mice. Naturally occurring peptides, eluted from HLA-DR1 molecules were in majority sequences from self HLA-A2 molecules which were naturally processed.[70] A similar feature was found in mice, were class II (I-E) derived peptides were naturally occurring in class II (I-A) molecules.[71] In a murine transplantation model Benichou et al[72] showed that allo-MHC class II peptides presented in host class II molecules can induce skin graft rejection. In clinical transplantation definitive evidence for the involvement of indirect allo-antigen presentation pathway has not been reported yet.Experiments reported by the group of Shearer [73,74] suggesting that the indirect pathway could be measured in kidney transplant patients after depletion of donor APC in the stimulating spleen cell population could not be confirmed.[75]

1.4.2.2 Presentation by MHC class I molecules

The requirement of professional APC to pick up extracellular antigen, degrade it into peptides and present it to CD_4^+ T cells in association with MHC class II antigens, has been accepted for many years. However, until recently, the requirement for such a pathway in MHC class I restricted responses was rejected by most immunologist because of conceptual difficulties. [76] MHC class I-associated antigenic peptides would mark the presenting cell for a lethal attack by CTLs. And, more importantly, there was no established pathway by which APC would process antigens leading to presentation in MHC class I molecules. Though there were reports showing that donor MHC class I derived peptides, in association with host class I molecules, could be recognized by allo-reactive CTL. The first reports suggesting this possibility came from Shihanora et al[77] and Song et al[78] who made murine CD8 positive CTL clones that were reactive with peptides derived from allo MHC Class II or Class I molecules, in a self-class I-restricted fashion. Also in humans evidence supporting the view that indirect presentation via class I molecules is important in alloresponses was reported. Parham et al showed that peptides derived from the alpha 2 domain of HLA-A2 could block cytolysis by human CTL clones raised against HLA-A2.[79] Breur-Vriezendorp and Ivanyi showed that in humans a large proportion of allo-class I reactive CTLs are selfrestricted.[80] Furthermore it is evident that this pathway is responsible for the presentation of organ specific antigens[81] and for initiating the rejection of MHC matched bone marrow transplants.[82,83] The most important conceptual problem was eliminated recently, when agreement was achieved that professional antigen presentation must occur for class I-restricted responses (reviewed by Rock[41] and Bevan[76]). Different forms of professional presentation have been suggested: a) the existence of a pathway in phagocytes to shunt protein from the phagosome into the cytosol. Here it enters the normal MHC class I-associated pathway of antigen processing; b) heat shock pro-

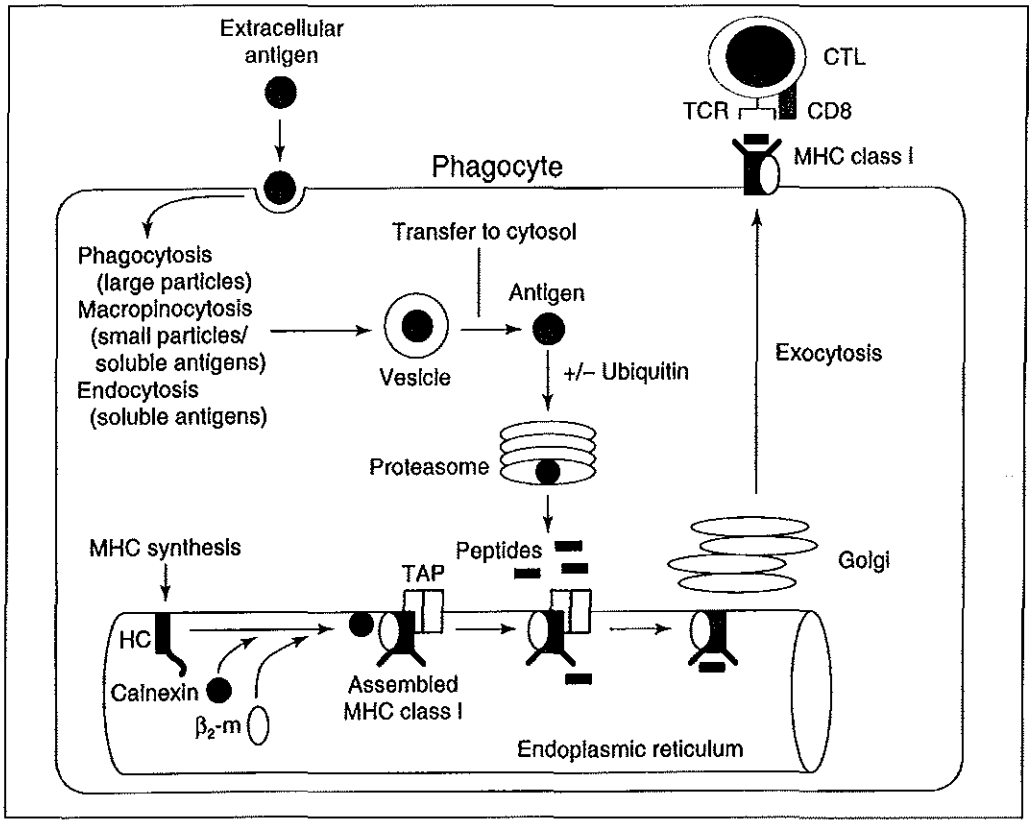


Figure 1.5 *The cytosolic pathway for presenting exogenous antigens on MHC class II molecules. HC = MHC class I heavy chain;β2-M = β2-microglobulin.*
(adapted from KL Rock, Immunol Today 1996; 17:131)

teins (HSPs), which participate in the normal transfer of proteasome-derived peptides to MHC class I, are released by dying cells, these peptide carriers can access the cytosol of professional APCs; c) phagocytes may digest ingested material in lysosomes and regurgitate peptides to load onto surface MHC class I (*Figure 1.5*).

1.5 Allograft rejection

After allografting four types of rejection can be distinguished based on the time of appearance.
1. **Hyper acute rejection** occurs within the first 24 hours after transplantation and is induced by pre-existing anti-donor antibodies[84] which cause obliteration of the interstitial capillaries by aggregation of platelets and destruction of the vascular endothelium by complement.[85] In clinical allografting hyperacute rejection is reduced to a very low incidence by the introduction of pretransplant crossmatching.[86]
2. **Accelerated acute rejection** starts within 5 days after transplantation and may be due to humoral and/or cellular immune reactivity. The incidence is rather low. In the first 200 heart transplants in Rotterdam 3 patients experienced this type of rejection, resulting in death of these patients.[87] The pathogenesis of this type of rejection is not clear but may involve anti-endothelial antibodies.
3. **Acute rejection** due to cellular effector mechanisms is commonly seen within the first three months after transplantation. However, at lower frequency, it can occur later. In kidney

transplant patients acute rejection is diagnosed by a deterioration of the renal function with increasing creatinine levels in the blood. Heart transplant (HTx) patients usually do not exhibit clinical signs of acute rejection until irreversible damage occurs. Endomyocardial biopsies (EMB) have to be taken and histologically examined at regular intervals to monitor acute rejection. Dense interstitial and perivascular infiltration of mononuclear cells with myocyte damage and interstitial edema is typical for acute rejection after heart transplantation.[88,89]

4 **Chronic rejection** is characterized by a gradual loss of graft function late after transplantation. In kidney transplant recipients a rather slow, progressive increase in blood creatinine levels in combination with proteinuria is seen. In heart transplant patients the diagnosis of chronic rejection largely depends on evaluation of coronary angiographs. With this method irregularities in the wall of the epicardial vessels are scored.[90,91] Systematic angiographic studies showed vascular abnormalities in approximately half of the grafts within the first 5 years after transplantation.[90,92] The narrowing of graft arteries and microvasculature is the result of a diffuse and concentric intimal thickening. This is caused by migration and proliferation of smooth muscle cells and fibroblast, from media to intima of the vessel wall, which is followed by deposition of extracellular matrix material in the intima.[93] The pathogenesis is still poorly understood and probably multifactorial. The process is thought be initiated by a combination of non specific factors e.g. reperfusion damage and cellular immune reactions evoked by the allograft. Cytokines may play a crucial role in the initiation and maintenance of this process.[94-97]

1.5.1 Inflammatory mechanisms in acute rejection

Morphological characteristics of the acute allograft rejection were first reported by Medawar in 1944 and 1945[98,99] from studies on skin grafts in rabbits. In the early sixties Waksman[100] described in detail the development of the histological characteristics during the rejection process of rat skin allografts. The essential structural changes in unsensitized recipients in the studies of both investigators were preceded by a diffuse cellular infiltration consisting of lymphocytes and histiocytes (now known as macrophages). In the same period Porter et al[101-103] stressed the significance of lymphoid cell infiltration in human and canine renal allotransplants, while Mitchison[104] and Billingham et al[105] showed that lymphoid cells could transfer transplantation immunity.

1.5.2 The role of cytotoxic T cells in allograft rejection

Already in 1960 Govaerts showed that sensitized lymphocytes were able to destroy allogenic target cells in tissue cultures in the absence of complement. The sensitized lymphocytes were obtained from the thoracic duct of dogs that had rejected a kidney graft with the same MHC specificity as the targets cells used in vitro.[106] The specificity of the cytotoxic reactivity was demonstrated in more detail by Rosenau and Moon.[107] Subsequently it has been assumed for many years that the cytotoxic T cell is the principal effector in graft rejection.[108-111] Delayed Type Hypersensivity (DTH), suggested by Billingham et al in 1954[105] as an alternative destruction mechanism in acute allograft rejection, became a popular concept in the eighties.[112-118] This DTH response was thought to be mediated by T helper cells.[112-118] McKenzie suggested that the production of cytokines, such as IL-2, resulted in the DTH response responsible for acute allograft rejection, since the kinetics of IL-2 production paralleled that of graft rejection.[118]

After the introduction of the hybridoma technique by Kohler and Milstein,[119] monoclonal antibodies (mAb) against lymphocyte subpopulations became available. Based on surface antigens, recognized by mAb, T cells in rats and man were divided into helper/inducer T lymphocytes (HTL) and cytotoxic/suppressor T lymphocytes (CTL).[120-124] In international Leucocyte Typing conferences all antibodies recognizing

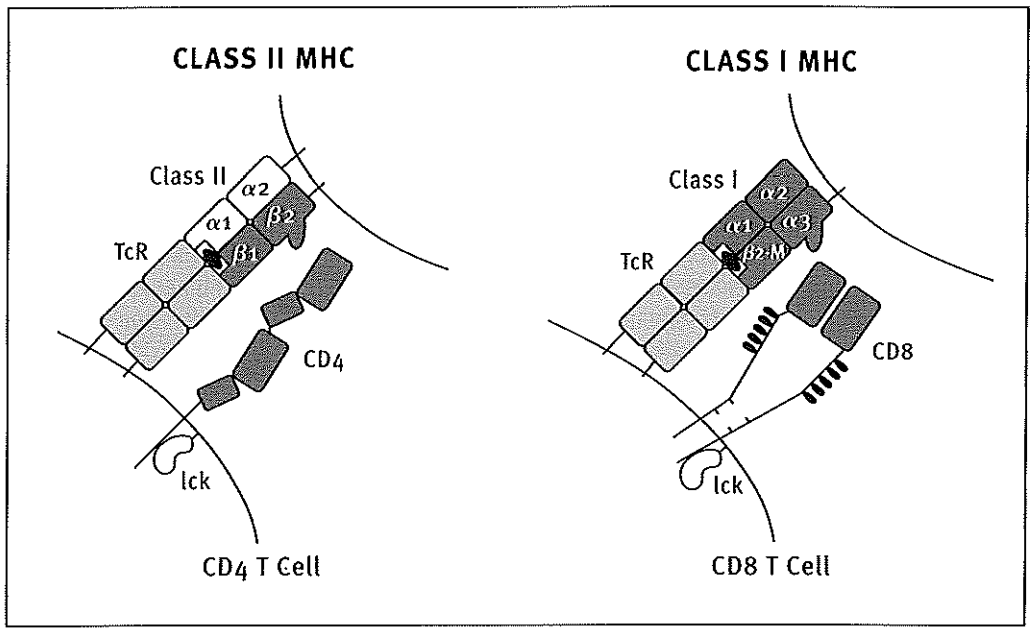


Figure 1.6 *Schematic presentation of the CD8 and CD4 molecules, interacting with MHC class I and CLass II, respectively.*

the same glycoprotein were grouped into a so called cluster of differentiation (CD). Initially the HTL became known as CD_4 positive T cells and CTL as CD8 positive T cells.[124]

The use of monoclonal antibodies against the T cell subpopulations in flow cytometric cell sorting, facilitated the studies of T cells involved in different immune responses considerably. The DTH concept as major effector mechanism in allograft rejection was mainly based on studies in lethally irradiated, thymectomised, bone marrow reconstituted (ATxBM) mice, restored with $Lyt1^+,2^-,3^-$ (CD_4-like) or $Lyt1^-,2^+,3,^+$ (CD8-like) T cells (113-115) and ATxBM rats restored with $W3/25^+$ (CD_4) or MRC-$Ox8^+$ (CD8) positive T cells (117). In the mouse model, sensitized $Lyt1^+$ cells but not $Lyt2^+,3^+$ cells could adoptively restore skin and tumour allograft rejection. In ATxBM rats $W3/25^+$ and not MRC-$Ox8^+$ T cells restored skin graft rejection. However the graft was in both cases infiltrated with MRC-$Ox8^+$ cells and macrophages of host origin.

Investigators[125,126] interpreted the results of this type of studies differently while some suggested a more regulatory role for HTL and a destructive role for CTL, others[127] criticized the results of the mouse experiments for a number of reasons. An important argument was the fact that Lyt1 serum recognizes CD_5 and not CD_4 as initially thought. Depletion with anti-sera to Lyt1 may also led to depletion of $Lyt2^+,3^+$ (CD8) cells, which also express Lyt1 albeit to a lesser degree than CD_4 ($L3T4^+$) cells. Furthermore ATxBM mice restored with sensitized $Lyt1^+,2^-,3^-$ (CD_4) cells had $Lyt1^-,2^+,3^+$ (CD8) specific CTL in the graft.[128]

Based on those early and more recent data, some investigators still believe in a limited role for cytotoxic T cells in graft rejection.[129-132] Recently VanBuskirk et al[129] reported that non cytotoxic, IL-4 producing CD_4^+ cells that were sensitized to a specific allo-antigen (B6), injected in Balb/c SCID mice, were able to reject the B6 allograft as fast as cytotoxic, non IL-4 producing CD_4 cells. Those results are confusing since they suggest that non cytotoxic Th2 (IL-4 producing) cells alone are able to mediate graft

rejection, whereas it has been postulated by others that IL-4 producing Th2 cells in mice are responsable for the induction and maintenance of antigen specific tolerance.[133,134]
An explanation for this discrepancy might be that the mice in the experiments of VanBuskirk[129] et al were depleted for $CD8^+$ cells, whereas the mice in the studies of Takeuchi[133] and Powell[134] were not. In mice depleted for $CD8^+$ T cells, eosinophils accumulate in the allograft, and cause graft rejection as shown by studies of the group of Bishop.[132,135] This accumulation and subsequent graft rejection by eosinophils seems only to occur in Th2 dominated alloresponses in CD8 depleted animals and not when $CD8^+$ T cells were present. This indicates that situations in which CTL seem not important for allograft rejection are not quite physiological ones.
More recent experiments supporting a limited role for CTL are clearly misinterpreted, in that all $CD4$ T cells were considered as being of the HTL type. No cytotoxic activity of these $CD4^+$ T cells was detected, which is not surprising when PHA blasts are used as targets.[131,132]
Several studies have shown clearly that $CD4$ T cell are able to kill MHC class II positive target cells.[136-139] Furthermore $CD4^+$ CTL can recognize allo-class I peptides in association with self-class II MHC, suggesting a much wider role for $CD4^+$ CTL in allograft rejection.[140,141]
Also in clinical studies the importance of class II directed cytotoxic T cells has been demonstrated in relation to allograft rejection.[142,143]
The absolute functional separation between $CD8^+$T cells as CTL and $CD4^+$T cells as HTL is not valid anymore, since it has been shown that also $CD8^+$T cells can function as HTL by producing cytokines. Moreover recently it became clear that based on their cytokine production pattern $CD8^+$T cells can be divided into Tc1 and Tc2 cells, like $CD4^+$ Th1 and Th2 cells.[144,145] The concept of Swain,[146] that $CD8^+$ T cells are associated with MHC class I restricted and directed responses and $CD4^+$ T cells with MHC class II restricted and directed responses is now established for cytotoxic activity as well as for cytokine production. For the inflammatory response it implies that both $CD4^+$ and $CD8^+$T cells may have regulatory functions by means of their cytokine production. Both subpopulations are able to destruct transplanted tissue too as both may exert cytotoxic activity in which $CD4^+$T cells react to class II antigens and $CD8^+$T cells to class I antigens. Both subsets also can recognize and react to minor antigens presented by self MHC class II and I antigens respectively. So there is ample evidence that both T cell populations are important, and may complement each other (reflecting the redundancy of the immune system).
Although there is now compelling evidence that cytotoxic T cells can destroy allografts, CTL can be recovered from well functioning allografts too.[147-152] Mason[150] gave two possible explanations for this enigma. 1. It may be that cytotoxic T cells are responsive to peptides (present in association with graft class I MHC antigens) that are present on the *in vitro* targets but not in the allografts. 2. Active inhibition *in vivo*, but not *in vitro*, possibly by cytokines. However non of these possibilities have been established yet. Wood and Streilein suggested a qualitative difference between CTL harvested from tolerant mice and those propagated from normal rejecting animals. CTL present in the graft from rejecting animals had high avidity for the donor antigen whereas CTL in the graft of tolerant animals had low avidity.[151] We have used this concept to study the difference between heart transplant patients that never had a rejection and patients that experienced one or more rejection episodes. In cardiac allografts with histological signs of acute rejection predominantly CTL with high avidity for donor class I antigens were found. In grafts from patients that never had acute rejection the majority of the $CD8^+$ CTL had low avidity for donor antigen,[152] (*Chapter 4 of this thesis*).

5.2.1 The avidity of CTL

The avidity of a T cell for antigen is determined by a:the affinity of the TCR for the class I/antigen peptide or TCR-class II/antigen peptide

interaction and the MHC-CD8 or MHC-CD4 interaction respectively; b:the density of the TCRs on the surface of the T Cell, and c:MHC/peptide density on the target cell or the MHC/peptide concentration.

CD8 and CD4 serve as associative recognition elements that stabilize TCR-antigen interaction by binding to non-polymorphic regions of class I and class II molecules respectively. CD8 coreceptors interact with MHC class I molecules through an acidic loop within the membrane proximal α_3 domain of class I.[153-156] In human MHC molecules, three clusters of amino acids in the region of residues 222-247 have been implicated in this interaction, with an exposed, negatively charged loop including residues 223-229 playing the dominant role.[154,155] Mutations in this region reduced lysis by CD8-dependent CTL .[154,155] Especially substitution of lysine for glutamic acid at position 227 produced a most striking reduction in both binding and cytotoxicity. CD4 interacts with the residues 134-148 of ß2 domain of the class II molecule, a region highly homologous to the residues 223-229 of MHC class I binding site for CD8.[157,158]

The structural information on both CD4 and CD8 are compatible with data suggesting that simultaneous binding of TCR and CD4 or CD8 to the same MHC/peptide antigen complex is required for maximal T cell stimulation.[156,159] Both CD4 and CD8 seem capable of spanning the length of the TCR and a part of an MHC molecule to bring their NH_2-terminal domains into contact with the membrane proximal domains of the MHC molecule on the apposing target cell or APC (*Figure 1.6*).

CD8 and CD4 perform a cosignalling role in T cell activation, in parallel with TCR-CD3 complex (reviewed in the references 160,161,162). The src-like tyrosine kinase, $p56^{lck}$, associated with the cytoplasmic domain of both CD4 and CD8 may be involved in the transmembrane signalling.[163,164] However, in some transfection experiments enhancement of the response was observed, even when TCR with specificity for class I antigens were transferred to CD4 cells or TCR with specificity for Class II to CD8 cells.[165,166] Therefore, expression of these coreceptor molecules can confer benefits which cannot be explained alone by co-localization of lck and CD3zeta/ZAP-70 complex amplifying signal transduction. Hence it is postulated that there is a significant contribution to the stability of the interaction between a T cell and its stimulator or target cell from CD4 or CD8 molecules binding MHC. It is generally assumed that T cells which benefit the most from such interactions, and which are most dependent on CD8 or CD4, express low affinity receptors for antigen, those T cells are known as low avidity cells.[138,165,167-176]

McDonald et al[167] were the first who suggested that the avidity of a $CD8^+$ CTL population or clone can be determined by the addition of anti-CD8 mAb in the cytotoxic assay. In the presence of anti-CD8 mAb, $CD8^+$ CTL with low avidity are not able to lyse their targets anymore, whereas $CD8^+$ CTL with high avidity still do. Biddison and coworkers showed the same for the Class II directed CTL, the function of $CD4^+$ CTL with high avidity could not be inhibited by monoclonal anti-CD4 antibody, whereas $CD4^+$ CTL with low avidity could.[138,175]

1.5.3 TCR γ/δ⁺ T cells in allograft rejection

TCR γ/δ^+ T cells can be divided into two major, mutually exclusive, subsets. The Vδ1 population expresses a TCR using Vδ1 gene products rearranged to Jδ1 in association with members of the Vγ1 gene family, and can be identified by the mAb δTCS-1.[177,178] The Vδ2 population carries a TCR composed of a Vγ9-JγPCγ1-positive γ-chain associated with a $V\delta2^+$ δ chain, and can be identified by the mAbs TiγA recognizing the Vγ9 gene product, and by BB3 or 15D recognizing the Vδ2 gene product.[179-183] The Vδ2 cells are the major population in PBL of most human adults, and comprise ± 70% of the TCR γ/δ^+ T cells.[177,178]

Although clones has been described that recognize classical serologically defined MHC antigens[184-186] the frequency of such TCR γ/δ^+ clones derived from a mixed lymphocyte culture is

very low.[186,187] Similarly, while it has been possible to obtain γδ clones by immunization with peptide antigens, recognition of these peptides is usually not MHC restricted (reviewed in 188). However, intact rather than processed polypeptides appeared to be recognized by γδ T cells.[189]

TCR γ/δ cells have been cultured from heart-,[180,191,192] kidney-,[193] and lung-allografts.[194] Although a relation with acute[193] and chronic[191] rejection is suggested, their function in the rejection process is not clear since no donor specific reactivity could be found. The infiltration of TCR $\gamma\delta^+$ T cells in the transplanted heart, the subpopulation involved and their significance for graft rejection is an object of studie in this thesis.

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CHAPTER 2

BACKGROUND AND AIM OF THE STUDY

The main objective of the studies described in this thesis is to analyze type and nature of T cell populations present in the graft and peripheral blood of heart transplant recipients. The final aim of these studies is to monitor immunological processes leading to acute rejection or indicating stable engraftment after clinical transplantation, allowing reduction of immunosuppressive load in the latter.

2.1 Peripheral blood

Many attempts have been made to correlate immunological parameters in PBL with the rejection status of the graft. The mixed lymphocyte reactivity (MLR), often in combination with the cell-mediated lympholysis assay (CML), first demonstrated Brunner et al,[1] is widely employed as *in vitro* test to detect alloreactivity. When correlating the outcome of these assays with the occurrence of early acute rejection or stable engraftment variable results were obtained.[2-9] In several studies a relation was found between MLR or CML hyporesponsiveness and longterm graft survival.[4-7] However others showed that CML hyporesponsiveness disappeared when performed at higher effector-to-target cell ratios[9] or when, instead of PHA-blasts, B-LCL were used as targets in the CML assay.[10]

The main problem with MLR and CML assays is that they are not quantitative at the cellular level and therefore falls negative results may be obtained, especially when no class II differences are present between donor and acceptor. To overcome this problem, limiting dilution assays (LDA) are employed to determine the frequency of donor directed CTL precursors (pCTL), HTL precursors (pHTL) and in vivo activated or committed donor specific CTL (cCTL) and HTL.

With LDA, the frequency of proliferating alloreactive T cells[11,12] pCTL,[11-14] cCTL[15] pHTL[16-18] and cHTL[19] has been determined in several transplant models.

The frequency of *in vivo* activated donor directed cCTL can be measured after in vitro stimulation with autologous instead of donor cells and the use of donor cells as target.[15] In the presence of IL-2, autologous cells only support the proliferation of T cells that express activation markers (e.g.IL-2 receptor) obtained due to previously encountered allo-antigen. In this system no *de novo* activation of pCTL will take place *in vitro*. Subsequently pCTL frequencies (pCTLf) can be determined by subtracting cCTLf from the total pool of CTL (tCTL), determined after stimulation with donor cells that will give *de novo* activation *in vitro* of pCTL as well as growth support to already activated cCTL.

The read out system for HTL frequency (HTLf) measurement is IL-2 production. In mice the frequency of *in vivo* activated, donor specific cHTL can be determined when the responder cells are mildly (2Gy) irradiated, before donor alloantigen is added.[19] cHTL are still able to produce IL-2 after mild irradiation, while pHTL (non IL-2 producing) can not be induced to do so by donor antigen after irradiation. pHTL can be calculated by subtracting cHTLf from the total HTLf determined after stimulation of non irradiated responder cells with the same alloantigen. However, in humans the mild irradiation technique to measure cHTLf seems not to be applicable.[20]

In PBL of humans it was found that each individual has a different pCTLf for every known HLA class I and II-specificity [20-24]. It has also been shown that high pCTLf are associated with in a more potent immune response after an antigenic challenge.[25] In line with this observation is the correlation between high pCTLf and the incidence of severe acute graft versus host disease after bone marrow transplantation.[26,27]

After solid organ transplantation the correlation between pCTLf in PBL and transplantation outcome is still a matter of debate.

Several groups studied the relation between the level of donor specific pCTL in PBL and acute rejection,[28-31] or during stable engraftment.[32-37] Only Reader et al[31] claimed a significant increase of pCTL frequencies during AR, whereas results concerning relation between stable engraftment and the frequency of alloreactive cells was very heterogeneous. In these

studies peripheral blood lymphocytes were stimulated with donor cells. In this way both *in vivo* activated cCTL as well as their immature precursors (pCTL) are enumerated. In the peripheral blood pCTL might be more abundant and will mask the probably more relevant cCTL when stimulated with donor cells. Studies in mice indeed have shown that cCTL, responsible for the effector function in graft rejection, are present at very low frequency in PBL.[19] In humans no reports are available that studied the relation between rejection and the frequency of cCTL in graft and PBL..

2.2 Intragraft

Immunohistochemical studies have provided valuable information about the phenotypic nature of cellular infiltrates present in the transplanted human heart during allograft rejection.[38-41] However, just phenotyping can not address questions about function and specificity of the infiltrating T cells. Functional determination of primed, donor-committed T cells may help to differentiate between allogenic processes involved in acute and chronic rejection and inflammatory processes due to infection, preservation or reperfusion injury. Moreover, studying the function of cellular infiltrates that are found at the time of stable engraftment may inform us on processes leading to non-responsiveness or perhaps tolerance.[42,43]

A direct approach to study the functional characteristics of infiltrating T cells is to isolate cells directly from the graft. Lymphocytes, mechanically or enzymatically isolated from rejected kidneys were studied in the 1970s (reviewed in 44). A major drawback of this type of investigations is the rather large amount of tissue needed, which implicates that only T cell functions can be studied in the endstage of the rejection process.

In 1983, Oka et al successfully cultured functionally active, donor-directed T lymphocytes from small biopsy pieces of canine renal allografts.[45] The biopsy pieces were grown in medium enriched with supernatant of mitogen stimulated T cells. During the next years several groups started to propagate and characterize lymphocytes from clinical renal,[46,47] liver-[48-51] and heart transplants[42,52-57] and chapter 3. Especially the studies in heart transplant patients are of interest. Since clinical heart allografts are monitored for acute rejection by serial endomyocardial biopsies taken weekly during the first 6 weeks posttransplant twice a month up to 10 weeks and with increasing intervals thereafter, biopsy material became available independent of the rejection status. This provided the opportunity to study quantitative and qualitative differences in phenotype and function of graft infiltrating cells obtained during acute rejection and stable engraftment. The studies showed that growth correlated well with the histological rejection grade in cardiac[42,53-57] and liver transplants.[49,50] From biopsies with a more profuse lymphocyte infiltrate, lymphocyte cultures could be established more often. It was also reported that growth from biopsies with no or small infiltrates was associated with a higher incidence and occurrence of a subsequent rejection episode,[43] although this was not a general finding.[57,58] Most studies have shown that infiltrates propagated from the biopsies predominantly consisted of T cells. Both $CD8^+$ $CD4^+$ T lymphocytes were found that were able to recognize mismatched donor HLA- antigens in cell mediated lympholysis assays and/or primed lymphocyte tests. Some studies showed that HLA class-I specific cells were more predominant in earlier biopsies, followed by mixed HLA class I/II or class II specific cells in later biopsies.[59,60] Those studies did not address to questions such as differences between infiltrates propagated from EMB taken in an early post operative period with relative frequent episodes of acute rejection and EMB taken in later post-transplant periods when acute rejections are rarely seen. Also the difference between infiltrates found in patients with at least one acute rejection and patients that never had a rejection were not systematically studied.

From the above reviewed literature it is obvious

that additional studies were necessary to clarify the differences between infiltrates obtained from EMB during rejection or stable engraftment. In particular the difference in avidity, and the specificity range of the CTL populations propagated from grafts in both situations was not known. Also the appearance in peripheral blood of pCTL with high avidity and cCTL in relation to rejection was not studied. Furthermore, the role of TCR-$\gamma\delta^+$ T cells in transplant rejection and acceptance was never subject of investigation in human transplant patients when we started the studies described in this thesis.

2.3 The objectives

The first study of this thesis (*Chapter 3*) concerns the characterization of growth pattern, phenotype and functional capacity of lymphocytes propagated from EMB taken early and late after clinical heart transplantation, both in patients that never had an acute rejection episode, and in patients that experienced one or more acute rejection episodes. Special attention was given to the difference in specificity of the cytotoxic T cells. In *chapter 4* a more detailed analysis is made of the dynamics of the T cell infiltrate during a rejection process, based on the TCR-Vα and TCR-Vβ gene usage of donor-specific CTL.
In *chapter 5* the presence of cCTL in the peripheral blood is described in relation to acute rejection.
In *chapter 6* qualitative characteristics, such as cyclosporin A resistance and the avidity of pCTL and cCTL propagated from EMB with myocytolysis (acute rejection) or obtained from patients that never had an acute rejection, are described.
The analysis in *chapter 3* revealed the presence of TCR-γ/δ T cells in several cultures in particular those propagated from EMB taken more than 1 year after HTx. In *chapter 7* the function of these TCR-$\gamma\delta$ cells is analyzed in more detail, and their relation to acute and chronic rejection is studied. In *chapter 8* their appearance in the peripheral blood, and the possible influence of the cyclosporin A medication on the differentiation of TCR-$\gamma\delta$ T cells was subject of study.
Finally, in *chapter 9* a pilot study is described in which the frequencies of pCTL with high avidity for donor HLA-antigens and of donor-specific IL-2 producing HTL in PBL of non rejectors and rejectors during a rejection episode are compared.

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CHAPTER 3

ALLOREACTIVE LYMPHOID INFILTRATES IN HUMAN HEART TRANSPLANTS

Abstract

From 535 endomyocardial biopsies (87 heart transplant recipients) 283 cell cultures could be generated. All cultures tested contained T lymphocytes, CD4 was the predominant phenotype in the first 6 months after transplantation. A significantly higher proportion of CD8 dominated cultures was found among cultures from biopsies without myocytolysis.

Propagation kinetics in this study revealed that donor-specific CTL are relatively late released from the biopsy fragments during culture. Most GIL cultures established in first 4 days after onset of the EMB culture were not cytotoxic for donor cells. GIL cultures established between 6 and 8 days after start of the EMB culture predominantly contained CTL, specific for donor antigens.

In the first three months after transplantation 57% of cultures showed cytotoxicity against both class I and class II mismatched donor MHC antigens, decreasing to an incidence of 33% more than 3 months after transplantation. This proved to be due to a significant decrease ($p=0.01$) in the number of cultures with HLA class II directed cytotoxicity.

This study shows that early after transplantation a heart transplant is infiltrated with activated donor specific cytotoxic T cells that recognize a broad spectrum of mismatched donor MHC antigens, and that in time this reactivity spectrum becomes more restricted.

3.1 Introduction

The diagnosis of rejection after clinical heart transplantation is based on histological criteria.[1] Therefore endomyocardial biopsies (EMB) are taken at regular intervals after transplantation. This provides the opportunity to culture graft infiltrating cells, and enables us to evaluate growth patterns, phenotypic composition and function of these cells both in periods of stable engraftment and during periods with acute rejection.

The biopsies were cultured in IL-2 conditioned culture medium in the presence of irradiated autologous PBMC as feeder cells, thereby assuming that only in vivo activated lymphocytes will proliferate.[2,3,4] Neither donor nor third-party cells were added within the first 3 weeks of culture to avoid de novo activation in vitro against mismatched donor antigens.

In this report, we describe the phenotypic and functional characteristics of lymphocytes grown from EMB, and the relation of these findings with time after transplantation and histopathological diagnosis. Additionally we tried to expand all T cells present in the biopsies of two patients, by polyclonal stimulation of the cultures with PHA in the presence autologous feeder cells. By seeding the newly propagated cells every other day during the first ten days of culture, we tried to obtain monoclonal cultures and to study the kinetics of the outgrowth of the infiltrated cytotoxic T lymphocytes (CTL) from the EMB pieces during the first 3 months after HTx.

3.2 Materials and Methods

3.2.1 Patients

We studied 535 biopsies from 87 heart transplant recipients. All patients had received preoperative blood transfusions and all received cyclosporin A and low dose prednisone as maintenance immunosuppression. Acute rejection episodes, i.e. biopsy proved myocytolysis, were treated with bolus steroids or with a two weeks course of a polyclonal rabbit anti-thymocyte globulin preparation in case of ongoing rejection. Their actuarial graft survival at three years was 89%.

During this transversal study we received two to eighteen EMB from each patient (median 4). EMB were taken 5 - 1587 (median 114) days after transplantation. In the early post transplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to once every four months at one year. After an acute rejection episode the next biopsy was taken one week following cessation of the rejection therapy. During right ventricular catheterisation four or five biopsy samples were obtained. Three or four were used for histologic evaluation, and one was placed in RPMI-1640 for tissue culture. The histological rejection grade was assessed according to Billingham's criteria:[1] Grade 0: no evidence of rejection, no infiltrate. Grade 1: mild infiltration of mononuclear cells. Endocardial and interstitial edema; diffuse perivascular and endocardial infiltration with pyroninophylic lymphocytes. Grade 2: moderate rejection. Perivascular, endocardial and interstitial infiltrates with pyroninophylic lymphocytes. Focal myocytolysis (necrosis). Grade 3: severe rejection. Vessel wall- and myocyte necrosis with interstitial bleeding. Interstitial infiltrates with polymorphonuclear cells and pyroninophylic lymphocytes. (This rejection grade was not observed in our study) Grade 4: resolving rejection, Active fibrosis, some small, non-pyroninophylic lymphocytes, some plasma cells and haemosiderin.

3.2.2 Culture method

Each biopsy was divided into two or more fragments and placed into two or more wells of a 96-well roundbottom microtitre culture plate (Costar 3799, Cambridge, MA) with 200 μl culture medium in the presence of 105 irradiated (40 Gy) autologous PBMC as feeders. Culture medium (CM) consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100

μg/ml streptomycin. PBMC were isolated by Ficoll-Isopaque (δ = 1.077) density gradient centrifugation. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Half the culture medium was refreshed every 2-3 days. When growth was observed the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5 - 10^6 cells/ml). When growth was slowing down or cell death was observed the cultures were restimulated by adding either 10^5 irradiated (40 Gy) donor spleen cells/well or, when available, 10^4 EBV transformed donor cells/well (irradiated with 60 Gy). This was not done in the first three weeks of culture.

For EMB from 2 patients (PATA and PATC) a different procedure was followed. EMB pieces were cultured in one well of a microtiter plate (Costar) in CM containing 0.1μg/ml PHA (Wellcome, Beckenham, England), after two days of incubation biopsy fragment was removed. The GIL were seeded over 96 wells of a microtitre plate with CM, and 10^5 (irradiated) autologous PBMC and 5.10^3 (irradiated) third party B-LCL as feeders, this culture was called GIL day 2 culture (GCD2). The biopsy fragment was washed to remove adhering cells and cultured for another two days in the presence of irradiated autologous PBMC and third party B-LCL in CM with 0.1μg/ml PHA. After two days the biopsy fragment was removed again and the propagated GIL day 4 (GCD4) were seeded over 96 wells, similar as described for day two. This procedure was repeated two times more (GCD6 and GCD8). After ten days of culture no more T cells could be propagated from the biopsy fragment. Two weeks after restimulation of the mini bulk cultures GCD2, GCD4, GCD6 and GCD8, the number of growing wells was determined. Growing wells were split in two, and restimulated with random PBMC and third party B-LCL in CM. This procedure was repeated every 2 weeks till 8 daughter, mini-bulk cultures, were obtained from every original well. Those mini-bulk cultures were phenotyped and assayed for cytotoxicity against donor B-LCL, third party B-LCL and K562.

B lymphoblastoid cell lines (B-LCL) originated from infection of fresh PBMC or spleen cells with Epstein Barr Virus (EBV) obtained from the marmoset cell line B95-8 as described by Moreau et al.[5] These cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated bovine supplemented calf serum (High-Clone, Logan Utah).

T lymphoblastoid cell lines were obtained by culturing PBMC or spleen cells in RPMI 1640 supplemented with 5% pooled human serum, 5% v/v Lymphocult-T (Biotest) and 1% PHA.

K562 was cultured in RPMI 1640 with 10% heat-inactivated supplemented Bovine calf serum.

3.2.3 Phenotype analysis

Surface differentiation antigens were analyzed by two colour flow cytometry after staining with monoclonal antibodies directed against CD3 (anti-leu4) as a pan mature T cell marker, WT31 as a marker for the α/ß chain of the TCR and CD8 (anti-leu2) and CD4 (anti-leu3) as T cell subset markers. CD16 (anti-leu 11) and CD56 (anti-leu 19) were used as markers for NK cells. Antibodies were directly conjugated to fluoresceine (FITC) or phycoerythrin (PE) (Becton & Dickinson, Mountain View, CA). The presence of γ/δ T cells was demonstrated by the monoclonal anti-TCR-γ/δ-1 (clone 11F2)[6] by an indirect fluorescence technique. Cells were stained by incubating 0.5 - 1 x 10^5 cells in 50 μl Hanks Balanced Salt Solution (Biochrom KG, Berlin) supplemented with 1 % bovine serum albumin and 0.1 % sodiumazide for 30 min at room temperature with 2 antibodies conjugated to different fluorochromes. After washing, cells were analyzed on a FACScan flow-cytometer (Becton and Dickinson).

3.2.4 Lymphocyte Mediated Cytotoxicity

Cytotoxicity was tested against donor cells and a panel of unrelated target cells sharing one or more HLA antigens with the donor. 59 bulk cultures could be tested before restimulation with allogenic cells. The remaining cultures had to

Days after HTx	# Patients	# EMB Cultured	Growing	(%)	Phenotyped	Tested In CML
0-10	30	31	7	(23)	6	4
11-30	31	80	46	(58)	39	24
31-90	32	125	91	(73)	74	49
91-180	34	96	55	(57)	36	25
181-365	32	83	31	(37)	21	7
>365	50	120	53	(44)	24	17
Total	**87**	**535**	**283**	**(53)**	**200**	**126**

Table 3.1 *Culture results in relation to time after transplantation. Expressed as the number of growing cultures and cultures analysed for T cell phenotype expression and cytotoxic capacity.*

be restimulated in order to obtain sufficient numbers of cells. No effect of restimulation on CML specificity could be demonstrated after repeated testing. Three types of target cells were used: PHA-blasts (HLA class I targets), B-LCL (class I and II targets) and the K562 cell line for assessment of NK cell activity. A standard 4-hour ^{51}Cr-release assay was performed[7] with one effector-target ratio of 20:1. When possible E:T ratios varying from 1.25:1 up to 80:1 were used. Target cells were incubated for 1 1/2 hours at 37°C with 200μCi ^{51}Cr (Na_2CrO_4 5 mCi/ml, specific activity 350-600 mCi/mg chromium, Amersham, UK). 2.5 x 10^3 ^{51}Cr labelled target cells were mixed with effector cells in 0.2 ml of culture medium in 96 well roundbottom tissue culture plates. The plates were centrifuged at 600g for 1 minute, incubated at 37°C and supernatants were collected after 4 hours (Skatron AS, Lier, Norway). Spontaneous chromium release was determined by incubation of targets in 0.2 ml culture medium, and maximum release was obtained by adding 10% Triton X-100 detergent (5% v/v solution in 0.01 TRIS buffer) to the targets. Experimental release was measured, and specific lysis was calculated with the following equation:

$$\% \text{ Specific lysis} = \frac{\text{exp. release - spontaneous release}}{\text{max. release - spontaneous release}} \times 100$$

A CML assay was considered positive when the percentage specific lysis of donor antigen bearing target cells exceeded 10% and the slope of a graph was positive.[7] Series of double dilution studies revealed that lysis percentages of less than 10% are within the variation range of the assay (data not shown).

3.2.5 Statistical analysis

For statistical analysis of all data a Fischer's exact test, with Yates correction, was performed.

3.3 Results

3.3.1 Generation of lymphocyte cultures

In total 283 lymphocyte cultures were established from 535 EMB (53%). From the majority of the patients (72/87) cells could be successfully grown from at least one biopsy. From the

Days post HTx	Grade 0			Grade 1			Grade 2		
	# EMB	Growing	(%)	# EMB	Growing	(%)	# EMB	Growing	(%)
0-10	26	3	(12)	5	4	(80)	0		
11-30	25	10	(40)	46	30	(65)	9	6	(67)
31-90	25	16	(64)	83	60	(72)	17	15	(88)
91-180	26	14	(54)	59	32	(54)	11	9	(82)
181-365	42	9	(21)	41	22	(54)	0		
>365	75	29	(39)	44	23	(52)	1	1	
Total EMB	219	81	(37)	278	171	(62)	38	31	(82)
# Patients	78	52		64	53		23	20	

Table 3.2 *Relation between succesfull cell growth from EMB and histological rejection grade (Billingham's criteria) in different time intervals after transplantation.*

remaining fifteen patients only a few biopsies were available. Cell growth was most successful from EMB taken in the second and third postoperative months (*Table 3.1*). After three months a significant ($p<0.001$) decrease in successful propagation of lymphocytes from the EMB was observed when all EMB taken in the first three months were compared with all EMB taken more than 3 months after HTx. Table 3.2 shows that the rate of establishing cultures is positively correlated with increasing histological rejection grade. When no mononuclear cells were detected histologically (grade 0), cell growth was obtained in 37% of the cases. EMB showing infiltrates histologically (grade 1), yielded significantly ($p<0.0001$) more cultures than grade 0 biopsies, independently of the time after transplantation. Grade 2 biopsies gave significant ($p=0.018$) more cultures than grade 1 biopsies. For grade 0 and grade 1 biopsies the highest growth percentages were found between 11 and 90 days after transplantation with a peak in the second and third postoperative months (respectively 64% and 72%). After the first 90 days a decline of the growth rate was observed (for grade 1 biopsies $p=0.033$, for grade 0 not significant). For grade 2 biopsies growth rate was always high, independently of the time after transplantation (*Table 3.2*).

3.3.2 Phenotypic analysis

Flow cytometric analysis of cell surface molecules of 200 cultures revealed that the majority (89.5%) exclusively consisted of cells carrying the CD3 determinant. Almost all $CD3^+$ cells were $TCR\alpha/\beta^+(WT31)$. In 29 cultures only $CD4^+$ T cells were present, 15 consisted exclusively out of $CD8^+$ cells and in 3 cultures only $CD3^+WT31^-CD4^-CD8^-$ cells were found (TCR γ/δ^+ cells,see also below). All except three recipients whose EMB cultures could be phenotyped had multiple mismatches for HLA class I and II with their donors. No significant difference in the number of mismatches between donor and recipient could be demonstrated between patients supplying pure CD8 or pure CD4 cultures (Mean number of mismatches: 2,80 vs 2,85 for HLA A, B and 1.60 vs 1.45 for HLA DR respectively). In the remaining 153 cultures both $CD4^+$ and $CD8^+$ cells were found. CD4 was the predominant sub population (>60% of the

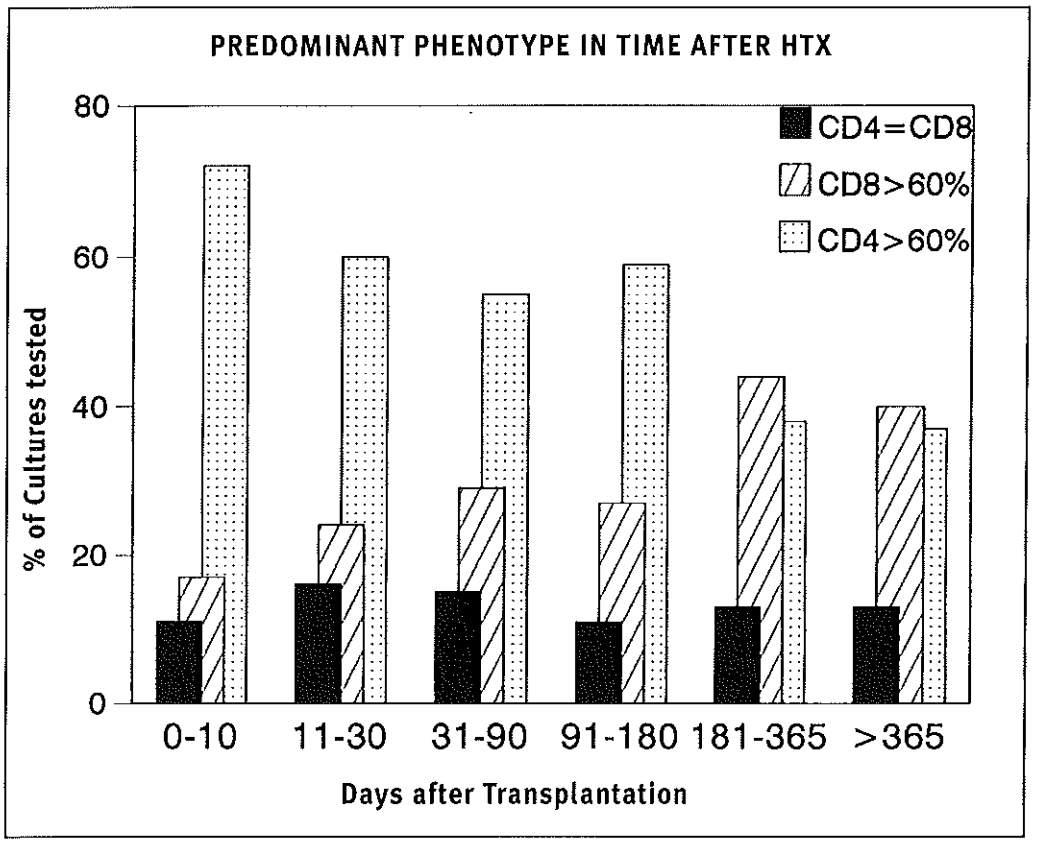

Figure 3.1 *Proportion of cultures dominated (>60 % of the cells) by $CD4^+$ or $CD8^+$ T cells, or containing nearly equal amounts of $CD4^+$ and $CD8^+$ T cells (CD4=CD8) in relation to time after transplantation. In the first 6 months after transplantation most cultures are dominanted by CD4 cells, after 6 months $CD4^+$ T cell dominated significant (p = 0.02) less cultures.*

T cells were CD4 positive) in most cultures obtained up to 180 days after transplantation (*Figure 3.1*). In the cultures propagated from EMB taken more than 6 months $CD8^+$ T cells dominated the same fraction of the phenotyped cultures as $CD4^+$ T cells. Again, no significant relation with the number of mismatches was found. In the cultures originating from biopsies with myocytolysis the preponderance of CD4 cells was more striking and a significantly lower proportion of CD8 dominated cultures was found (*Figure 3.2*, p = 0.01).
In 16 cultures from 13 patients $CD3^+WT31^-11F2^+$ cells (TCR γ/δ^+) were found in amounts varying from 5-100% of the cultured cells (median 27%). The EMB were taken between 29 and 1324 days post transplant (median 624 days). For further analysis of the TCRγ/δ T cells see chapter 7.
$CD3^-WT31^-$ cells were found in 21 cultures (19 patients). In these cultures 7% to 96% of cells expressed CD16 and/or CD56 antigens (median 24%), and EMB were taken from 6 to 1587 days post transplant (median 148 days). In seven cultures from various patients the NK cells were dimly CD8 positive, six of them were grown from EMB taken in the first post transplant year. We detected significantly more NK

	CML specificity	Grade 0 @ n (%)	Grades 1 and 2 n (%)	All GIL n (%)
< 90 days#		n* = 18	n = 59	n = 77
	Class I only	7 (39)	10 (17)	17 (22)
	Class II only	1 (6)	5 (8)	6 (8)
	Both class I and II	5 (28)	39 (66)	44 (57)
	negative	5 (28)	5 (8)	10 (13)
> 90 days#		n = 16	n = 33	n = 49
	Class I only	4 (25)	14 (42)	18 (57)
	Class II only	2 (13)	3 (9)	5 (10)
	Both class I and II	4 (25)	12 (36)	16 (33)
	negative	6 (38)	4 (12)	10 (13)
All GIL		n = 34	n = 92	n = 126
	Class I only	11 (32)	24 (26	35 (28)
	Class II only	3 (9)	8 (9)	11 (9)
	Both class I and II	9 (26)	51 (55)	60 (48)
	negative	11 (32)	9 (10)	20 (16)

More than 90 days after transplantation a significant decrease in the number of cultures with broad CML reactivity was found. This was due to a decrease in the number of GIL cultures with HLA class II-directed cytotoixicity propagated from grade 1 and grade 2 EMB (from 44/59 to 15/33 after 90 days, p=0.007). Most negative cultures were found among GIL cultures from grade 0 EMB (p=0.005) compared to grade 1 and 2 EMB.

@ histological rejection grade, Billingham's criteria; n*= number of cultures; # days after HTx.

Table 3.3 *CML reactivity of 126 bulk cultures (47 patients) against panel cells sharing either HLA class I or class II antigens with the donor.*

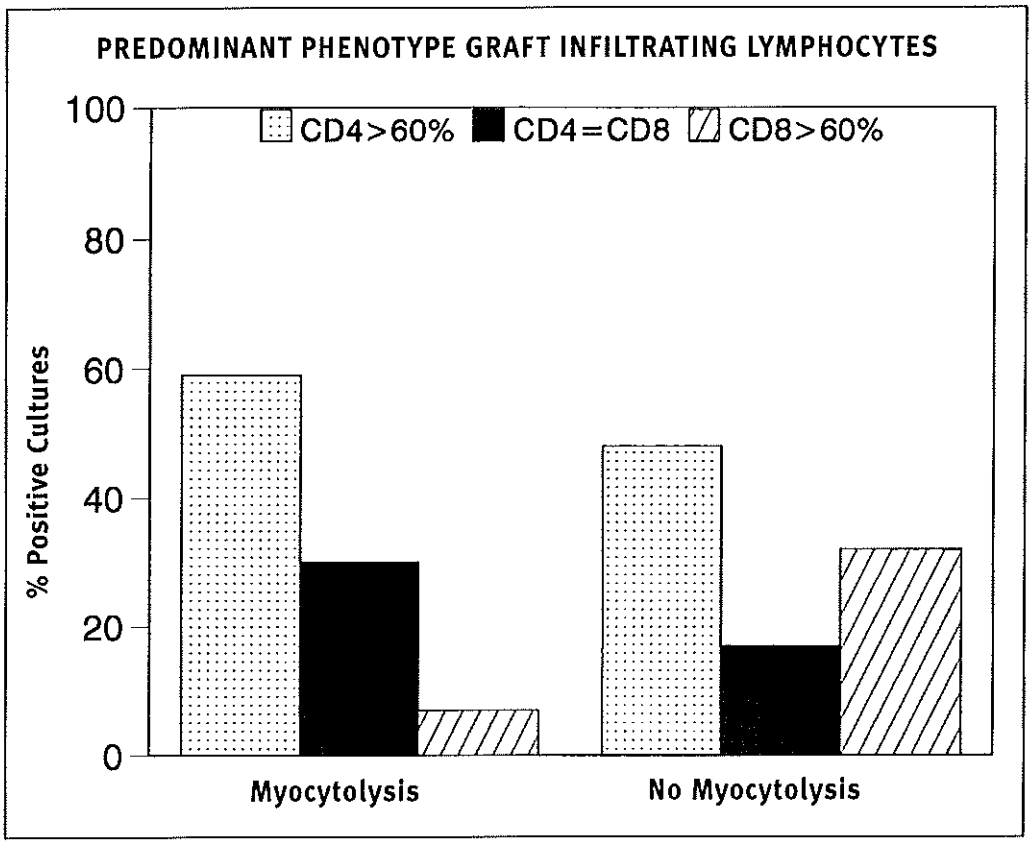

Figure 3.2 *Proportion of cultures dominated by (>60%) $CD4^+$, $CD8^+$ or containing equal amounts of $CD4^+$ and $CD8^+$ cells. The predominant T cell subpopulation in 173 GIL cultures derived from EMB without myocytolysis (histology grade 0 and 1) was compared with that of 27 cultures from EMB with myocytolysis (grade 2). During acute rejection a decrease in the number of cultures containing predominantly $CD8^+$ cells was observed (p = 0.01).*

cells in cultures from biopsies taken more than one year after transplantation compared to the first post transplant year (8/24 vs 13/176, p = 0.001). These cells were never grown from biopsies with myocytolysis.

3.3.3 Cell mediated cytotoxicity

From 126 EMB (47 patients) sufficient cells were generated to test cytotoxic function (*Table 3.1*). Lysis of donor antigen bearing target cells was found in 106 cultures. Table 3.3 shows the results of the specific CML reactivity against donor Class I and Class II antigens in relation to histological rejection grade, and time after HTx. Donor specific cytotoxicity against HLA class I antigens (either alone or in combination with reactivity against class II) was found in 75% of the cultures analyzed, and against class II antigens (alone or in combination with class I) in 56%. Reactivity against both class I and class II antigens was detected in 48% of the bulk cultures and restricted reactivity (i.e. against either class I or class II antigens only) in 37%. Cultures propagated from grade 1 and 2 EMB significantly more often showed multi-specific CML reactivity when compared to grade

o EMB cultures (*Table 3.3*, p = 0.005), while most negative CMLs were found among grade o EMB cultures (p=0.005, compared to grade 1 and 2 EMB cultures).
In time a significant shift was observed from a predominantly multispecific to a more restricted CML pattern when the cultures from EMB taken before and after three months were compared (*Table 3.3*). This proved to be due to a significant decrease in the number of cultures with HLA class II directed cytotoxicity derived from grade 1 and 2 EMB (p = 0.01).
Lysis of the K562 cell line was observed in 26/107 tested bulk cultures. In 22 of these also allospecific reactivity was found. NK reactivity did not show a significant relation with time after transplantation or histological rejection grade. Only 7/26 cultures contained cells with the CD16 and/or CD56 phenotype of which two had 70% and 64% $CD8^{dim+}$ NK cells next to $CD4^{bright+}$ T cells. One of the latter cultures showed donor specific lysis and lysis of K562, the other culture did not show donor specific lysis, but only killed K562. Five of eight tested $CD8^-$ NK cell containing cultures showed NK reactivity, two did not lyse donor antigen bearing panel cells. All four tested cultures with high percentages of NK cells (>55%) killed K562.

3.3.4 Rejectors versus non-rejectors

The group of 87 heart transplant recipients consisted of 59 patients who had experienced one or more rejection episodes (rejectors) and 28 patients who never had an acute rejection period. (non-rejectors). There was no significant difference in the number of HLA mismatches between donor and recipient in both populations (mean number of mismatches 2.78 vs. 2.79 for HLA A,B and 1.41 vs 1.21 for HLA DR respectively).
Generation of lymphocyte cultures from EMB from both patient groups showed similar growth patterns in relation to time after transplantation and histological rejection grade (*Table 3.4*). Cultures from biopsies from non-rejectors more often showed a predominance of $CD8^+$ T cells than EMB cultures from rejectors (*Table 3.5*, p = 0.012). This was due to the significantly higher proportion of CD8 dominated cultures derived from histology grade 1 EMB in the non- rejectors (p = 0.002) compared with grade 1 EMB cultures from rejectors), especially in grade 1 EMB preceding a grade o biopsy. No significant relation with time after transplantation could be demonstrated.
CML reactivity of GIL cultures from rejectors changed from a predominantly multispecific pattern in the first three months to a more restricted pattern thereafter (*Figure 3.3.*) In the first three months after HTx 67% of cultures were cytotoxic for both, target cells carrying mismatched donor HLA class I, and target cells mismatched for class II antigens, this decreased to 35% of the GIL cultures taken more than 3 months after HTx (p = 0.005). This decrease was mainly due to a loss of HLA class II directed cytotoxicity. In the GIL cultures derived from EMB taken from non-rejectors in the first three post-operative months, the number of cultures exhibiting cytotoxicity against both HLA class I and II antigens was significantly (p=0.035) lower than in the rejectors in the same period. The fraction of GIL cultures showing broad reactivity, obtained from the non-rejectors in the first three months, was comparable with that of the rejectors after three months. In the non-rejector group no decline in the percentage of GIL cultures with broad reactivity was found more than three months after HTx . The cytotoxic patterns of the GIL cultures obtained from rejectors after three months and from non-rejectors in the whole post-transplant period were comparable (*Figure 3.3*).

3.3.5 Propagation kinetics

From the EMB of PATA and PATC GIL cultures were established every two days, during the first ten days of culture, to obtain information about the time necessary to propagate donor specific CTL from the biopsy. From Table 3.6 and 3.7 it is obvious that the cultures in general were not monoclonal but poly or oligoclonal,

	Rejectors				Nonrejectors			
Days after HTx	# Patients	# EMB	Growing n	(%)	# Patients	# EMB	Growing n	(%)
0-10	17	17	5	(29)	13	14	2	(14)
11-30	18	44	25	(57)	13	36	21	(21)
31-90	20	72	45	(74)	12	53	38	(38)
91-180	24	73	52	(58)	10	32	13	(13)
181-365	23	59	25	(42)	9	24	6	(6)
>365	36	87	41	(47)	14	33	12	(12)
Total	59	352	191	(54)	28	183	92	(50)

Table 3.4 *Culture results of EMB from patients with at least one acute rejection episode (rejectors) and patients without rejection (nonrejectors).*

	Rejectors				Nonrejectors		
	Rejection Grade			Total	Rejection Grade		Total
T subset	0	1	2		1	2	
>60% CD4	13	39	16	68	14	17	31
>60% CD8	13	16	2	31	4	22	24
CD4=CD8	3	20	8	31	1	6	7
>60% WT31	3	2	1	6	0	0	0
Total	32	77	27	136	19	45	64

Table 3.5 *Predominant phenotype of GIL cultures from EMB of 59 HTx patients that expirienced at least one acute rejection episode (rejectors) and HTx patients that never had an acute rejection (nonrejectors).*

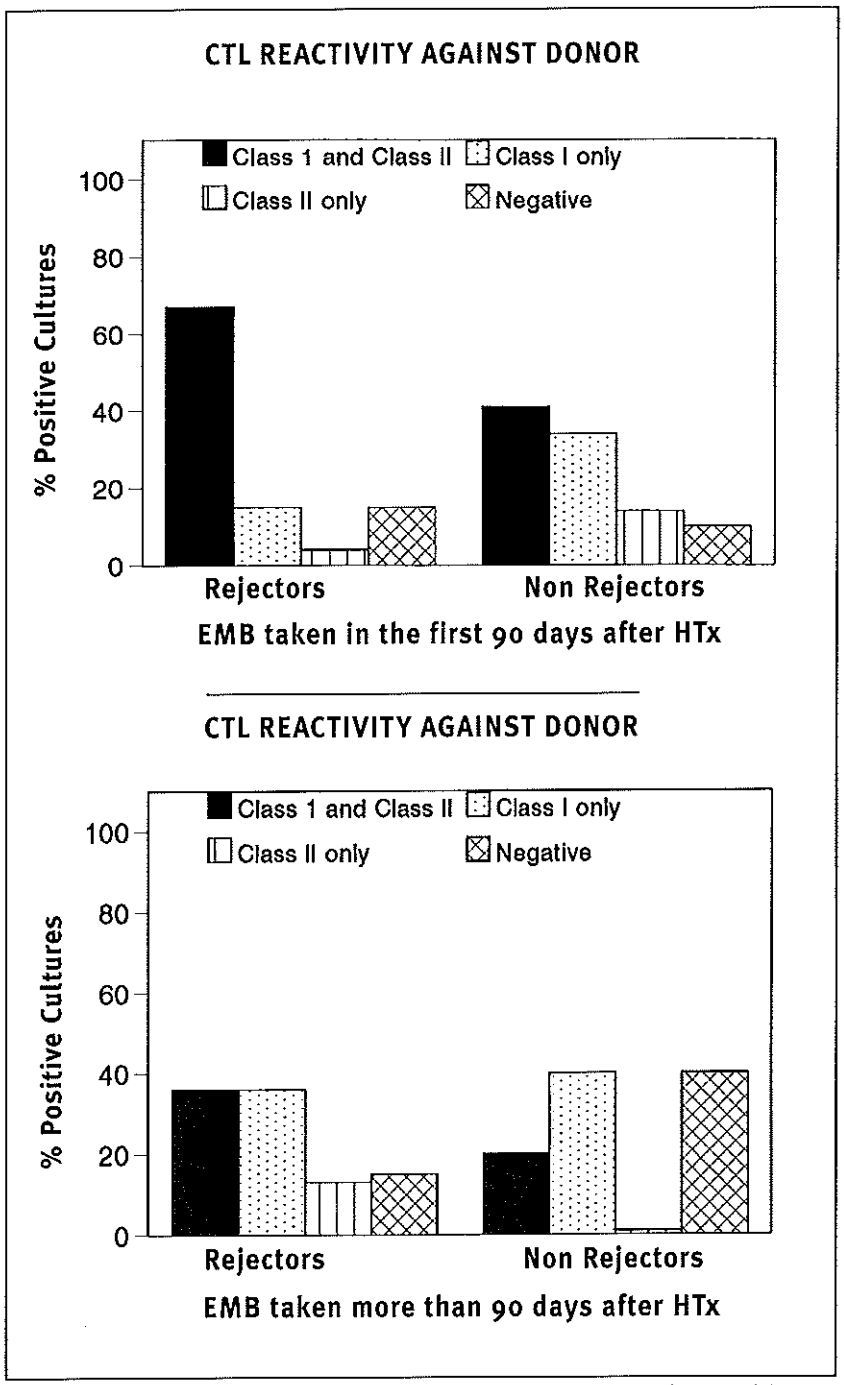

Figure 3.3 *Donor specific CML reactivity of 87 bulk cultures propagated from EMB of patients that experienced one or more acute rejection episodes (rejectors, n=33) and the CML reactivity of 39 cultures obtained from patients that never had an acute rejection (nonrejectors, n=14).*

In the first 3 months after HTx in the nonrejector group a significant (p=0.035) lower proportion of the GIL were cytotoxic for both class I and class II antigens than in the rejector group.
After 3 months the proportion of CIL cultures wth broad reactivity declined significant (0.005) to the level of GIL of nonrejectors before and after 3 months after HTx.

to distiguis them from the cultures described above we refer to these cultures as mini-bulk cultures. From Table 3.6 it is evident that despite the presence of a polyclonal T cell mitogen very few GIL cultures could be established from the first three EMB of PATA. Although most of the mini-bulk cultures contained predominantly CD8 positive TCR-αß T cells, non of them showed specific cytotoxic activity against donor antigens. This was also observed for PATC (*Table 3.7*): 59 out of the 96 day-2 subcultures of the first EMB (PATC-1GCD2) contained αß T cells and only 2 of them displayed donor directed cytotoxicity.

For the EMB with myocytolysis (grade 2), EMB5 for PATA, and EMB3 for PATC, essentially the same pattern of outgrowth was observed. From the mini-bulk cultures initiated 6 and 8 days after culture onset of the EMB, all 96 wells contained T cells and most of them were cytotoxic against donor derived B-LCL (*Table 3.6 and 3.7*). From the mini-bulk cultures established on day 2 from rejection EMB 45% (PATA, 5GCD2), and 76% (PATC, 3GCD2) of the wells contained T cells, but only 1 resp 3 mini-cultures were cytotoxic for donor cells. From the on day four established cultures of those rejection biopsies (PATA 5GCD4 and PATC 3GCD4), a smaller number of wells (± 30%) had T cells when compared to the previous point. However, 30% of the wells contained donor directed cytotoxic T cells. Unfortunately, because of infection in the mini-cultures, PATC-3GCD4 cultures could not be assessed for cytotoxicity. This relation between increase in the number of wells with T cells cytotoxic for donor antigens and later propagation time was for PATA also observed for the biopsy preceding the rejection biopsy (EMB4, *Table 3.6*).

Although both patients, after successful rejection treatment with RATG, did not encounter a second acute rejection period, growth and cytotoxicity patterns were remarkably different. From the biopsies 7 and 8 of PATA growing cultures could not be established and none of the mini-bulk cultures derived from the biopsies 6 and 9 showed donor directed cytotoxicity (*Table 3.6*).

For PATC (*Table 3.7*) the situation was completely different: from all biopsies up to 232 days post transplantation (EMB12) T cell cultures could be established, mostly at high frequency. The majority (93%) of the cultures (4GCD2-8) obtained from the biopsy directly preceding RATG treatment, were not cytotoxic for donor antigens. This was also the case for the EMB9 and EMB11 *Table 3.7*). EMB5 showed another pattern than the other biopsies in that most cultures with cytotoxic T cells were now found in the first mini-bulk (5GCD2, *Table 3.7*) instead of cultures which were started later as seen for the EMB 6,7,8,10 and 12 (*Table 3.7*).

The contents on T subsets of the cultures was diverse. For PATA most mini-bulks of EMB4 and 5 contained predominantly CD8 T cells. From the mini cultures GCD2 of both EMB 4 and 5, approximately 50% contained predominantly CD4 T cells, whereas GCD8 of the rejection EMB most mini-bulks contained nearly equal amounts of CD8 and CD4 T cells. For PATC in only four timepoints (8GCD8, 9GCD6, 9GCD8 and 12GCD8) mini bulk cultures in which CD8 positive T cells predominated, formed the majority of the cultures established. At 8 timepoints (3GCD2, 3GCD4, 4GCD8,5GCD8, 7GCD4, 8GCD2, 8GCD4 and 10GCD4) cultures with predominantly CD4 cells formed the majority. For the other culture timepoint of this patient all three possibilities: predominantly CD4 cells; predominantly CD8 cells and mixed cultures were nearly equally represented. No clear pattern could be extracted from these data.

3.6 Discussion

Graft-infiltrating T lymphocytes, that were polyclonally propagated from sequentially taken EMB from 2 heart transplant patients showed a consistent growth pattern. In general, most T cell cultures established in the first 4 days of culture did not recognize donor specific HLA-antigens as determined by cytotoxicity assays. Those cytotoxic T cells however, were abundantly present in the majority of the later established cultures. Most T cells propagated in the

HIST[1]	EMB minibulk	Growth	Phenotype			Donor specific CML
		#[2]	$WT31CD4^+$ #[3]	$WT31CD8^+$ #[3]	$WT31CD4^+$ $WT31CD8^+$[4]	#[5]
1	1GCD2	1		1		0
	1GCD4	2		1		0
	1GCD6	0				
	1GCD8	0				
1	2GCD2	2	1	1		0
	2GCD4	0				
	2GCD6	3		3		0
	2GCD8	0				
1	3GCD2	1		1		0
	3GCD4	1		1		0
	3GCD6	3	2	1		0
	3GCD8	0				
1	4GCD2	27	10	15*		1
	4GCD4	34	6	28		10
	4GCD6	96	0	89	7	43
	4GCD8	96	2	90	4	27
2	5GCD2	44	28	9	4*	1
	5GCD4	31	6	20	3*	9
	5GCD6	96	9	68	14*	70
	5GCD8	96	2	42	52	90
1	6GCD2	4	2	2		0
	6GCD4	11	4	6	1	0
	6GCD6	25	1	23	1	0
	6GCD8	4	3	1		0
1	9GCD2	0				
	9GCD4	3	nt	nt	nt	0
	9GCD6	16	nt	nt	nt	0
	9GCD8	42	nt	nt	nt	0

Characteristics of the mini-bulk cultures established from seven EMB of PATA at four different propagation times. At all time points 96 cultures were started in a 96 well tissue-culture-cluster.
[1] Rejection grade according to the Billingham criteria. [2] Number of wells with growing lymphoid cells.
[3] Number of wells in wich 90% or more of the TCR-αß T cells were of the CD4 or CD8 subpopulation.
[4] Number of wells in which none of the two subsets enclose more than 89% of the TCR-αß T cells.
[5] Number of well with T cells cytotoxic for donor B-LCL. *In the other wells, NK or TCR-γδ T cells were the predominant (90% or more) lymphoid cells.

Table 3.6 *Mini Bulk-cultures propagated from PATA.*

first 4 days of culture may represent cytotoxic T cell precursors (CTLp) with donor specificity or with specificity for irrelevant antigens. In another study it is demonstrated that CTLp with donor specificity were present at rather high frequency in T cell cultures propagated from EMB, even when they were cultured only in the presence of IL-2 and autologous feeder cells (*See chapter 5*). Also CTLp without specificity for donor HLA antigens were found to be present.[8,9] Those CTLp may reflect PBMC passing the grafts capillary system at the moment the EMB was taken or may be attracted "aspecific" to the graft as a result of the donor directed immune response as shown by Orosz et al.[10] Since CTLp are not activated they are not directly engaged in the binding of donor MHC/peptide complexes in the EMB and as a consequence could leave the biopsy earlier during culture when compared to the activated donor directed CTL which is engaged in active binding to their target structure. Biopsy 5 of PATC was the only exception, from this biopsy the day 2 culture gave most cytotoxic mini bulks. Since this was the biopsy following the control biopsy for rejection treatment, in which only a few mini bulk cultures showed cytolytic activity against donor cells, we interpreted this as activated donor directed CTL, that just repopulated the graft. In the following biopsies the donor directed CTL were again predominantly present in the minibulk cultures propagated at 6 days or later.

This study further demonstrates that lymphocytes can be grown at high incidence (82%) from endomyocardial biopsies with histological signs of rejection at any time after heart transplantation. Though at a lower incidence, from EMB without histological signs of rejection lymphocytes can be grown too, especially in the first three months after transplantation. We could not confirm the finding of Weber et al.[11] that cell growth from histologically negative EMB obtained in the first postoperative month had a positive correlation with the cumulative incidence of subsequent histological rejection The majority (68%) of the GIL cultures contained CTL with specificity for donor HLA antigens. In GIL cultures from patients that experienced at least one acute rejection episode, CML reactivity pattern changed in relation to time from a predominantly multispecific to a more restricted pattern. This proved to be due to a significant decrease in the number of cultures with CTL directed to mismatched donor HLA class II antigens. No corresponding change was observed in the phenotypic composition as CD4 remained the predominant phenotype in most cultures at the same time after transplantation. This could mean that only a small fraction of the lymphocytes in the bulk cultures is responsible for the CML specificity. It is also possible that $CD8^+$ CTL recognize HLA class II peptide in HLA class I molecules as $CD4^+$ CTL can recognize class I peptides in Class II molecules.[12] Another finding in the present study was the higher incidence of NK cells and TCR γ/δ^+ cells among cultured graft infiltrating cells at one year after transplantation which originated from EMB without myocytolysis. The role of these cells in an allograft is not clear. They might play a role in maintaining graft stability or just be attracted to the site by lymphokines produced by MHC restricted cells, macrophages or other types of cells. See chapter 7 for a more detailed study of TCR $\gamma\delta$ T cells propagated from EMB.

Several comparative studies of the CD4/CD8 distribution within the T cells seen in the graft with immunoperoxidase staining, and that in lymphocyte cultures propagated from the graft showed that the CD4/CD8 distribution in the cultures had a good correlation with the actual situation in the graft.[13,14] Most cultures studied yield mixtures of $CD4^+$ and $CD8^+$ cells with CD4 as the predominant phenotype in most cases in the first 6 months after HTx, which is in agreement with data of Fung et al.[15] In a more limited number of observations others found CD8 as the predominant T cell subset in cultures from renal or cardiac biopsies.[3, 14, 16] This might be due to the use of azathioprine in their immunosuppressive protocols. No relation was found between predominant phenotype

HIST[1]	EMB minibulk	Growth	Phenotype			Donor specific CML
		#[2]	WT31CD4$^+$ #[3]	WT31CD8$^+$ #[3]	WT31CD4$^+$ WT31CD8^{+4}	#[5]
0	1GCD2	59	28	22	7*	2
	1GCD4	6	3	3		0
	1GCD6	0				
	1GCD8	7	7			
0	2GCD2	5	4	1		0
	2GCD4	0				
	2GCD6	0				
	2GCD8	0				
2	3GCD2	73	38	23	11*	3
	3GCD4	31	18	10	2*	nt
	3GCD6	96	3	34	59	81
	3GCD8	96	11	16	69	84
0	4GCD2	47	22	20	5	0
	4GCD4	16	13	3		2
	4GCD6	23	8	14	1	1
	4GCD8	96	75	6	15	6
1	5GCD2	91	43	19	29	36
	5GCD4	56	22	22	10*	19
	5GCD6	96	24	43	24*	23
	5GCD8	91	61	5	23*	14
1	6GCD2	9	5	4		0
	6GCD4	10	9	1		0
	6GCD6	96	39	20	36*	70
	6GCD8	96	48	20	27*	63
1	7GCD2	43	19	8	17	18
	7GCD4	50	37	11	11	10
	7GCD6	94	45	4	45	90
	7GCD8	94	46	0	46	84

Table 3.7A *Mini Bulk-cultures propagated from PATC.*

HIST[1]	EMB minibulk	Growth	Phenotype			Donor specific CML
		#[2]	$WT31CD4^+$ #[3]	$WT31CD8^+$ #[3]	$WT31CD4^+$ $WT31CD8^{+4}$	#[5]
1	8GCD2	96	58	6	7*	2
	8GCD4	21	14	6		0
	8GCD6	65	34	18		
	8GCD8	93	4	77		
1	9GCD2	48	19	18		0
	9GCD4	14	4	8		
	9GCD6	24	4	20		
	9GCD8	96	1	95		
1	10GCD2	72	26	35	11*	3
	10GCD2	78	48	15	2*	nt
	10GCD2	96	43	1	59	81
	10GCD2	96	7	3	69	84
1	11GCD2	0				
	11GCD4	3	2	1		1
	11GCD6	13	13	0		0
	11GCD8	26	22	3	1	0
0	12GCD2	91	17	33	41	11
	12GCD4	92	32	28	32	3
	12GCD6	92	17	31	44	25
	12GCD8	96	9	62	25	6

Characteristics of the mini-bulk cultures established from seven EMB of PATA at four different propagation times. At all time points 96 cultures were started in a 96 well tissue-culture-cluster.
[1] Rejection grade according to the Billingham criteria. [2] Number of wells with growing lymphoid cells.
[3] Number of wells in wich 90% or more of the TCR-αß T cells were of the CD4 or CD8 subpopulation.
[4] Number of wells in which none of the two subsets enclose more than 89% of the TCR-αß T cells.
[5] Number of well with T cells cytotoxic for donor B-LCL. *In the other wells, NK or TCR-γδ T cells were the predominant (90% or more) lymphoid cells.

Table 3.7B *Mini Bulk-cultures propagated from PATC.*

and the number and type of HLA mismatches between donor and recipient, this is in agreement with results of other investigators.[3,14,16] A lower rejection grade was associated with a higher number of CD8 dominated cultures. Also in patients who never experienced acute rejections significantly more $CD8^+$ cells were found among infiltrating cells. These observations could suggest that these cells may play a mitigating role in the rejection process. An alternative explanation for the more prominent predomination of $CD4^+$ cells during rejection might be that it is a consequence of higher HLA class II expression on graft tissue in combination with a more pronounced proliferative capacity of the $CD4^+$ T cells compared to $CD8^+$ cells during rejection. The lower incidence we found for HLA class II-directed cytotoxicity after the first 3 months is in agreement with an extensive survey among renal transplant recipients.[17] This survey showed that the effect of DR matching on the relative risk for graft failure was high in the first 5 months, thereafter the effect disappeared. On the other hand the matching effect of HLA class I antigens was evident during the whole follow up period. We found that class I directed cytotoxicity remained relatively constant in time after transplantation. Several investigators have demonstrated the induction of MHC class I antigens on myocytes and increased expression of class I and II on interstitial structures during rejection,[18-20] which makes the graft tissue more susceptible to specific cell-mediated lysis.[21] In some studies MHC expression in the allograft returned to normal after successful rejection treatment,[19] although others found persistence of expression of donor type class II determinants on interstitial structures of the donor heart 1 and 2 years after transplantation.[18,21] It has been shown that to a certain degree HLA class II expressing dendritic cells of donor origin are replaced by recipient, bone marrow derived, dendritic cells.[18,23,24] This may contribute to the lower incidence of donor class II-directed cytotoxicity after 3 months and a decreased fraction of cultures predominated by $CD4$ cells more than 1 year after HTx. *In vitro* experiments have shown that lymphokines, particularly interferon-γ, regulate the induction and upregulation of MHC expression on graft tissue and enhances the leucocyte binding and penetration through the endothelium[25,26] and that in the early posttransplant period, when the incidence of acute rejection is high, lymphokine producing cells are numerous in the graft.[27] This is in agreement with our finding that the highest growth rates of alloactivated lymphocytes were found in the second and third months after transplantation. This peak in growth was observed in the group of patients that experienced at least one acute rejection episode as well as in the patients that never had an acute rejection. Apparently the presence of activated lymphocytes in the graft does not always lead to allograft destruction. This might be due to qualitative or quantitative differences between CTL present in the graft of rejectors and non-rejectors. We found that cultures propagated from EMB taken from non-rejectors, in the first three postoperative months, generally had a more restricted cytotoxicity pattern than those from rejectors. Suggesting that in this period the number of alloreactive CTL clones in vivo in non-rejectors is lower than in rejectors, which might have consequences for the development of myocyte injury. An additional mechanism involved may be that CTL present in the graft of non-rejectors have low avidity for antigen and can not lyse myocytes in the graft because they express class I and class II antigens at low density.[28,29] Whereas CTL present during rejection might have high avidity.

About possible mechanisms involved in stabilisation of the graft, and on the role different kinds of cells and lymphokines play in this process, many speculations have been made.[30-33] Mechanisms that have been proposed to be involved are clonal deletion of donor-reactive cells, specific suppression of alloreactive T cells and clonal anergy. From earlier studies on circulating mononuclear cells CML hyporesponsiveness[30,31] and a reduction in the fre-

quency of donor-reactive CTL-precursors[32] have been reported in patients with well functioning grafts. In contrast, another study on a patient with a well functioning kidney graft showed that the frequency of donor specific CTL-precursors was still high, but these cells were not operational *in vivo*.[33] We showed that biopsy grown lymphocytes from allografts without acute rejection often still contain donor directed cytotoxic cells. The mechanism that plays a major role in controlling the immune response *in vivo* is still unclear. Further investigations will address the question whether specificity of graft infiltrating cells becomes more restricted because of diminished MHC class I and/or class II expression on donor heart
tissue, if it is caused by low avidity of the CTL or by deletion or suppression of certain allospecific CTL clones, irrespective of the degree of allograft-MHC expression.

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CHAPTER 4

STRUCTURE OF T CELL RECEPTOR Vα AND Vβ CHAINS EXPRESSED BY T-LYMPHOCYTES IN CARDIAC ALLOGRAFT DERIVED CELL LINES

Abstract

Cellular rejection of a cardiac allograft is mediated by T-lymphocytes. To study the function of these T cells, graft infiltrating T-lymphocytes propagated from endomyocard biopsies were analyzed for their capacity to lyse donor derived target cells. Subsequently, the structure of T cell receptor V-regions was analyzed to determine the nature of the cardiac alloresponses. Our studies have revealed that donor-specific cytotoxic T cell lines can be established from endomyocard-infiltrating T-lymphocytes. T cell clones established from these donor-specific T cell lines displayed multiple specificities against donor encoded cell surface antigens. Some of these allospecific T cell clones expressed identical T cell receptors, revealing that they were derived from the same progenitor. T cell clones specific for different donor allo-antigens expressed multiple TCRAV and TCRBV regions. Also, T cell clones, reactive against the same donor allo-antigens, but established from sequentially taken biopsies from the same patient displayed a different TCRAV and TCRBV repertoire. Taken together, our studies suggest that within a given patient the allo-specific T cell repertoire of T cells which have accumulated into the cardiac allograft is compressed.

4.1 Introduction

The immunological mechanisms that mediated allograft rejection are not yet fully understood, but it is well established from both experimental and clinical transplantation that T-lymphocytes play an important role in the initiation and mediation of allograft rejection.[1,2] Graft-infiltrating T-lymphocytes can easily be detected via morphometric techniques[3] and it is possible to propagate graft-infiltrating T-lymphocytes from allograft tissue by *in vitro* cell culture (*See chapter 3*). The majority of T cells that infiltrate the allografted heart express the $\alpha\beta$ T cell receptor and is composed of both $CD4^+$ and $CD8^+$ T cells. Among these $CD4^+$ and $CD8^+$ graft-infiltrating $\alpha\beta$ T-lymphocytes T-cells can be found that are capable of recognizing specific MHC class I and class II mismatches on donor-derived Epstein-Barr Virus transformed B cell lines (B-LCL)([4-9] and (*Chapter 3*).

T cell activation is initiated following specific recognition by the T cell receptor of MHC molecules and/or complexes formed by MHC and antigenic peptides on the APC. Within the TCR α and ß chains the so called complementarity determining regions (CDR) are directly involved in contacting the MHC/peptide complex.[2,10-13] The CDR 1 and 2 are comprised within the TCR V genes whereas the CDR3 region is the direct product of the TCRV, (TCRD), and TCRJ joinings. There are several lines of evidence which suggest that the CDR3 region interacts with the peptide presented by the MHC. In this regard it was shown in various studies that modifications in the CDR3 region of the TCR affects the recognition of the MHC/peptide complex.[13,14] Moreover the amino acid composition of the presented peptide seems to direct the composition of amino acids in the CDR3 loops of TCRs.[15,16] In a number of studies involving specific antigenic peptides both in the MHC class I as well as MHC class II system it was found that in humans the MHC/peptide complex mediated selection of TCR V regions. These studies showed restricted TCR V gene usage and shared amino acid motifs in the CDR3 regions by TCRs of T cells specific for the given MHC/peptide complexes.[17-23] These observations suggest that MHC/peptide complexes are able to select for TCR V regions that optimally fit their counterstructures. In this light, selective accumulation and expansion of T-lymphocytes can be determined through the analysis TCR V regions of graft-infiltrating T-lymphocytes in comparison of similar analysis of paired samples of peripheral blood derived T-lymphocytes.

To determine the nature of the alloresponse in heart-transplant patients with respect to the level of clonality of the responding T-lymphocytes and whether in the alloresponse T cell receptors use specific TCR V genes and CDR3 regions, we have performed a number of studies both at the functional and molecular level. For these studies T-lymphocytes were propagated from endomyocard biopsies and were analyzed for T cell receptor V-gene usage and structure of the TCRVα and Vβ chains. Where possible the studies were performed in relation to function.

4.2 Materials and Methods

4.2.1 Patients and biopsies

HLA-class I and -class II typing of the patients (PATA,PATB and PATC) and their donors used in this study are tabulated in Table 4.1. In all cases, patients received a preoperative blood transfusion and were transplanted with HLA-mismatched donor hearts. All patients received immunosuppressive drugs (Cyclosporin A and a low dose of prednisone). Endomyocardial biopsies (EMB) were taken according to protocol. In the first 6 weeks posttransplant at weekly intervals, the next 4 weeks every 2 weeks, once every 4 weeks up to 18 weeks, from 18 to 30 weeks once in 6 weeks and between 30 and 52 weeks posttransplant an EMB was taken once in 2 months, declining to once every four months more than one year after heart transplantation. After an acute rejection episode the next biopsy was taken one week following rejection therapy. During right ventricular catheterisation four or five biopsy samples

	HLA-A	HLA-B	HLA-C	HLA-DR
PATA	2,11	35,62(15)	3,4	4,-
donor	1,29(19)	8,44(12)	7,-	3,15(2)
PATB	1,3	7,8	7,-	3,-
donor	2,11	60(40), 62(15)	1,3	1,4
PATC	2,3	7,57(17)	3,4	1,7
donor	1,2	8,62(15)	3,7	1,13(6)

Table 4.1 *HLA-class I and -class II typing of the three donor/recipient combinations.*

were obtained. Of these EMB samples, 3-4 were used for histological evaluation and 1 was used for the propagation of the T cell infiltrate. Administration of rabbit antithymocyte globulin (RATG) or corticosteroids was given as rejection therapy when rejection grade 2 was observed.

4.2.2 Generation of T cell lines from EMB

From all biopsies of PATA, 2 fragments were obtained, and cultured differently. From one fragment T-lymphocytes were propagated, and after 8 days of culture in CM supplemented with 30 units IL-2, PHA and irradiated (30 Gy), autologous feeder cells, further expanded by restimulation with irradiated random PBMC and third party B-LCL until sufficient cells were obtained for analysis (32 - 70 days). The graft-infiltrating lymphocyte (GIL) cultures obtained in this way were considered as bulk cultures. From the other fragment mini-bulk cultures were established (*See chapter 3*).
From the biopsies of PATB bulk cultures were prepared with the standard method described in chapter 3, while every 2-3 days half of the medium was replaced by fresh CM supplemented with ± 30 Units IL-2. When cell growth was observed the wells were pooled and further expanded when sufficient cell density was reached (10^5 - 10^6 cells/ml). After three weeks of culture, when growth was slowing down or cell death was observed the cultures of PATB were restimulated by adding 5.10^3/well irradiated (50 Gy) third party B-LCL, until enough cells were obtained for analysis.
From all EMB of PATC mini-bulk cultures were established as described in chapter 3.

4.2.3 Generation of T cell clones

Randomly stimulated GIL cultures of PATA and PATB were washed and cloned by limiting dilution at 0.3 cells per well in a 96 well U-shaped tissue culture plate in 200 μl culture medium containing 0.1 μg PHA and 20 U/ml rIL-2 in the presence of 10^5 irradiated (30 Gy) random feeder cells and 10^4 irradiated (50 Gy) B-LCL. After 10-14 days the growing cultures were transferred to a 24 well plate (Costar) and restimulated with PHA and rIL-2 in the presence of third party B-LCL and random PBMC.

4.2.4 Monoclonal antibodies

Cultured cells were phenotyped by flow cytometry using 1.10^5 cells for each labelling. The following purified, PE or FITC labelled monoclonal antibodies (Mab) were used: anti-Leu-2 (anti-CD8), anti-Leu-3 (anti-CD4), anti-Leu-4 (anti-CD3) and WT31 (anti-$\alpha\beta$TCR) (Becton Dickinson, Mountain View, CA). ßV5a (anti-TCRBV5S2/3), ßV5b (anti-TCRBV5S3), ßV6 (anti-TCRBV6S7), ßV8 (anti-TCRBV8), ßV12 (anti-TCRBV12), αV2 (anti-TCRAV2S3), αV12 (anti-TCRAV12) (T Cell Sciences, Inc. Cambridge, MA) and TCRBV2S1, TCRBV3S1, TCRBV5S1, TCRBV8S1, TCRBV13S6, TCRBV17S1, TCRBV18S1 (Immunotech, Marseille, France). Inhibition studies were performed using the

following mAb: W6/32 and B1.23.2 (both directed against monomorphic HLA class I structures)[24] B1.1G6 (anti-ß2-microglobulin),[25] PdV5.2 (directed against a monomorphic epitope shared by DR, DP and most of the DQ alleles),[26] WT32 (anti-CD3),[27] FK18 (anti-CD8[30] and RIV6 (anti-CD4, Dr M. Leerling, RIVM, Bilthoven, The Netherlands).

4.2.5 Cytotoxicity assay

Four daugther plates of the four seeding points (GCD2, GCD4, GCD6, GCD8) from each growing biopsy of patients PATA and PATC from chapter 3 were used.
To two plates 3.10^3 51Cr-labelled donor B-LCL were added. The 2 other plates were used to determine the specificity of the cytotoxicity, therefore one plate was incubated with 3.10^3 51Cr-labelled third party B-LCL (different from the one used for restimulation) and one with 3.10^3 51Cr-labelled K562 as a control on LAK and NK activity of the cultures.
The plates were centrifuged (600g, 1 min) and incubated for 4 hours at 37°C in a humidified atmosphere with 5% CO_2.
Supernatants were collected with a Skatron harvesting system (Skatron-AS, Lierse, Norway) and counted in a gamma counter for 3 minutes. From each well the % lysis was calculated according to the formula:

$$\%\text{lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

Maximal release was determined in six-fold from a Triton X100 (5% v/v solution in 0.01 M TRIS-buffer) lysate of the target cells. Spontaneous release was determined in six-fold, by incubation of target cells in medium (RPMI-1640-Dutch modification, supplemented with 1% heat inactivated human serum) only. The mean lysis % was then determined for the two corresponding wells. Mini-bulk-cultures that lysed donor target for more than 10% and did not show activity against third party and K562 were considered positive.

The bulk cultures from all three patients were assayed for cytotoxicity as follows:
2.10^4 effector cells (T cell lines or T cell clones) were mixed with 2.10^3 ^{51}Cr-labelled target cells (B-LCL) in 200 μl medium (RPMI-1640-Dutch modification, supplemented with 1% heat inactivated human serum) in U-shaped microtiter wells. The plates were centrifuged (600g, 1 min) and incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2.
The supernatants were harvested using a Skatron harvesting system and counted in a gamma counter.
Inhibition of cytotoxic activity with mAb was carried out as follows: an appropriate dilution of the mAb containing ascites was pre-incubated with 2.10^3 target cells or 2.10^4 effector cells in 0.1 ml medium at 37°C. After 30 minutes 2.10^4 effector or 2.10^3 target cells were added without prior washing of the pre-incubated cells.

4.2.6 RNA extraction and transcription

5 to 10.10^6 cultured T-lymphocytes were purified by centrifugation through a Ficoll Isopaque gradient. Following washing with HBSS (Gibco, Paisley, Scotland) the cells were pelleted by centrifugation and stored at -70°C for the molecular analyses. Total RNA was extracted using the RNAzol method (Cinna/Biotecx, Laboratories Inc, Houston, TX). 5μg of total RNA was transcribed into first strand cDNA in 25 μl reaction mixture by reverse transcriptase using oligo dT as a primer (Promega Corporation, Madison, WI).

4.2.7 PCR amplification

TCR α and ß chain encoding cDNAs were amplified by using 22 or 28 different TCRAV and 19 or 25 different TCRBV family specific oligonucleotides using the methodology as described by Hawes et al.[29] For these polymerase chain reactions 0.5-1.0 μl of cDNA was added to a PCR mixture containing 10 mM Tris-HCl pH 8.4, 50 mM KCl, 4 mM MgCl2, 0.06 mg/ml BSA, 0.5 mM of each dATP, dCTP, dGTP and dTTP, 2.5 units of Taq DNA polymerase (Boehringer,

Mannheim, FRG), 20 pmol of a TCR 3' C-region primer and 20 pmol of a TCR V-family specific 5' primer in a final volume of 100 μl. As an internal control for total amplification, a reaction tube containing a 3' and 5' C-region primer was included. The sequences of these primers were derived from Lambert et al.,[30] Wucherpfennig et al.,[23] Oksenberg et al.,[31] and Hawes et al.[29] Each reaction mixture was overlaid with 50 μl of mineral oil (Sigma, St. Louis, MO) prior to PCR reaction in a thermal cycler (Biomed, Thermocycler 60). TCRAV and TCRBV specific sequences were amplified for respectively 30 and 25 cycles. Each cycle consisted of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C.

4.2.8 Detection and Quantification

PCR products were size fractionated by electrophoresis on 1% agarose gels in Tris-acetate/EDTA buffer, visualized by staining with ethidiumbromide and in case of PATA subsequently transferred to nylon membranes (Biotrace, Gelman Sciences, Ann Arbor, MI). TCRAV and TCRBV specific sequences were detected by hybridization with ^{32}P-labelled TCRAC or TCRBC probes respectively according to the Biotrace protocol. Autoradiograms were analyzed by densitometry (LKB 2220-020, Ultrascan XL, Laser Densitometer, Pharmacia LKB Bio-technology, Uppsala, Sweden) to measure the intensity of the bands. To achieve a relative value for the amount of amplification the densitometry values were normalized with respect to the C-region internal controls of the GITL and PBMC as follows:

$$\text{measured V gen family intensity } X = \frac{\text{measured C gene intensity of PBMCPATA.o}}{\text{measured C gen intensity}}$$

4.2.9 DNA cloning and sequencing

5' TCRAV or TCRBV and 3' TCRAC or TCRBC sequence specific primers were used to generate PCR products from the T cell clones and the EMB derived T cell lines PATA.4 and PATA.5. PCR products of PATA were purified through Qiagen columns (Diagen GmbH, Düsseldorf, FRG). The purified PCR products and Sma I digested pUC 19 plasmids were tailed with dATP and dTTP respectively (Boehringer, Mannheim, FRG). dA-tailed PCR products were ligated into the dT-tailed pUC 19 plasmids overnight at 16°C and subsequently transfected into E. coli JM101. Positive colonies were expanded, purified through Qiagen columns, and the DNA sequence was determined by using the T7 DNA Polymerase Sequencing System (Promega Corporation, Madison, WI). PCR products of patient PATB were size fractionated by low melting agar and subsequently purified using PCR 'Wizard' columns (Promega, Madison, WI). The DNA sequence of the purified PCR products was determined by using the Circumvent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA).
The sequencing products were resolved on polyacrylamide gels and detected by autoradiography.

4.2.10 CDR3 detection

To detect the presence of allo-specific TCR ß-chain sequences an oligonucleotide was synthesized on the basis of the CDR3 sequence as detected in the HLA-A29 specific T cell clones of PATA. This TCR ß chain CDR3 oligo (5'- CATAAGCAGGCCCTACACTC-3') was labelled with ^{32}P γATP and used for detection of homologous sequences by hybridization of TCRBV20 family specific PCR products in the PATA.4, PATA.5 and PBMC derived PATA T cell lines.[32]

4.3 Results

To elucidate the T cell-mediated mechanisms involved in allograft rejection, we have performed functional and molecular studies on T lymphocytes infiltrating the endomyocardium after cardiac transplantation from three diffe-

	EMB	days[1]	hist.[2]	imm.[3]	CD4	CD8	cyt.[4]
PATA	1	8	1		3	97	-
	2	15	1		5	95	-
	4	29	1		10	90	+
	5	36	2	RATG	14	86	+
	6	58	1		11	89	-
	9	129	1		1	99	-
PATB	1	91	2	predn.	nd5	19	+
	2	137	2	predn.	nd	59	+
	3	147	2	RATG	nd	91	+
	4	210	2	predn.	nd	58	+
PATC	1	7	0		60	40	-
	2	15	0		81	10	-
	3	22	2	RATG	80	20	+
	4	42	0		89	10	-
	5	49	1		71	29	+
	6	57	1		81	18	+
	7	70	1		8	91	+
	8	87	1		0	99	+
	9	112	1		0	99	-
	10	140	1		95	5	+
	11	192	1		92	8	-
	12	232	0		16	84	+

1 days posttransplantation 2 histological evaluation according to the Billingham criteria
3 immunosuppressive treatment of rejection (RATG: Rabbit anti-thymocyte globulin, predn.:methylprednisolon). 4 cytolytic activity as defined by cell mediated lymphocytotoxicity against a panel of EBV transformed B-cell lines sharing at least one of the MHC-class I mismatches with the heart transplant donor.
5 not determined.

Table 4.2 *Histological evaluation, phenotypic characterization by surface staining and cytolytic capability of the EMB derived bulk T-cell lines.*

rent individuals. For these studies endomyocardial biopsies, sequentially taken before, during and after a histologically determined rejection episode, were used. T lymphocyte cell lines, both bulk cultures and mini-bulk-cultures were established, phenotyped, and assessed for their cytolytic capacity against donor B-LCL and/or B-LCL sharing 1 or more HLA antigens with the donor.
In the bulk cultures all cells expressed the CD3-protein ensemble and the $\alpha\beta$ T cell receptor. All T cell lines derived from PATA were predominantly $CD4^-CD8^+$. In contrast, the T cell lines propagated from the EMB of PATB and PATC exhibited a different phenotype (*See table 4.2*). In PATB and PATC a number of cell lines exhibited a mixed $CD4^+CD8^-$ and $CD4^-CD8^+$ character, whereas both in PATB and PATC cell lines were established in which $CD4^+CD8^-$ T cells predominated. Independent of the outcome of the histological evaluation, the established bulk T cell lines exhibited cytolytic activity against donor or HLA matched B-LCL. Bulk T cell lines established from grade 1 biopsies did not always

	Target cells							
HLA	**E143**	**COR**	**JVG**	**LAG**	**CAA**	**PLI**	**HEY**	**K562**
A	1,29	2,29	2,29	2,3	1	1,26	2,11	
B	8,44	44,62	44,49	18,44	8	7,8	51	
Cw	7	3,7		5,7	7	7	2	
DR	3,15	3,11	5,7	9,13	3	1	7,14	
Cell line	**Percent lysis of target cells**							
PATA.5	60	68	68	0	2	4	0	0
clone BS2	54	93	nd	1	nd	1	nd	5
clone BS42	80	83	nd	0	nd	nd	0	0
clone CS1	80	67	72	1	0	1	3	nd
clone CS15	80	78	60	0	0	0	0	0

Donor-shared MHC class I and Class II antigens are underlined.

Table 4.3 *Cell mediated Lymphocyto-toxicity of the EMB derived GIL line PATA.5 and 4 clones generated from this line against a panel of B-LCL and the NK sensitive cell line K565 at an E:T ratio of 10:1.*

manifest cytolytic activity, while bulk T cell lines established from biopsies with myocytolysis (rejection grade 2) were always cytolytic against donor and/or donor HLA matched B-LCL. From 3 T cell lines derived from rejection biopsies T cell clones were established.
The cytolytic T cell lines and T cell clones of the patients were further investigated against a panel of allogenic EBV-transformed B cell lines. An example of such panel study is shown for GIL line 5 and its clones of PATA (*Table 4.3*). Several clones of PATA.5 shared reactivity against the HLA-A29 antigen, while clones of PATB.3 showed specific reactivity against the HLA-B60, HLA-B40, HLA-B62 and HLA-A11 antigens. Four out of six of the seeded T cell lines established from patient PATC (PATC.6, PATC.7, PATC.9 and PATC.12) only lysed B-LCL that shared the HLA-B62 antigen. Inhibition experiments with monoclonal antibodies directed against HLA class I and CD3 could inhibit the cytolytic activity of the HLA-A29 specific clones, confirming the TCR/CD3 mediated reactivity against this class-I antigens. Anti-CD8 did not inhibit the lysis which is indicative for the high affinity of the T cell receptor for this allo-MHC/peptide complex.

4.3.2 TCR V-region analysis of allo-MHC class I specific CTL lines

Using a number of the currently available monoclonal antibodies directed against defined TCR V gene segments, we could only detect a few of the TCR V family gene segments in the T cell lines propagated from the graft-infiltrating T lymphocytes when compared to T cell cultures generated from samples of peripheral blood mononuclear cells of patient PATA (PBMCPATA.6 *Table 4.4*). In some of the T cell lines derived from different biopsies a predominance of defined T cell receptor V regions was noted (for example see patient PATA which expressed almost exclusively the TCRBV6S7 gene segment in biopsy derived T cell line PATA.6). Similarly patient PATB expressed relative high levels of TCRBV8S1 in the T cell line PATB.2 and of TCRBV2S1 and TCRBV5S2/3 gene segments in the T cell line PATB.4 (*Table 4.4*).
Since these types of analysis do not allow the description of the complete T cell receptor gene repertoire we have expanded these studies and have analyzed T cell receptor V-gene use in the various endomyocard biopsy derived T cell lines at the transcriptional level by RT-PCR. As can be seen from Table 4.5 these analyses showed that each graft-infiltrating T cell line exhibited a different pattern of TCRBV gene usage. In general, the number of TCRBV gene segments which were used by the T cell lines, propagated from endomyocardium infiltrating T-lymphocytes were restricted when compared to the T cell lines derived from PBMC. In the PBMC derived T cell lines all TCRBV genes could be detected. Despite different patterns of TCR V gene usage in the different graft-infiltrating T lymphocyte cell lines there is an apparent sharing of defined TCR V gene families by some of the T cell lines tested in the individual patients. This is exemplified by the sharing of TCRBV 6, 7, 14, 17 and 18 in the different graft infiltrating T lymphocyte cell lines of patient PATA. Similarly, the various T cell lines of patient PATC, which were selected on basis of their cytolytic activity, shared the TCRBV4 gene family in five out of six T cell lines. It should be noted that each patient exhibited an individual specific pattern of the TCRBV gene expression and sharing of these TCR V-regions among the various graft-infiltrating T lymphocyte cell lines. Subsequent analysis to the level of TCRAV and TCRBV expression in patient PATA, as defined by densitometry, showed that some of the TCRBV as well as the TCRAV gene segments including the shared TCR V genes were used at high frequencies in the various T cell lines when compared with the other TCR V genes used by the same T cell lines (*See figure 4.1 and 4.2*). In general, the various TCRAV and TCRBV genes were expressed at different levels in the T cell lines propagated from graft-infiltrating T-lymphocytes.
In case of PATA an expansion of the TCRAV and TCRBV repertoires was noted in time, which

Cell line	Percentage stained cells													
	TCRVB													
	2	3	5.1	5.2/3	5.3	6.7	8.1	8	12	13	17	19	2	12
PATA.4	nd	nd	nd	8	0	0	nd	0	0	nd	nd	nd	0	nd
PATA.5	nd	nd	nd	0	0	0	nd	13	0	nd	nd	nd	0	nd
PATA.6	nd	nd	nd	0	0	88	nd	0	0	nd	nd	nd	0	nd
PBMCPATA.6	nd	nd	nd	5	1	2	nd	5	2	nd	nd	nd	4	nd
PATB.1	1	0	0	0	0	nd	1	1	1	0	0	0	8	3
PATB.2	18	0	0	6	0	nd	34	14	1	0	0	0	0	3
PATB.3	0	0	9	0	1	nd	0	0	2	1	0	0	3	0
PATB.4	30	0	0	30	27	nd	0	0	1	1	0	1	3	0

Table 4.4 *Cell surface expression of TCR V gene segments as determined by FACS analysis of some of the T cell lines of PATA, the PBMC derived T cell line of PATA and all T cell lines of PATB.*

seems to be associated with the rejection crises as determined by the Billingham criteria. Treatment with anti thymocyte globulin (RATG) resulted in a clear declination in the spectrum of TCR V gene families used in patient PATA (*See figure 4.1 and 4.2*).
The T cell lines derived from patient PATB, which were all derived from grade 2 biopsies, exhibited in general a more polyclonal character as deduced from the number of TCRBV families which could be detected by RT-PCR in the T-cell lines. However, not all T cell lines derived from grade 2 biopsies exhibit this polyclonal character. In PATC we observed a paucity in the number of TCRBV gene families employed by grade 2 derived T cells in PATC.3.

4.3.3 Structure of the TCR α and β chains of T cell clones specific for allo-MHC

To investigate the structure of the TCR α and ß chains used by allo-specific T cell clones in more detail, we have analyzed 26 T cell clones which were derived from cytolytic graft-infiltrating T lymphocyte cell lines from two different cardiac transplant patients. As can been seen from Table 4.2, not all clones manifested cytolytic activity against donor B-LCL. It is to note that the majority of these non-cytolytic clones were $CD4^+CD8^-$, whereas all cytolytic T cell clones exhibited the $CD4^-CD8^+$ phenotype with the exception of two clones established from PATB.3 which expressed simultaneously both the CD4 and CD8 antigens.
Panel analyses, to determine the HLA specificity of these various cytolytic T cell clones derived from graft-infiltrating T-lymphocytes, showed that in PATA the cytolytic clones recognized HLA-A29 (*Table 4.3*) whereas the cytolytic clones from PATB.3 and PATB.4 recognized the HLA-B62, B40 and HLA-B62, B40, B60 and A11 respectively. The analysis of the TCRAV and TCRBV gene family usage of the various cytolytic clones revealed an heterogeneous usage pattern as tabulated in Table 4.7. The HLA-A29 restricted cytolytic T cell clones from PATA expressed the TCRAV10 and TCRBV20 genes. Depending on the allo-antigen that was recognized, the various T cell clones employed different combinations of TCRAV and TCRBV genes within each patient. In patient PATB, the cytolytic T cell clones, that were established from two different grade 2 biopsy T cell lines that showed reactivity against the same HLA-B62 or HLA-B40 antigen used different TCRAV/TCRBV gene family combinations. Furthermore, the HLA-B62 and HLA-B60 specific T cell clones that were derived from T cell line PATB.4 both used the TCRBV5S3 gene segment. Comparison of the TCRV gene family analysis as presented in Table 4.5 and 4.6 shows that the TCR V genes used in the allo-specific cytolytic T cell clones were readily detectable in the endomyocard derived cell lines.
To gain more insight in the structural aspects of the TCRs used in allo-specific recognition, we have determined the nucleotide sequence of the TCR V regions and analyzed the deduced amino acid composition including the CDR3 region of both the α and ß chain of TCRs used by the different cytolytic T cell clones and non-cytolytic T cell clones. As can been seen from Table 4.6, the TCR ß chain CDR3 region amino acid composition of different T-cell clones, including T cell clones with the same HLA class-I specificity, showed a diverse usage pattern of amino acids. Similarly, analysis of the TCR α chain CDR3 regions of T-cell clones of PATA and PATB, both cytolytic and non-cytolytic also revealed a heterogeneous usage pattern of amino acids in the CDR3 region. As can been seen from Table 4.7, the CDR3 region of the HLA-B40 restricted cytolytic T cell clones derived from PATB.3 and PATB.4 differed also.
The question, whether the cytolytic T cell clones established from grade 2 biopsies could also be detected in previously taken biopsies, was addressed in more detail in PATA. The presence of the CDR3 region of the TCRBV20S1 gene segment, used by the HLA-A29 specific T-cell clones derived from PATA.5, in the TCRBV20 PCR product of cell line PATA.4, was examined taking advantage of an oligonucleotide specific for the CDR3 region. These hybridization analysis revealed that the cytolytic CDR3

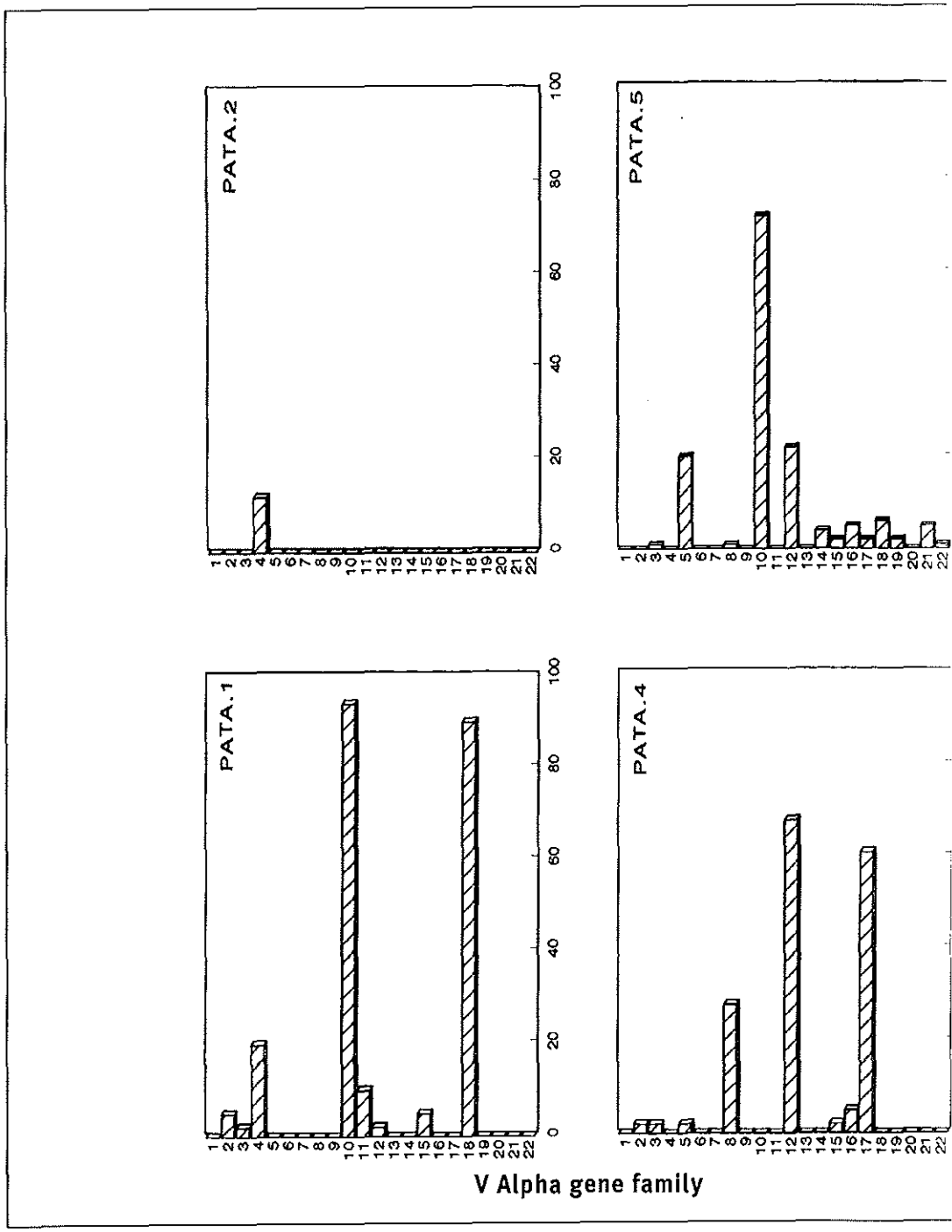
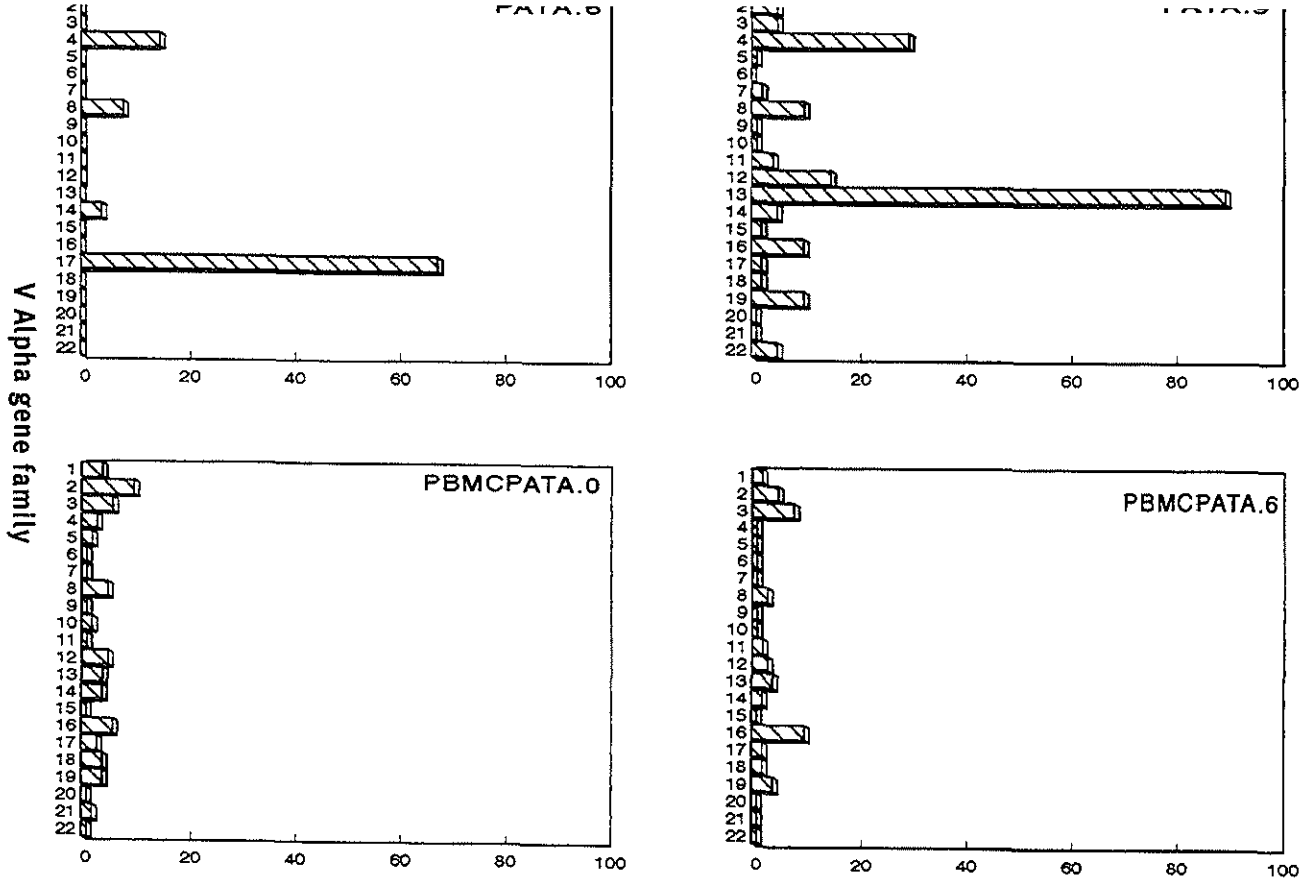


Figure 4.1 *TCRAV gene segment usage of GITL cell lines derived from six EMBs of PATA and two T cell lines generated from PBMC taken before transplantation (PBMCPATA.0) and at time of EMB PATA.6 (PBMCPATA6).*


PBMCPATA.0
PBMCPATA.6
V Alpha gene family
Relative intensity

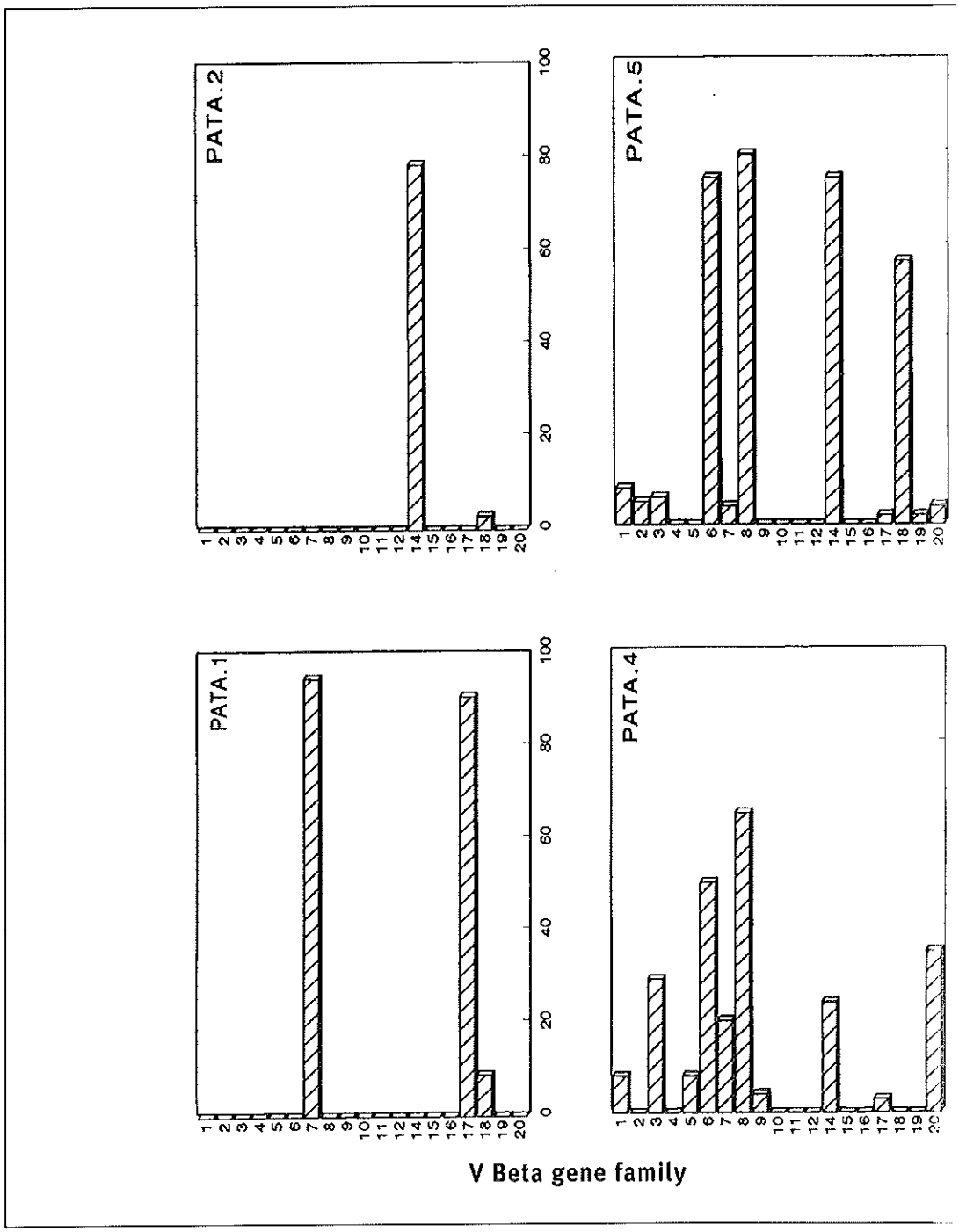
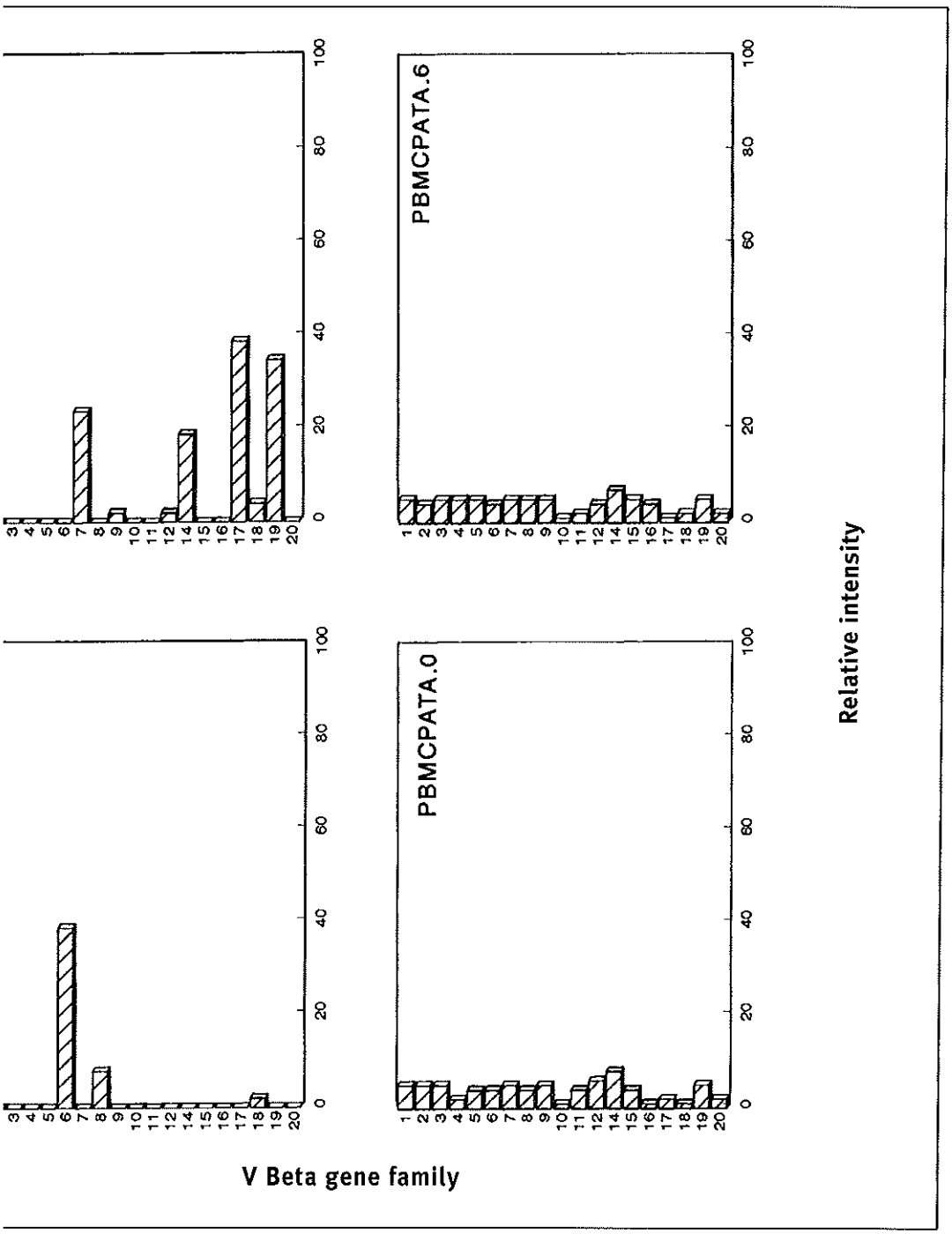


Figure 4.2 *TCRBV gene segment usage of GITL cell lines derived from six EMBs of PATA and two T cell lines generated from PBMC taken before transplantation (PBMCPATA.0) and at time of EMB PATA.6 (PBMCPATA6).*


PBMCPATA.6
PBMCPATA.0
Relative intensity
V Beta gene family

	Vß family used																							
T cell line	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
PATA.1							+						nd				+	+			nd	nd	nd	nd
PATA.2													nd	+				+			nd	nd	nd	nd
PATA.4	+	+	+		+	+	+	+	+				nd	+			+			+	nd	nd	nd	nd
PATA.5	+	+	+			+	+	+					nd	+			+	+	+	+	nd	nd	nd	nd
PATA.6						+		+					nd					+			nd	nd	nd	nd
PATA.9							+		+			+	nd	+			+	+	+		nd	nd	nd	nd
PBMCPATA.0	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	nd	nd	nd	nd
PBMCPATA.6	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	nd	nd	nd	nd
PATB.1	+	+	+	+	+	+	+	+	+		+		+	+			+	+	+		+	+		
PATB.2	+	+	+		+	+	+	+	+			+	+	+	+			+	+		+	+	+	
PATB.3				+	+			+			+	+	+	+										
PATB.4		+			+	+		+					+		+				+		+			
																			+					
PATC.3					+		+	+						+										
PATC.6			+	+										+										
PATC.7			+	+									+	+				+			+			
PATC.8				+																				
PATC.10		+		+	+				+															
PATC.12	+			+			+					+						+	+					

PBMCPATA.0 was taken before transplantation; PBMCPATA.6 was taken at the same time as biopsy PATA.6 (nd: not determined).

Table 4.5
TCRBV gene segment usage of T-lymphocyte cell lines derived from EMBs taken from three different heart transplant patients and of two PBMC derived T cell lines of PATA.

Patient	spec.[1]	no.[2]	CD	TCRAV	TCRBV
PATA.5	A29	4	8	10	20
	nc[3]	6	8	5	6
	nc	2	4	21	14
	nc	1	4	4	6
PATB.3	B62	1	8	2	4
	B40[4]	2	8/4[5]	6	14
	nc	2	4	14,16	12
PATB.4	B62	2	8	6	5
	B40	2	8	11	2
	B60	1	8	7	5
	A11	1	8	16	21
	nc	2	4	20	13

1 HLA specificity as determined by panel analysis.
2 no. of clones analyzed.
3 no cytotoxicity.
4 T cell clones recognized both the HLA-B40 splits (HLA-B60 and HLA-B61).
5 The double positive nature of these clones was confirmed by staining with anti CD8 ß-chain monoclonal.

Table 4.6 *Specificity, phenotype and TCRAV and TCRBV gene segment usage of T cell clones generated from three EMB derived T cell lines.*

regions were not present in the cell line from PATA.4 (See Datema et al.[33]). Also, we were not able to detect these sequences among peripheral blood mononuclear cells, taken at the same time as PATA.5, indicating that these effector
T cells were present at extremely low concentrations in the periphery. The occurrence of identical V, N-D-N and J regions revealed that a number of T cell clones derived from the graft infiltrating T-lymphocyte cell lines were derived from the same progenitor. Whether this progenitor T cell clone was already amplified in the endomycard biopsy or whether this progenitor as a consequence of previous activation in the endomyocard was preferentially amplified during the generation of the graft-infiltrating T lymphocyte cell line remains to be investigated.

4.4 Discussion

Both the TCRAV and TCRBV repertoires in the various cytotoxic T cell lines were extensive though smaller than the peripheral TCRAV and TCRBV repertoires on the basis of the numbers of expressed TCR V-gene families. Subsequent establishment of allo-specific cytolytic T-cell clones revealed that various donor encoded HLA specificities could be detected among these clones. The nucleotide sequence analysis of the TCRAV and TCRBV regions of these cytolytic T cell clones established from the same T cell line has shown that a number of cytolytic T cell clones displaying shared specificities expressed identical TCRAV and TCRBV regions. This suggests that these T cell clones were all derived from the same progenitor. However, cytolytic T cell clones, established from different T cell lines but sharing HLA-specificity, expressed different TCRAV and TCRBV regions. Propagation and sequencing of T cell clones isolated from GIL cell line PATA.5 showed that all T cell clones that recognized donor B-LCL in a MHC restricted fashion were using identical

Clone	HLA-spec	TCRVA					TCRVB				
		V		N	J		V		NDN	J	
PATA.5	A29	10	CA	AD	GGS	42	20	CAWS	VGPA	YGY	1.2
	nc						6.4	CASSL	DRP	QYF	2.5
PATA.4	nc						6.6/7	CASSL	LPL	NEQ	2.1
PATB.4	B62	6	CA	MMRT	NDY	20	5.3	CASS	IRQY	TGE	2.2
	B60	7	CA	RA	TTD	24	5.3	CASS	PGQGAV	DTQ	2.3
	B40	11	CAV	KG	YGQ	26	2	CSAR	DPSGR	SYE	2.7
	A11	16	CAVR	DLVD	SGY	41	21	nd			
PATB.3	B62	2	nd				4.1	CSV	DSAITT	FG	1.2
	B40	6	CA	MRRD	FKK	21	14	CASSL	SGAIV	NQP	1.5
	nc	14	CA	YRSVD	SGT	40	12.2	CAIS	TGTRPL	ETQ	2.5
	nc	16	CAVR	DA	GNQ	49					

Table 4.7
V,D,J and C region determination by sequence analysis of T cell clones.

TCR A and B V-regions. The TCRBV20 gene product used by these clones is detectable in cell line PATA.5 albeit at a relative low frequency. This in contrast to the other TCR V-genes that were detected in PATA.4 which exhibited relative higher expression patterns. However, T cell clones employing TCRBV6S4 genes did not manifest donor specific reactivity. Furthermore, using a CDR3 region specific oligonucleotide we were able to demonstrate that this sequence could only be detected in the rejection biopsy of PATA (PATA.5) and not in the preceding PATA.4 without signs of acute rejection, showing that among the relatively more abundant TCRBV20, as detected by PCR, the cytotoxic T cell clone progenitor of PATA.5 was not present.

However, it is possible that T cells which initiate the rejection episode are detected in GIL cell line PATA.4 and may be represented by the T cells that employ TCR V-regions that differ from the TCR V regions employed by the effector T cells that are responsible for the tissue damage. This is supported by the observation made in PATB. In PATB (PATB.3 and PATB.4) the cytolytic T cell clones with shared specificities displayed usage of different TCRAV and TCRBV regions. These clones were derived from cytotoxic T cell lines established from sequentially taken grade rejection, suggesting that indeed the effector functions can be performed by different T cell clones. Alternatively, this observation could also imply that T cells that are mediating tissue damage are unique at a certain timepoint following transplantation and as a consequence, these effector T cells might not be identifiable at other timepoints after transplantation.

The function of the other T cells that use TCR V-genes like BV6, 8 and 14, which are predominant in the GIL cell line PATA.4 and PATA.5 for instance, remains to be investigated further. These $\alpha\beta$ TCR might recognize the HLA alloantigens only in combination with endothelial or endomyocard specific peptides which are also possible targets for an alloreactive response causing tissue destruction.[34]

T cell clones established from patient PATB (PATB.3 and PATB.4) showed identical HLA class I specificities (HLA-B62 and HLA-B40), despite the fact that they used different TCRAV/TCRBV gene combinations.

In the biopsies taken at earlier time points following transplantation of PATA only a limited number of TCRAV and TCRBV genes could been detected. This was seen particular in GIL cell lines derived from grade 1 EMB that exhibited no cytolytic activity *in vitro*. In GIL cell lines derived from rejection EMB that exhibited specific cytolytic activity *in vitro*, the TCR V-gene repertoire was more extensive. However, when compared to T cell lines derived from purified samples of PBMC, the TCR V-gene repertoire in these EMB was limited. Since the cultures used for TCRAV and TCRBV repertoire analysis from PATC were day 6 or day 8 mini-bulk cultures the more restricted pattern in TCR V gene usage might represent more accurately the donor directed T cells. From PATA and PATB bulk cultures were used for these analysis in which beside donor directed T cells also T cells might be present with irrelevant specificities as discussed before. This may lead to a more broad type of TCRVB repertoire.

An other explanation for the apparent utilization of only a limited number of TCR V-genes by GIL cell lines might be that the starting number of T cells present in the original biopsy is below the amount of T cells that is needed to describe a complete T cell repertoire allowing the detecting of all TCR V-genes. Wyngaard et al.[35] showed that the maximal number of $CD3^+$ cells detected in grade 1 biopsies by immunolabeling did not exceed 200 cells/mm2. However, the observed sharing of certain TCR V family genes by some of the GIL cell lines supports the idea of a restricted usage of TCR V-gene segments by GIL. Also, the apparent restriction in the number of T cell receptor V-gene families in the T cell lines established from endomyocard infiltrating T cells might be the results of selection in vivo for allo-specific T cell clones. In a study by Hall et al.[36] it was shown that the T cell receptor repertoire of unstimulated

peripheral blood lymphocytes became compressed in a longterm mixed lymphocyte reaction as a consequence of repeated allogenic stimulation *in vitro*. These observations suggests that the T cell receptor repertoire of allo specific T cell clones could be restricted in nature. The presence of a relative extensive TCR V gene repertoire both in PATA.4, PATA.5, PATB.1 and PATB.2 could be the result of attraction to the site of inflammation of non-specific T cells,[37] by events such as increased production of cytokines and growth factors by graft infiltrating regulating T cells[38,39] or, through presentation of novel antigenic peptides as a consequence of tissue damage by cytotoxic T cells.[40]

In conclusion, our study indicates that in GIL propagated from biopsies, taken at different time points from three cardiac transplant patients, in general fewer TCR V gene families could be detected as found among PBMC. Some of these TCR V genes were shared by different GIL cell lines which is indicative of a restricted usage of TCR V genes by GIL, However, T cells that were able to lyse donor B-LCL at high avidity, in a MHC class I restricted fashion, as determined by T cell cloning and sequence analysis, were only detected in grade 2 biopsies taken at the time of rejection.

No evidence was found for the presence of identical cytolytic T cells in multiple biopsies taken at different time points, suggesting that T cells, responsible for myocytolysis have been recently recruited towards the endomyocard.

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CHAPTER 5

ACUTE REJECTION IN HEART TRANSPLANT PATIENTS IS ASSOCIATED WITH THE PRESENCE OF COMMITTED DONOR SPECIFIC CYTOTOXIC LYMPHOCYTES IN THE GRAFT BUT NOT IN THE BLOOD

Abstract

In vivo activated, committed, donor specific cytotoxic lymphocytes (cCTL) can be propagated and expanded from endomyocardial biopsies (EMB) in IL-2 enriched medium especially during an acute rejection episode. We here report our efforts to detect these cCTL's by the same technique in peripheral blood at the moment of rejection and when no rejection was diagnosed. During or just before rejection significantly less often ($p<0.01$) donor reactive cCTL were found in PBL samples (2/20) than in the simultaneously taken EMB samples (13/19). Donor B-LCL and/or third party B-LCL were lysed by 15 PBL samples. Inhibition studies revealed that this lysis was due to LAK-like cytotoxicity. The results show that peripheral blood does not reflect intra graft events, which is probably the reason for the ir reproducible results of diagnosis of rejection by monitoring immunological parameters in the peripheral blood.

5.1 Introduction

In the last decade several attempts have been made to correlate immunological parameters of peripheral blood lymphocytes (PBL) with acute cellular rejection after solid organ transplantation. Recently, investigators have tried to demonstrate the presence of activated T cells in the PBL or increased levels of soluble IL-2 receptor (sIL-2R) as a marker of T cell activity during acute rejection. Activated T cells can be distinguished from resting T cells by their morphological appearance and by their expression of IL-2 receptors and HLA-DR molecules. Several investigators reported that neither flow cytometry, nor cyto-immunological monitoring based on morphology (CIM) or serial sIL-2R monitoring were suitable tools to diagnose intragraft events reliably in the peripheral blood.[1-6] In other studies however, activated T cells were found in PBL during graft rejection.[7-10] We wondered if these activated T cells are *in vivo* activated, committed, donor directed cytotoxic T cells (cCTL) which are responsible for rejection. Such cCTL can be propagated from kidney biopsies and from endomyocardial biopsies (EMB) after transplantation and their presence seems to correlate with histological rejection.[11-13]

In this study our efforts to culture these donor reactive cCTL from PBL are described. The functional capacity of these cells was compared with the reactivity of biopsy derived graft infiltrating lymphocytes.

5.2 Materials and Methods

5.2.1 Patients

In our centre a total of 535 EMB from 87 patients obtained at several time intervals after HTx were cultured in IL-2 containing medium.[13] From most patients 40 ml peripheral blood was taken simultaneously with each biopsy. For the current study we selected 39 PBL and 38 EMB samples from 20 cardiac transplant recipients. From 13 patients EMB and PBL were studied in the first half year after heart transplantation (HTx). Six patients were studied during and around a biopsy proved

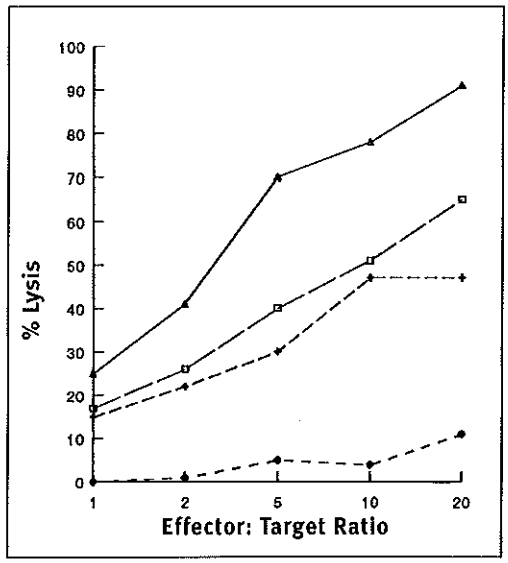

Figure 5.1 *A representative experiment showing the LAK-like cytotoxicity pattern of PBL culture WL132 grown for 12 days on IL-2, tested in a 4 hour ^{51}Cr release assay against donor T-BL (●) and B-LCL (+), third party B-LCL (□) and K562 (▲).*

rejection episode and seven at a period that no rejection was monitored. From nine of these patients 2 to 7 consecutive time points and from 4 patients one time point was studied. From 7 patients EMB and PBL taken more than 1 year after HTx were studied. All patients had received preoperative blood transfusions and were under immunosuppression with cyclosporin A and low dose steroids. Rejection was monitored by means of histological examination of EMB, the histological rejection grade being assessed according to Billingham's criteria.[14] Grade 0: no evidence of rejection; Grade 1: mild rejection, perivascular and endocardial infiltration with pyroninophylic lymphocytes, endocardial and interstitial edema; Grade 2: moderate rejection, more dense perivascular interstitial infiltrates, and focal myocytolysis (necrosis). Grade 3: severe rejection, was never observed in our study. Rejection therapy, methylprednisolone, OKT-3 or rabbit-anti-thymocyte-globuline was only instituted in case of moderate or severe rejection.

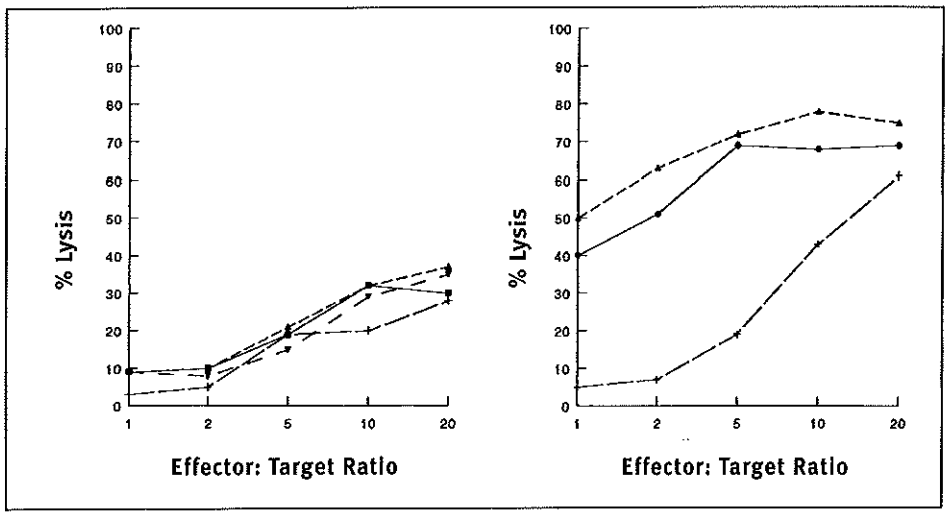

Figure 5.2 *The donor specific cytotoxicity pattern of PBL culture KU42 grown for 14 days on IL-2. (A) Cytotoxicity against ^{51}Cr-labelled donor B-LCL(■) Could not be inhibited by a 10-fold excess unlabelled K562 (+), third party T-BL-710 (▲) or third-party B-LCL-VE(▼); (B) Cytotoxicity against labelled K562 (●) was inhibited by cold K562 (+) and not by cold third party T-BL-710 (▲).*

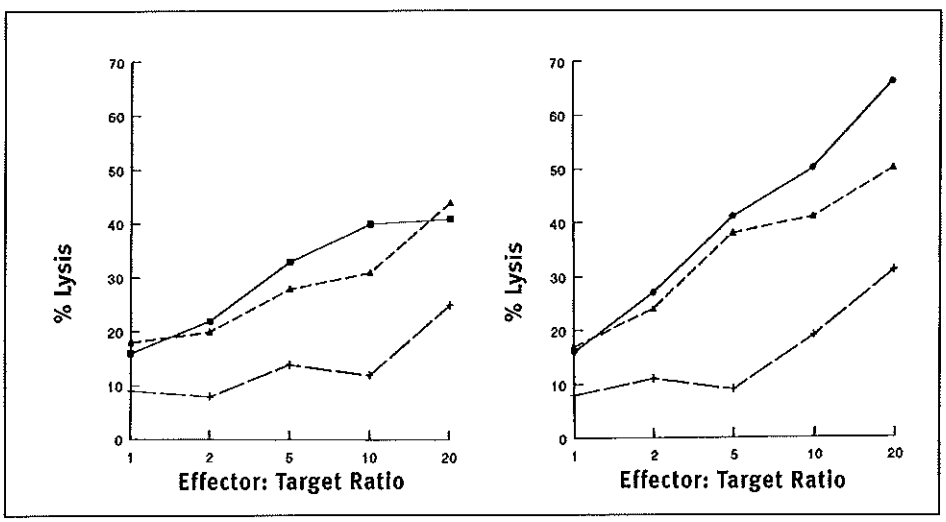

Figure 5.3 *LAK-like cytotoxic activity of culture WL132 against (A) labelled donor B-LCL (■) and (B) labelled third party B-LCL(●) could not be inhibited by a 10-fold excess unlabbelled K562(+) and not by a 10-fold excess cold thirdparty T-BL-78 (▲).*

		EMB		PBL		
		CML[c]		CML[c]		
Sample ID[a]	Rejection grade[b]	Do-BLCL	3P-BLCL	Do-T-BL[d]	Do-BLCL[e]	3P-BLCL[e]
KU22	1	83	38	0	19(0)	15(0)
KU29	0	71	4	0	25(0)	-(-)
KU42	2	78	6	20	32(20)	0(-)
KU52	1	74	5	0	25(0)	30(5)
KU57	1	NG	-	20	-(-)	-(-)
KU66	1	NG	-	0	0(-)	90(-)
KU94	2	48	8	0	31(4)	30(-)
RO07	0	NG	-	0	15(5)	20(0)
RO14	2	46	3	0	0(-)	0(-)
RO28	1	46	2	0	0(-)	0(-)
RO33	2	74	3	0	0(0)	0(0)
RO40	1	75	9	0	0(0)	0(0)
RO47	1	75	7	0	0(0)	4(10)
OO102	2	-	-	0	10(0)	0(0)
OO149	1	NG		0	45(6)	42(5)
EN41	1	NG	-	0	0(-)	0(-)
EN51	2	64	1	0	0(-)	0(-)
WO13	1	NG	-	0	0(-)	0(-)
WO18	2	36	5	-	-(-)	-(-)
KO57	2	40	6	0	-(-)	-(-)

[a] *Patient identification, follwed by the number of days after transplantation.*
[b] *Billingham's rejection grade.*[14]
[c] *Percentage of donor-specific lysis (Do) and lysis of third-party cells (3P), at an Effector:Target ratio of 20:1.*
[d] *Percentage lysis of donor PHA-T blasts at an E:T ratio of 20:1.*
[e] *Percentage lysis of donor B-LCL (Do-BLCL) or unrelated third party B-LCL (3P-BLCL) at an E: T ratio of 20:1 before and,in parenthesis, after inhibition with 10-fold excess of cold K562 at an E: hot-Target ratio of 5:1.*
- No sample or not tested.
NG: no or insufficient growth

Table 5.1 *Cytotoxic activity of EMB- and PBL- derived cell lines obtained in the first half year after heart transplantation during the period in which patients had an acute rejection episode*

		EMB		PBL		
		CML[c]		CML[c]		
Sample ID[a]	Rejection grade[b]	Do-BLCL	3P-BLCL	Do-T-BL[d]	Do-BLCL[e]	3P-BLCL[e]
CL159	-	-	-	0	25(0)	54(15)
CL177	1	NG	-	0	35(9)	15(0)
CL219	0	NG	-	0	52(2)	-(-)
LA117	0	0	0	-	0(-)	30(-)
LA132	-	-	-	0	0(-)	70(-)
LA186	0	61	6	0	30(-)	40(-)
WL97	1	44	4	0	0(-)	0(-)
WL132	0	NG	-	0	48(-)	65(-)
JU26	1	42	7	0	0(-)	0(-)
JU32	0	NG	-	0	0(-)	0(-)
BN25	1	88	0	0	30(-)	60(-)
JO29	0	NG	-	0	22(0)	0(0)
BU149	1	56	2	0	0(-)	0(-)

[a] *Patient identification, follwed by the number of days after transplantation.*
[b] *Billingham's rejection grade.*[14]
[c] *Percentage of donor-specific lysis (Do) and lysis of third-party cells (3P), at an Effector:Target ratio of 20:1.*
[d] *Percentage lysis of donor PHA-T blasts at an E:T ratio of 20:1.*
[e] *Percentage lysis of donor B-LCL (Do-BLCL) or unrelated third party B-LCL (3P-BLCL) at an E:T ratio of 20:1 before and,in parenthesis, after inhibition with 10-fold excess of cold K562 at an E:hot-Target ratio of 5:1.*
- No sample or not tested.
NG: no or insufficient growth

Table 5.2 *Cytotoxic activity of EMB- and PBL- derived cell lines obtained in the first half year after heart transplantation during a period in which patients had no acute rejection episode.*

5.2.2 T cell lines from EMB

T cell lines were generated from EMB as described before. [13] In brief, biopsy fragments were placed in 200 μl culture medium (CM) containing 10 % v/v (± 60 units IL-2) lectin free Lymphocult (Biotest, Dreiech, Germany), (CM-IL2), in the presence of 10^5 irradiated (40 Gy) autologous PBL. CM consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% human serum, L-glutamine, penicillin and streptomycin. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Cultures were assayed for phenotypic expression of differentiation antigens and donor specific cytotoxicity within 4 weeks after initiation of the culture.

5.2.3 T cell lines from PBL

Buffy coats were harvested from blood after centrifugation at 600 g during 7 min. and diluted with an equal volume of Hanks Balanced Salt Solution (HBSS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. This suspension was layered on a Ficoll-isopaque gradient (δ= 1.077) (Pharmacia, Uppsala, Sweden) and centrifuged during 20 min at 800 g at room temperature. Lymphocytes were harvested from the interface, washed three times and cultured in 200 μl CM-IL2 in 96 well round bottom tissue microtiter plates (Costar 3799, Cambridge, MA, USA). From each sample 10^5 20 wells containing 10^5 cells were grown during 10 -14 days at 37°C in a humidified 5% CO_2 incubator. Half the culture medium was changed every 2-3 days.

5.2.4 Target cells

To determine allospecific cytotoxicity PHA transformed T-blasts (T-BL) and EBV transformed B lymphoblast cell lines (B-LCL) from donor origin were used. T-BL were used as targets for HLA class I specific cytotoxicity. Since HLA-class II antigen expression on T-BL is usually low, B-LCL were used as targets for HLA class II specific reactivity. Third party B-LCL or T-BL (sharing no HLA antigens with the donor) served as negative controls. As control for non-MHC-restricted (NK and/or LAK) cytotoxicity, the proerythro-blastic tumour cell line K562 was utilized.

For the generation of T-BL 10^7 nucleated donor spleen cells/ml were stimulated for 3 days with 1% PHA-M (DIFCO, Detroit, Mi, USA) in CM and then cultured for 3 more days in CM supplemented with 5% Lymphocult (Biotest) as a source of IL-2. Generation of B-LCL was done as described elsewhere.[15] In brief, lymphocytes isolated from donor spleen or PBL were resuspended in culture supernatant of the marmoset cell line B95-8 and incubated for 4 hours at 37°C in 5% CO_2. After washing cells were resuspended in CM containing 1% PHA and 5% heat-inactivated FCS and cultured.

5.2.5 Cytotoxicity assay

Cytotoxic capacity of the cultures was measured as described before.[13] In brief, 2.5 x 10^3 ^{51}Cr labelled PHA-blasts or B-LCL were mixed with effector cells in effector-to-target (E/T) ratios ranging from 40/1 to 1/1 in 200μl/well CM in 96 well U-bottom microtiter plates (Costar). The plates were centrifuged (60 g, 1 min) and incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were collected with a Skatron harvesting system (Skatron-AS, Norway). The percentage of specific lysis was calculated according to the formula:

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Maximal release was determined from a Triton X100 lysate of the target cells. Spontaneous release was determined by incubation of target cells in CM only.

A CML was considered positive when the percentage specific lysis exceeded 10% and the slope of the ratio curve was positive.

5.2.6 Cold target inhibition

To discriminate between *in vivo* induced MHC-restricted donor- specific reactivity and culture induced non specific cytotoxicity (LAK), cold target inhibition studies were performed. Unlabelled (cold) K562 cells were mixed in 10 fold excess with ^{51}Cr labelled B-LCL target cells or ^{51}Cr labelled K562. Control values were established by adding donor or third party T-BL as cold competitors.

5.3 Results

After culture in CM with 60 units IL-2 during 10 to 14 days PBL samples were harvested and analyzed for cytotoxic function. Cultures propagated from EMB in the same medium were analyzed after 3 to 4 weeks of culture.

5.3.1 Cytotoxic activity

PBL and EMB cultures were tested in the CML-assay with donor T-BL, donor B-LCL, third party

		EMB		PBL		
		CML[c]		CML[c]		
Sample ID[a]	Rejection grade[b]	Do-BLCL	3P-BLCL	Do-T-BL[d]	Do-BLCL[e]	3P-BLCL[e]
PA742	0	NG	-	0	-(-)	-(-)
PJ844	0	NG	-	0	-(-)	0(-)
LI844	1	NG	-	0	0(-)	0(-)
ST662	0	NG	-	0	0(-)	-(-)
KR1290	0	NG	-	0	0(-)	0(-)
TW592	1	NG	-	0	71(9)	80(21)
BL1582	0	NG	-	0	20(0)	70(18)

[a] Patient identification, follwed by the number of days after transplantation.
[b] Billingham's rejection grade.[14]
[c] Percentage of donor-specific lysis (Do) and lysis of third-party cells (3P), at an Effector:Target ratio of 20:1.
[d] Percentage lysis of donor PHA-T blasts at an E:T ratio of 20:1.
[e] Percentage lysis of donor B-LCL (Do-BLCL) or unrelated third party B-LCL (3P-BLCL) at an E:T ratio of 20:1 before and,in parenthesis, after inhibition with 10-fold excess of cold K562 at an E:hot-Target ratio of 5:1.
- No sample or not tested; NG, no or insufficient growth

Table 5.3 *Cytotoxic activity of EMB- and PBL- derived cell lines obtained more than 1 year after heart transplantation, during a period in which patients had no acute rejection episode.*

B-LCL and T-BL, mismatched with the donor, and K562 as targets. As shown in the tables 5.1, 5.2 and 5.3 only two cultured PBL samples did lyse donor T-BL at a low but significant level. Third party T-BL were never killed (data not shown). The results obtained with B-LCL as targets had a more variable pattern. Twelve cultures lysed both donor and third party B-LCL, seven killed either donor B-LCL or third party B-LCL and 15 cultures did lyse neither. Five samples were not tested on all targets. Since the PBL samples that killed B-LCL always had high cytotoxicity against the LAK sensitive cell line K562 (*Figure 5.1*) B-LCL kill could be due to LAK activity induced by IL-2 during culture. As shown by Oshimi et al.[16] B cell blast are more sensitive to Il-2 activated LAK effectors than T cell blasts. Only one of the corresponding T cell cultures propagated from the EMB exhibited LAK-like cytotoxicity, a third party B-LCL and K562 were lysed at the same level (*Table 5.1*). All other EMB derived T cell lines tested neither lysed third party B-LCL (*Table 5.1 and 5.2*) nor K562 (data not shown).
To distinguish between aspecific LAK activity and donor specific class II allo-reactivity, cold target inhibition was performed with a 10 fold excess of unlabelled K562 added to ^{51}Cr labelled donor B-LCL, third party B-LCL or K562. Only in culture KU42 the reactivity against

donor B-LCL could not be inhibited by cold K562 (*Figure 5.2A*). This was one of the two cultures that specifically lysed donor T-BL (*Table 5.1*). The lysis of ^{51}labelled K562 by this culture was blocked by cold K562 up to a E/T ratio of 10, whereas a 10 fold excess of unlabelled third party T-BL had no influence (*Figure 5.2B*). In all other PBL cultures reactivity against B-LCL could be inhibited by cold K562 and not by cold third party T-BL. In figure 5.3A and 5.3B a representative example of these experiments is depicted. Inhibition was not always complete over the whole E/T ratio range because of high LAK activity. Cultures were considered to be not donor specific when the percentage lysis at an E/T ratio of 5 was reduced by cold K562 to 10% or less. The PBL cultures BN25, PA741, PJ844, and TW592 contained to few effector cells to perform these control tests. Since reactivity against donor T-BL was negative and the reactivity against third party B-LCL and K562 was high, we regarded the lysis of B-LCL by the PBL cultures BN25 (*Table 5.2*) and TW592 (*Table 5.3*) as caused by aspecific LAK-activity.

5.3.2 Relation between cCTL and rejection

In order to clarify whether donor reactive cCTL were detectable in PBL during rejection, we in vitro expanded PBL from a group of 6 patients during an episode in which they showed a biopsy proved rejection crisis. During these episodes cCTL are generally found in the graft (*Table 5.1*). Donor reactive CTL were found significantly less often in PBL samples (2/20) than in simultaneously taken EMB samples (13/19) (X^2-test $p<0.01$).

It can be argued that at the time of a rejection crisis cCTL accumulate in the graft, which results in an extreme low cCTL frequency in the PBL. If cCTL circulate, it might well be that the frequency of donor specific CCTL in PBL will be higher during periods that less cCTL are present in the graft. We therefore analyzed PBL samples from a period that no rejection crisis occurred. Table 5.2 shows the results obtained with PBL and EMB taken from 7 other patients in a comparable period after HTx to the first group but this time during a period of freedom from rejection. However none of the PBL cultures lysed donor T-BL. Activity against donor B-LCL could always be inhibited by cold K562 and not by cold third party T-BL (*Figure 5.3A*). In table 5.3 the results are summarized obtained from cultured EMB and PBL taken from 7 additional patients, more than one year after HTx. Also in this period, when most patients do not encounter a rejection episode any more and EMB did not contain donor reactive cCTLs, no cCTL could be propagated from the blood.

5.4 Discussion.

Many attempts have been made to diagnose rejection using peripheral blood parameters and thus replace the need to biopsy the graft. Parameters such as changes of T cell subsets or their ratio, an increase in $CD25^+$ or $HLA\text{-}DR^+$ T cells, sIL-2R levels or morphological changes in PBL have been studied.[1-6,17-20] The results were variable and inconclusive.

The aim of our study was to verify whether donor specific immunological reactivity of primed cells in PBL reflects intragraft events. For that purpose we compared the occurrence of donor reactive cCTL in the peripheral blood and in the graft, both in episodes with rejection and of immunological quiescence. Activated, antigen specific, committed CTL are derived from precursor CTL (pCTL). The activation process resulting in proliferation and maturation of the CTL requires 2 signals, alloantigen and cytokines.[21] If pCTL have recently received the alloantigenic signal *in vivo* only IL-2 is required for subsequent *in vitro* growth during 3-4 weeks without loss of specificity.[11-13] Thus if recently allo-activated cCTL are present in the PBL it should be possible to expand them in the presence of IL-2 without loss of specific function. Our results however demonstrated that such cells were not detectable within PBL population of most patients studied. Neither at the time of a biopsy proved rejection when cCTL were present in the graft nor in the episode

just prior to rejection. In periods when no acute rejections were encountered and no cCTL were present in the graft also no donor directed reactivity could be detected in the PBL. Similar results were reported by Suitters et al.[22] for humans and Orosz et al. [23,24] for mice using limiting dilution assays (LDA). Both studies reported a higher frequency of donor reactive CTL within the graft than in PBL or lymphnodes. Orosz et al. [23] used LDA methods that allowed for discrimination between cCTL and pCTL. Their results showed that the majority of CTL in the sponge allo graft were cCTL whereas in the spleen and regional lymph nodes the majority were pCTL. In our patient group MLR reactivity and MLR induced CTL response against donor cells could be easily detected in PBL in the first 3 months posttransplant.[25] This indicates that although pCTL do circulate in PBL, cCTL are not detectable using the present approach. Reader et al. [26] suggested quantification of donor reactive CTL in blood by means of the LDA technique as an useful tool for noninvasive rejection monitoring in cardiac transplants. An increase in the donor reactive CTL frequency in the blood was associated with signs of rejection in the graft. However they could not discriminate between a histologic grade 1 (lymphocyte infiltrates) and grade 2 (infiltrates and myocytolysis) rejection. Most centres consider only grade 2 as clinically relevant and start rejection treatment when myocyte damage is seen in the EMB. Orosz et al. [23] suggested that enumeration of cCTL might be more informative. One major drawback for the use of LDA for peripheral rejection monitoring is the 10 day culture period. Another, more fundamental argument is that the peripheral blood may not be the preferential site for cCTL.

For the preferential accumulation of cCTL in the graft three mechanisms might be involved. 1. pCTL migrate to the graft were they become activated by alloantigen and subsequently receive the lymphokine signal to become mature cCTL. 2. After central sensitization as postulated by Larsen et al. [26] and Austen and Larsen [27] immature CTL migrate from the lymphoid organs to the graft were they receive their final maturation step by lymphokines. 3. pCTL become fully activated to cCTL in the lymphoid organs, cCTL migrate at a very low frequency and accumulate in the graft were they expand under influence of IL-2. It is as yet unknown which mechanism takes place, however the three possibilities are not mutually exclusive. The lymphokines may be produced in the graft by sensitized CD_4 positive T cells.

We previously reported [13] that CD_4 cells indeed are dominant especially in cultures propagated from EMB taken during acute rejection. From studies of Dallman et al. [28] it became apparent that a normal IL-2 pathway is required for allograft rejection. They showed that in the grafts of tolerant rats lymphocytic infiltrates were still present. However those cells did not produce biologically active IL-2 and do not respond normally to IL-2.

If pCTL indeed undergo their final activation in the graft to become cCTL it is obvious that monitoring of PBL can not be representative for intragraft events. Our present data support this which leads to the conclusion that at the moment EMB remain the only reliable source for the detection of cardiac graft rejection.

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CHAPTER 6

DIFFERENTIAL AVIDITY AND CYCLOSPORIN A SENSITIVITY OF COMMITTED DONOR SPECIFIC GRAFT INFILTRATING CYTOTOXIC T-CELLS AND THEIR PRECURSORS:

RELEVANCE FOR CLINICAL CARDIAC GRAFT REJECTION

Abstract

We have used limiting dilution analysis (LDA) to study the qualitative and quantitative differences between graft infiltrating cytotoxic T cell populations propagated from endomyocardial biopsies (EMB) of heart transplant patients that experienced one or more acute rejection episodes and patients who never showed signs of rejection. Limiting dilution cultures were stimulated with autologous or donor cells both in absence or in presence of Cyclosporin A (CsA) and of CD8 in the cytotoxic phase. Almost all antigen primed, committed cytotoxic T cells (cCTL) present in the graft of patients with rejections were CsA resistant. In contrast, in most patients of the non rejector group a substantial part of the cCTL could be inhibited by CsA. The CTL precursors (pCTL) in both groups were predominantly CsA sensitive. Addition of CD8 monoclonal antibody during the cytotoxicity phase of the LDA was used to differentiate between CTL populations with high avidity for donor antigens and populations with low avidity. The predominant subpopulation in the graft of rejectors was a CsA resistant cCTL with high avidity, while in the graft of most non rejectors cCTL with low avidity dominated. In most rejectors CD8 mAb had only a minor influence on the pCTL frequency estimates, and thus on had high avidity. This CsA sensitive pCTL with high avidity might represent an intermediate stage between the naive pCTL and mature, functional, CsA insensitive cCTL with high avidity for donor antigens.

6.1 Introduction

Although many cell types have been identified in rejecting grafts including B cells, natural killer (NK) cells, macrophages, neutrophils, and eosinophils [1,2] it is generally accepted that alloreactive T lymphocytes are the primary effector cells in the acute rejection process after organ transplantation.[3,4,5] During acute rejection donor reactive cytotoxic T lymphocytes (CTL) as well as CTL precursors with irrelevant antigen specificity accumulate in the graft as shown by conventional [6] and limiting dilution analysis.[7,8,9] Orosz and coworkers [7] demonstrated in an experimental model that donor reactive CTL can be subdivided in comitted (primed) cytotoxic T cells (cCTL), and their naive precursors (pCTL). High levels of donor specific, T cell-mediated cytotoxic activity were also found in grafts of blood transfused animals that never rejected their graft,[10,11,12] and in the graft of patients that never experienced an acute rejection.[13] Qualitative differences between CTL present in the graft of rejectors and non rejectors might be an explanation. Experiments in mice by MacDonald and coworkers [14,15] suggested that CTL clones that need the CD8 molecule for their effector function have a T cell receptor (TCR) with low affinity for antigen and CTL clones that do not require CD8 for their cytolytic function have a high affinity TCR for antigen. They also showed that in vivo activated CTL were predominantly cells with high affinity TCR, whereas in vitro activated CTL were mainly of the low affinity type. Similar qualitative differences in CD8 requirement were found for CTL clones from humans [16] and between CTL populations derived from neonatally tolerant animals and normal animals.[17] CTL from tolerant mice interacted with their target cells with a lower avidity than CTL from normal animals. In a previous study using LDA we described that donor-reactive CTLs derived from the graft of patients with acute rejection could not be inhibited by CD8 monoclonal antibody, while in non rejectors the cytolytic function of graft infiltrating CTL could be inhibited.[9] The experimental design of that study did not enable us to distinguish between cCTL and pCTL. It is conceivable that in non rejectors CD8 sensitive pCTL are the dominant population, whereas *in vivo* activated, CD8 resistant, cCTL is the predominant cell type in the rejectors. We tested this hypothesis in a modified LDA and investigated simultaneously whether pCTL and cCTL populations in those patients had a different sensitivity for cyclosporin A. CsA is known to impair the generation of alloantigen-specific CTL in murine and human mixed lymphocyte cultures (MLC). Added to LDA cultures of PBL from normal human individuals [18,19] or mice spleen cells,[20] CsA reduces the estimate of the CTL precursor frequency. The influence of CsA on cCTL, primed in MLC, is subject to controversy. Orosz and coworkers [18] found that 75 - 98% of the alloantigen primed CTL obtained from primary MLCs failed to respond to the same allo-antigen in LDA cultures supplemented with CsA. In contrast, in the studies of Kabelitz et al [19] CsA did not impair the function of primed CTL obtained from primary MLCs and thus did not reduce the frequency estimate of cCTL in these cultures.

6.2 Materials and Methods

6.2.1 Patients

We studied 18 heart transplant recipients of whom 8 had experienced one or more acute rejection episodes during the first post transplant year (rejectors), and 10 patients that never showed signs of rejection (non-rejectors). All patients had received preoperative blood transfusions. Cyclosporin A (CsA) and low dose prednisone were used as maintenance immunosuppression.

The mean number of HLA-mismatches between donor and recipient for the A, B, and DR-antigens was respectively 1.25, 1.50 and 1.37 for the rejectors, and 1.20, 1.40 and 1.10 for the patients without rejection. Before transplantation, in none of the patients HLA allo-antibodies were detectable in a screening in the standard National Institute of Health complement-dependent cytotoxicity assay against a panel of 53 lymphocyte donors.

Detection of acute rejection was performed by histological examination of endomyocardial biopsies taken by transvenous approach from the right ventricle. During each of the 15 biopsy procedures in the first post transplant year, four or five fragments of the endomyocardium were obtained. Three or four were used for histologic evaluation, and one was placed in interleukin-2 (IL2) conditioned culture medium for cell culture.
For the diagnosis of clinically relevant rejection, the coexistence of myocyte damage and mononuclear cell infiltrates was required. In these cases, anti-rejection treatment was instituted. From each of the patients series of biopsy-derived cultures were available. For the present study, we selected from each rejector a culture derived from an EMB taken before the first anti-rejection therapy was instituted. The cultures ME,KU,JA, and RO were obtained from EMB with signs of acute rejection (myocyte damage), the cultures JS, MO, PO, and HA were propagated from EMB taken respectively 35,7,6, and 5 days before acute rejection was diagnosed. The cultures grown from EMB from patients that never had an acute rejection, were taken 16 - 62 days, median 35 days after heart transplantation. This time period after transplantation was comparable with the period the biopsies were taken from the rejectors (range 16 - 98, median 32 days). The lymphoid cells propagated from EMB of rejectors were cultured for 16 - 35 days, median 24, and the cells grown from the non-rejectors for 16 - 34 days, median 24 before they entered the LDA.

6.2.2 Media
The culture medium (CM) used in this study was RPMI-1640-Dutch modification supplemented with 4mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, (all available from Gibco, Paisley, Scotland) and 10% pooled human serum. Approximately 750 ml lots of serum were prepared by mixing male sera that were tested for good growth support in a standard 7 day, one way, MLC.

For propagating lymphoid cells from EMB, CM was supplemented with 10% v/v lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL2 (CM-IL2)

6.2.3 Isolation of peripheral blood mononuclear cells (PBMC).
Mononuclear cells were isolated from heparinized venous blood of the transplant recipients by Ficoll-Hypaque density gradient centrifugation. The cells were washed two times with Hanks Balanced Salt Solution (Gibco) and resuspended in CM.

6.2.4 Lymphocyte cultures from EMB
GIL Cultures were established as described in chapter 3. In brief, each biopsy was cultured in a 96 well round bottom tissue culture plate (Costar, Cambridge, MA) containing 200 μl CM-IL2 per well in the presence of 10^5 irradiated (30 Gy) autologous PBMC as feeders. The plates were incubated in a humidified atmosphere at 37°C in 5% CO_2.

6.2.5 Allogenic target cells
T cell blasts (T-LCL) were obtained by culturing donor spleen cells for 4 days in the presence of 1% Phytohaemagglutinine-M (PHA) (Difco, Detroit, MI) in CM with 5% lymphocult-T (Biotest).

6.2.6 Phenotypic analysis
Lymphoid cell cultures were analyzed for the expression of TCR-$\alpha\beta$, CD3, CD4 and CD8 antigen by two-colour flow cytometry after staining with the monoclonal antibodies WT31, anti-leu4, anti-leu3a and anti-leu2 respectively. The antibodies were directly conjugated to fluoresceine (FITC) or phycoerythrin (PE) and were obtained from Becton Dickinson, Mountain View, CA. From each culture 10.000 cells were stained under standard conditions as described before [13] and 5000 cells were analyzed on a FACScan flowcytometer (Becton Dickinson).

6.2.7 Limiting dilution analysis
We employed a limiting dilution culture method

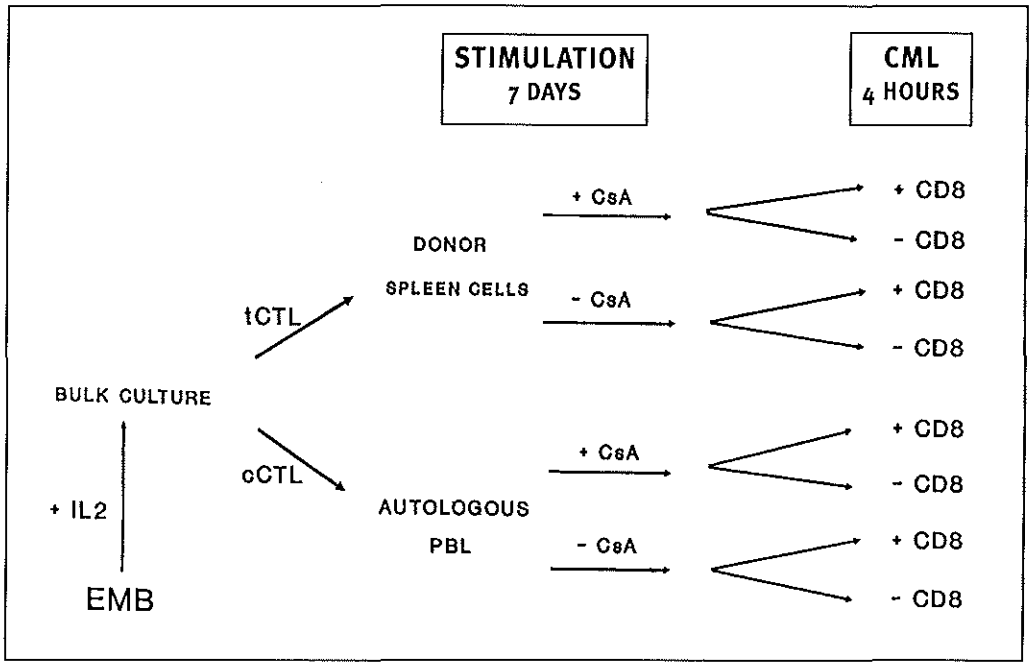

Figure 6.1. *Scheme of the LDA methods used to enumerate the frequency of the tCTL and cCTL populations in the lymphoid cultures propagated from EMB obtained from heart transplant patients.*

(*Figure 6.1*) that selectively enumerates CTL that have been stimulated following *in vivo* sensitization or the total pool of donor reactive CTL. According to Orosz et al [7] we refer to the subpopulation of CTL that have been stimulated *in vivo* by donor allo-antigens and subsequently *in vitro* (during LDA) by autologous cells, as "allo-antigen conditioned" or cCTL. The total pool of donor reactive CTL is quantified by *in vitro* stimulation (during LDA) with donor cells, will be referred to as tCTL (= antigen-conditioned cCTL + naive precursor, pCTL). The frequency of naive donor reactive precursor (pCTL) is calculated by subtracting the cCTL frequency from the tCTL frequency.

By adding CsA during the stimulation phase of the LDA and CD8 mAb in the cytotoxic phase we were able to determine frequency estimates of CsA and/or CD8 resistant cCTL, tCTL and indirectly of the pCTL (*Figure 6.1*).

Limiting dilution microcultures were set up in 96-well round-bottom microculture plates (Costar) by adding responder cells derived from EMB-cultures to either 5 x 10^4 irradiated (30 Gy) donor spleen cells or autologous PBMC as stimulator cells. EMB derived cells were titrated in 8 fold doubling dilutions starting from 5,000 to 15,000 per well, depending on the number of cells available. In 4 patients lower cell numbers were tested. Usually, 24 replicate microcultures were set up for each responder cell dilution in a total volume of 0.2 ml CM supplemented with 20 Units recombinant IL-2/ml (Biotest).

Limiting dilution microcultures were incubated in the presence or absence of 100 ng CsA/ml for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. At day 7 the microcultures were split in two by transferring 190 μl of the culture to 2 new wells, 95 μl in each well. Each well was individually tested for cytolytic activity against 2.5 x 10^3 ^{51}Cr labelled donor T-LCL in a 4 hour assay at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were harvested using a Skatron harvesting system (Skatron-AS, Norway), and the release of ^{51}Cr was assayed in a Cobra gamma-counter

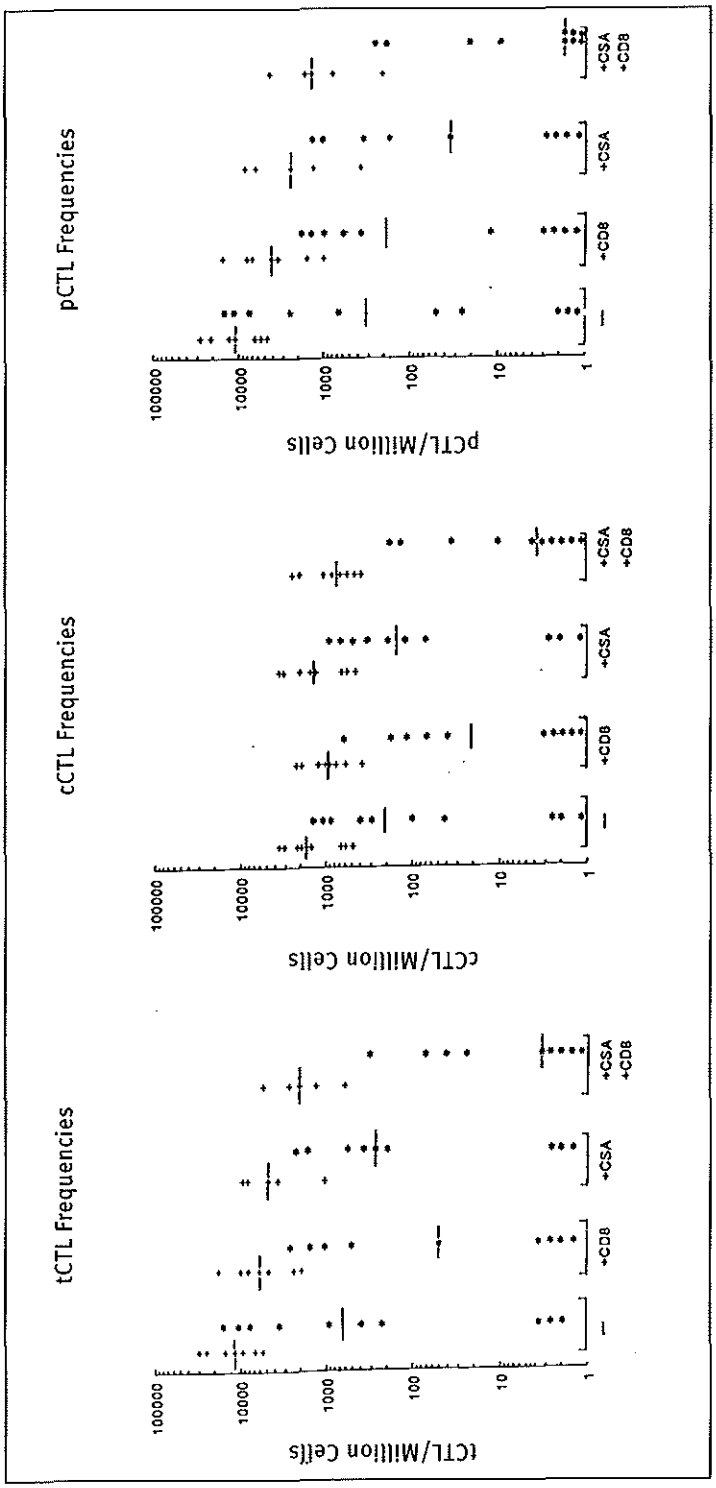

Figure 6.2 *tCTL (panel A) cCTL (panel B) and pCTL (panel C) frequencies in GIL cultures propagated from EMB of rejectors (+) or non-rejectors (*), in the presence of CD8, CsA, or both CsA and CD8 or without these agents. Horizontal bars represent the median value of the group.*

(Packard-Canberra).
Half of the split wells were tested for cytotoxicity in the presence of CD8 monoclonal antibodies, as described below. The other half of the wells was tested without monoclonal antibody or in the presence of control antibody (see below). Maximum and spontaneous release were determined in 5 fold. Microcultures were considered cytolytic when the experimental lysis percentage exceeded 10%. Cultures with the highest responder concentration were tested against autologous T-LCL, third party T-LCL and the K562 cell line as control for specificity.

6.2.8 CD8 inhibition studies

The CD8 monoclonal antibody FK18 (a mouse-anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule;[21,22] a gift of Dr. F. Koning, Department of Immunohaematology and Bloodbank, University Hospital Leiden, Netherlands) was used as a 1:500 dilution of ascitic fluid. Before addition of the targets, FK18 was added to the effector cells and cells were preincubated during 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2. As control antibody the mouse anti- rat Mab MRC-Ox8 (CD8) or mouse anti-human Mab RIV-6 (CD4) was used. RIV-6 was a gift of Dr. M.F. Leerling from the Lab. for controle of bacterial vaccines, National Institute of Public Health and Environmental Hygeine, Bilthoven, The Netherlands.

6.2.9 CsA inhibition studies.

Cyclosporin A (Sandimmune, Sandoz, Basel, CH), obtained as a solution of 5000 ng CsA/ml, was diluted in human serum to 1000 ng/ml. At the start of the 7 day incubation period of the LDA 20μl of this solution was added to the each well of a microtitre plate containing 180 μl CM (*See figure 6.1*).

6.2.10 Statistical analysis

Minimal estimates of the CTL frequencies were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the wells and the percentage of replicate cultures that failed to develop cytotoxicity.[23]
Frequency calculations were made using a computer program designed by Strijbosch et al.[24] The frequency, expressed as number of cytotoxic cells per 10^6 cells, was calculated with the maximum likelihood estimation, adapted with a jackknife method. Standard deviation and 95% confidence intervals were calculated as well. The calculated frequencies were accepted when the goodness-of-fit did not exceed 12.
To test significant differences between frequencies measured in absence or presence of CD8 and/or CsA the student T test was used.
The Wilcoxon rank sum test was used for comparing differences in CTL frequencies with and without CD8 and/or CsA within one patient group. The significance of differences between the groups of patients was analyzed with Fisher's exact test using STATGRAPHICS V4.0 software.

6.3 Results

We investigated lymphoid cultures propagated from the EMB of 8 heart transplant patients at the time of rejection or before rejection (rejectors) and of 10 patients that never had an acute rejection (non-rejectors) in the same time period after transplantation. The cultures propagated from EMB from rejectors contained 22 - 78%, median 54% CD8 positive cells, for non rejectors this was in a comparable range (17 - 88, median 55%). The intensity of the CD8 antigen expression on the cell surface was also comparable in both groups. When stained with CD8-PE the mean fluorescence intensity varied between channel 780 and 835. None of the cultures were restimulated with donor cells before analysis in LDA. To obtain sufficient cells for LDA after liquid nitrogen storage, cultures were stimulated for 7 days with third party B-LCL cells, fully HLA mis matched with both donor and acceptor.

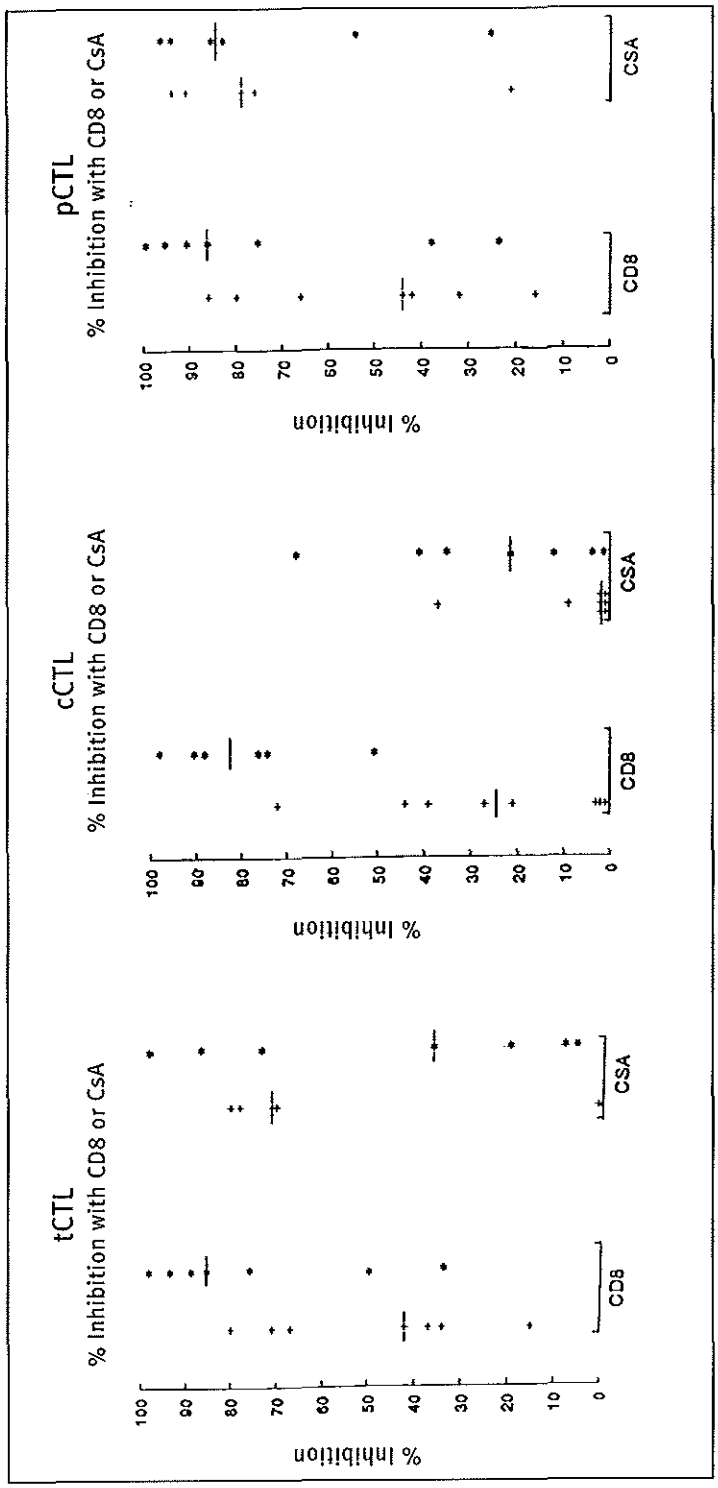

Figure 6.3. *The inhibition of donor specific tCTL (panel A) cCTL (panel B) and pCTL (panel C) by CD8 Mab or CsA in GIL cultures propagated from EMB of rejectors (+) and non-rejectors (*). Horizontal bars represent the median value of the group.*

ID	LDA	tCTL		cCTL	
	with	freq[2]	95% confidence interval	freq[3]	95% confidence interval
KU	-	30003	21518 - 38487	1908	1448 - 2386
	CD8	17513	12989 - 22036	1916	1456 - 2375
	CsA	8582	6496 - 10667	2005	1539 - 2471
	CsA+CD8	5778	4311 - 7244	1993	1436 - 2249
JS	-	11682	8698 - 14666	617	453 - 781
	CD8	2352	1743 - 2961	764	597 - 949
	CsA	9360	6992 - 11729	611	445 - 778
	CsA+CD8	2074	1572 - 2576	664	476 - 851
ME	-	ND[4]		473	350 - 596
	CD8	ND		372	270 - 473
	CsA	ND		477	368 - 606
	CsA+CD8	ND		375	276 - 473
MO	-	5456	4109 - 6803	587	450 - 723
	CD8	4649	3550 - 5748	597	441 - 718
	CsA	1038	779 - 1297	561	423 - 699
	CsA+CD8	1322	953 - 1690	524	400 - 648
PO	-	9471	7177 - 11764	3010	2214 - 3806
	CD8	5924	4398 - 7450	2189	1707 - 2670
	CsA	ND		3152	2767 - 3938
	CsA+CD8	ND		2421	1813 - 3028
JA	-	6512	4624 - 8397	1454	1091 - 1818
	CD8	1894	1400 - 2388	880	670 - 1089
	CsA	ND		1322	953 - 1690
	CsA+CD8	ND		823	628 - 1019
RO	-	2456	616345 - 32778	3453	2657 - 4250
	CD8	8065	6028 - 10101	961	718 - 1203
	CsA	4802	3662 - 5941	3452	2537 - 4367
	CsA+CD8	612	465 - 760	399	296 - 502
HA	-	13957	9559 - 18355	2122	1634 - 2610
	CD8	9281	6535 - 12027	1185	866 - 1504
	CsA	3854	2842 - 4866	1340	995 - 1685
	CsA+CD8	2743	2097 - 3390	1036	719 - 1353

[1] *The jackknife version of the maximum likelihood estimation procedure was used as the statistical method for calculation of the frequency.*
[2] *tCTL/10^6 cells, quantified after stimulation with donor spleen cells*
[3] *cCTL/10^6 cells, quantified after stimulation with autologous PBL.*
[4] *ND: not determined*

Table 6.1. *The influence of CD8 Mab and CsA on tCTL and cCTL frequency estimates against donor antigens in the graft of HTx patients with rejection* [1].

ID	LDA	tCTL		cCTL	
	with	freq[2]	95% confidence interval	freq[3]	95% confidence interval
WS	-	426	203 - 321	401	300 - 501
	CD8	<3	0 - 8	<3	
	CsA	33	6247 - 422	335	249 - 421
	CsA+CD8	<3	0 - 8	<3	
SA	-	14660	9875 - 19445	916	645 - 1187
	CD8	1558	1048 - 2069	164	113 - 214
	CsA	1805	1332 - 2278	802	601 - 1003
	CsA+CD8	38	11 - 64	134	80 - 188
BE	-	<3		<3	
	CD8	<3		<3	
	CsA	<3		<3	
	CsA+CD8	<3		<3	
HN	-	7726	5872 - 9580	993	741 - 1246
	CD8	1076	835 - 1317	119	77 - 161
	CsA	2029	1578 - 2481	588	413 - 762
	CsA+CD8	<4	0 - 11	<3	
HI	-	3688	2770 - 4606	1281	834 - 1728
	CD8	2463	1873 - 3053	630	467 - 794
	CsA	ND[4]		410	294 - 525
	CsA+CD8	ND		168	106 - 228
FO	-	9781	4914 - 14648	40	25 - 55
	CD8	527	402 - 653	<1	
	CsA	225	171 - 278	65	44 - 85
	CsA+CD8	24	13 - 35	<1	
BR	-	<3		<3	
	CD8	<3		<3	
	CsA	<3		<3	
	CsA+CD8	<3		<3	
LA	-	878	657 - 1081	282	208 - 356
	CD8	442	343 - 540	71	47 - 96
	CsA	556	356 - 756	182	125 - 238
	CsA+CD8	308	223 - 393	36	14 - 57
HS	-	212	145 - 278	161	113 - 209
	CD8	51	24 - 79	39	18 - 59
	CsA	193	141 - 246	126	88 - 163
	CsA+CD8	69	40 - 98	5	0 - 21
WE	-	<3		<3	
	CD8	<3		<3	
	CsA	<3		<3	
	CsA+CD8	<3		<3	

[1] *The jackknife version of the maximum likelihood estimation procedure was used as the statistical method for calculation of the frequency.*
[2] *tCTL/10^6 cells, quantified after stimulation with donor spleen cells*
[3] *cCTL/10^6 cells, quantified after stimulation with autologous PBL.*
[4] *ND: not determined*

Table 6.2. *The influence of CD8 Mab and CsA on tCTL and cCTL frequency estimates against donor antigens in the graft of HTx patients without rejection*[1].

6.3.1 Frequency estimates of CTL

As illustrated in Table 6.1 and Figure 6.2A, high tCTL frequencies with donor reactivity were measured in all graft infiltrating cell (GIL) cultures propagated from EMB from rejecting patients. In the EMB of non rejectors tCTL frequencies were more variable, and in most cultures lower than in those of the rejector group (*Table 6.2 and Figure 6.2A*). The LDA stimulated with autologous PBL to detect cCTL revealed for both groups a significantly ($p=0.05$) lower frequency than found for tCTL (*Tables, and Figure 6.2B*). Since tCTL = pCTL + cCTL it is obvious that in most patients from both groups, graft infiltrating CTL are predominantly of the precursor type (*Figure 6.2C*). For three cultures the tCTL and cCTL frequencies were below detection level.

As control for specificity of the cytotoxic response the highest responder cell concentration was tested on unrelated third party T-blasts and K562 as targets. Lysis of third party cells was between 5 and 11% for all cultures tested and lysis of K562 never exceeded 15%.

6.3.2 Frequency estimates after inhibition with CD8 mAb

In the next series of experiments we tested the avidity of the graft infiltrating CTL by inhibition with the CD8 Mab FK18. In the GIL cultures derived from rejectors frequency estimates of tCTL were 42% (median) lower (*Table 6.1, Figure 6.3A*) and for the non-rejectors 87% (median) lower (*Table 6.2, Figure 6.3A*) after the addition of mAb CD8. In the non-rejector group the percentage tCTL that could be inhibited (low avidity) was significantly ($p=0,02$) higher than in the rejector group.

Subsequently we studied whether this difference in frequency of high avidity tCTL between the two patient groups is located in pCTL, cCTL or in both populations. The experiments revealed that in most rejectors cCTL were predominantly resistant to inhibition with CD8 mAb (*Figure 6.2B*). The median frequency for this group was in the presence of CD8 not significantly lower than frequency estimates without CD8, while the median inhibition by CD8 was 25% (*Figure 6.3B*). In the non-rejector group a significant ($p<0.005$) decline was observed of cCTL frequencies after inhibition with CD8 mAb. In three patients no cCTL frequency was measurable (<3 cells/10^6)(*Table 6.2 and Figure 6.2B*). For the other 7 cultures the median inhibition found was 87% (*Figure 6.3B*).

In 6/7 non rejectors the fraction of cCTL which function was inhibited by CD8 was 75% or more (*Figure 6.3B*). In the rejector group cCTL from only 1/8 GIL populations could be inhibited by CD8 at that level. In this group cCTL in 3/8 cultures could not be inhibited by CD8 at all (*Figure 6.3B*).

The data reveal a significant ($p<0.005$) difference between the rejector and non rejector group for the presence of cCTL with high avidity.

In Figure 6.2C pCTL frequencies are depicted. These were calculated by substraction of the cCTL frequencies from the tCTL frequencies. From these data and those in figure 6.3C it is obvious that in the graft of rejectors compared to this of the non rejectors, significantly ($p<0.05$) more pCTL are present that differentiate *in vitro* into CD8 resistant CTL.

6.3.3 Controls

Addition of the CD4 Mab RIV-6 or the control mab MRC OX-8, a mouse-anti-Rat CD8 did not result in a reduction of the frequencies detected (data not shown). Since in our hands T-blasts are no suitable targets to assay CD4 positive T cell mediated lysis against HLA class II mismatched antigens, the results with the CD4 Mab were as expected.

6.3.4 Frequency estimates of CsA resistant CTL

In both patient groups frequency estimates of tCTL determined after stimulation with donor spleen cells, were significantly lower ($p<0.005$) in the presence of 100 ng/ml CsA during LDA (*Tables 6.1 and 6.2, Figure 6.2A*). In the rejectors tCTL frequency estimates were decreased with 75% (median) and in the non

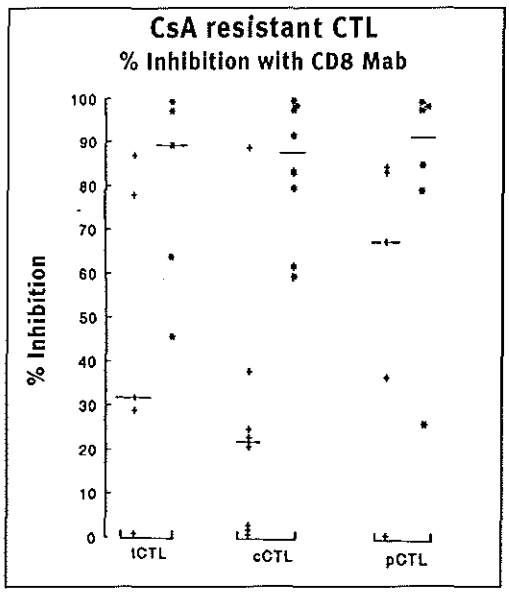

Figure 6.4. *The inhibition of donor specific, CsA resistant tCTL, cCTL and pCTL by CD8 Mab in GIL cultures propagated from EMB of rejectors (+) and non-rejectors (*). The percentage inhibition was calculated in relation to the frequency estimates determined in the presence of CsA only. Horizontal bars represent the median value of the group.*

rejectors 55% (median)(*Figure 6.3A*).
In the rejectors frequency estimates of cCTL, in the presence of CsA were not significantly different from those in the absence of CsA *(Table 6.1 and Figure 6.2B)*. Median inhibition was 0% *(Figure 6.3B)*. However in the non-rejectors cCTL frequency estimates declined significantly (p=0.04) under influence of CsA (*Table6.2, figure6.2B*) .Median inhibition was 25% (*Figure 6.3B*) Frequency of CsA resistant pCTL was again calculated from the measured tCTL and cCTL frequencies (*Figure 2C*). Since tCTL frequencies in most rejecting patients were lower under the influence of CsA and cCTL frequencies stayed within the same range, it is most likely that the differentiation from pCTL to cCTL is blocked by CsA. From Figure 3C it can be seen that from most patients in both groups more than 80% of the pCTL in the GIL cultures did not differentiate into functional CTL in the presence of CsA. In both groups there was one patient whose GIL derived pCTL in the graft could be inhibited by CsA less than 30%.

6.3.5 Influence of CD8 Mab on the CsA resistant CTL populations

In most rejectors cCTL were not sensitive for inhibition with CsA, consequently the frequency of CD8 resistant, CsA resistant cCTL was, as expected, comparable to the frequency of CD8 resistant cCTL frequencies in absence of CsA the stimulation phase (*Table6.1and Figure6.2B)* In the graft of the non-rejectors most CsA resistant cCTL could be inhibited with CD8 Mab ($p<0.005$), median inhibition 92% (*Figure 6.4*). CsA resistant pCTL frequencies declined after addition of CD8 in most GIL cultures, in the rejectors for 65% (median) and in the non rejectors for 94% (median) (*Figure 6.2C and 6.4*). Since CsA resistant cCTL with high avidity for donor antigen can be considered as the cell type relevant for rejection the two patient groups were compared for the presence of this cell type. It was found that in the patients that experienced one or more rejections the relative contribution of CD8 and CsA resistant cCTL was significantly ($p<0.005$) higher than in the non rejectors. In the rejectors JS, MO, PO and HA those CsA resistant cCTL with high avidity already could be detected in the graft before signs of acute rejection (myocytolysis) were found.

6.4 Discussion

LDA is a sensitive and the only available method for enumeration of allo-reactive cells. In an earlier study [9] we showed that this technique can be used to demonstrate qualitative differences between CTL populations. In the graft of rejecting heart transplant patients CTL with high avidity for donor antigens were predominant. In non rejectors the majority of the CTL propagated from the grafts had a low avidity. The experimental design of that study did not enable us to distinguish between *in vivo* activated cCTL and naive pCTL. MacDonald and coworkers [15,16] have shown that *in vivo* activation results in functional CTL resistant to inhibition with CD8 (high avidity), whereas *in vitro*

activated CTL are very sensitive to CD8 (low avidity). Now we investigated whether the CD8 sensitive CTL in the graft of non rejectors are the offspring of in vitro (during LDA) activated pCTL and whether CD8 resistant CTL in the rejectors are in fact *in vivo* activated cCTL. Based on the experiments of Orosz and coworkers[25] a modified LDA was employed that can discriminate cCTL from pCTL. Although we realise that the absolute frequency estimates might be biased by the culture period preceding the LDA, from the results of these experiments it is obvious that the pCTL is the major cell type in the graft of most patients irrespective of graft rejection. These results are consistent with those of Orosz et al [7] who found in allo-antigen loaded sponge grafts in mice that 13-53% of the tCTL were cCTL at the time "normal" allografts rejected.

pCTL and cCTL frequencies in EMB derived cultures of the rejector group were significantly ($p<0.005$) higher than in the non rejector group. The higher cCTL frequency in the rejectors might be expected assuming an active role for these cells in allograft rejection. The presence of high frequencies of pCTL with specificity for donor antigens in the cultures was not expected, since it was postulated that the culture method used would only propagate activated, IL-2 receptor expressing, T cells.[8,13,26,27] There are at least two possible explanations. First the concentration IL-2 (20-50 units) used may result in the propagation and expansion not only of activated lymphocytes, but of all lymphocytes present in the allograft biopsy. The other possibility is that the donor directed pCTL that accumulate at high frequency at the graft site are not completely naive. It might be that these donor reactive pCTL are already activated but not yet fully maturated into active cCTL. These activated pCTL might resemble the poised CTL (poCTL) stage as proposed by Ochoa and Gromo.[28,29] This cell expresses the IL2 receptor on its surface and is therefore, like cCTL, susceptible to expansion by IL2. pCTL without specificity for donor HLA-antigens, present at low frequency [8,9] may reflect PBL passing the graft capillary system at the moment the EMB was taken or may be attracted to the graft as a result of the rejection process as shown by Orosz et al.[30] In culture these cells are also expanded by the relative high concentration IL2. pCTL with donor specificity may preferentially accumulate in the graft in the poCTL stage, after centrall sensitization as postulated by Larsen and Austyn.[31,32] In the graft these poCTL become fully activated to cCTL under influence of cytokines like IL2,[28,33] IFN-γ[33] and cytotoxic T cell differentiation factor (CTDF)[34] in the presence of antigen. On the other hand it is thought that some graft reactive cCTL are fully activated in and then exported from lymphoid organs to the graft.[35] In an earlier study however, we were not able to detect cCTL in peripheral blood during or before graft rejection.[36] Others [7,37] found cCTL only at low frequencies in lymph nodes and spleen of allografted mice.

The inhibition studies with CD8 Mab showed a significant difference between patients that experienced one or more acute rejection episodes and those who did not reject at all. In the rejectors the majority of the cCTL could not be inhibited by CD8 Mab and therefore are thought to have high avidity for donor antigens. As we expected based on the data from MacDonald in mice [14,15] and Roelen et al in humans,[21] activation *in vivo* results in cCTL with high avidity. However in the non-rejectors *in vivo* activation did not result in cCTL with high avidity for donor antigen. Apparently the immune-state of these non rejecting patients differs in such a way from the rejectors that cells with high avidity can not be formed. This situation may resemble that found in tolerant mice, in which CTL interact with their target cells with a lower avidity than CTL from rejecting animals.[17] This unresponsiveness in the presence of CTL with low avidity might be caused by the blood-transfusion given prior to transplantation. [10,11] or by the immunosuppressive treatment. Surprising was the finding that, particularly in the graft of rejectors, pCTL were present that, after *in vitro* stimulation

with donor antigens (in LDA), gave rise to a rather large proportion of CTL with high avidity for donor antigens. We regard this as an indication that most pCTL accumulated in the graft of rejectors are not naive pCTL but already partially activated pCTL (poCTL) as discussed before. In rejectors these poCTL mature into high avidity cCTL. Obviously these poCTL can achieve final maturation when stimulated with donor antigen *in vitro* in the presence of cytokines. In most non-rejectors the offspring of nearly all pCTL propagated from the EMB had low avidity, which might implicate that these pCTL are all naive pCTL or consist of a mixture of both naive pCTL and poCTL with low avidity for donor antigens.

Finally we studied a possible differential effect of cyclosporin A on cCTL and pCTL in rejectors and non-rejectors. The differentiation and maturation of pCTL into functional CTL was largely inhibited by CsA in both groups. The 15-25% CsA resistant pCTL present in the cultures of most patients of both groups may resemble the CsA resistant CTL found in mice and rats. [20,38,39] In 2 patients, (one rejector, and one non rejector) pCTL were mainly CsA-resistant. These results are consistent with those of Kabelitz et al [19] who showed that CsA reduced the frequency of allo-antigen inducible pCTL. Also in their study 2 individuals had pCTL that were resistant to CsA, even a dosis of 1000 ng/ml did not block the differentiation from pCTL to functional CTL. Since we only used a clinical relevant plasma concentration of 100 ng/ml we do not know if pCTL in our two patients are also resistant to such a high dose of 1000 ng/ml.

In contrast to its effect on pCTL, CsA has in most rejectors no effect on the cCTL population. In the non-rejectors the effect of CsA on cCTL was more variable. In 5 cultures cCTL frequency estimates were 10 - 68% lower. Indicating that part of the cCTL indeed were inactivated during LDA in the presence of CsA, resulting in a lower frequency estimate. This phenomenon resembles the CsA sensitive cCTL that Orosz et al [18] found after in vitro priming of human PBL in a MLC.

In 7/8 rejectors most CsA resistant cCTL had high avidity for donor antigens, whereas in the graft of non-rejectors, CsA resistant cCTL had a low avidity for donor antigens. These CsA resistant, high avidity cCTL are already detectable in the graft before myocytolysis is detectable. We are currently investigating if the appearance of these cells is an early indication of an approaching rejection.

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CHAPTER 7

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF T CELL RECEPTOR $\gamma\delta$ BEARING T CELLS ISOLATED FROM HUMAN HEART ALLOGRAFTS

Abstract

Endomyocardial biopsies (EMB) from human heart transplant (HTx) recipients were investigated with respect to the occurrence of in vivo activated, alloreactive TCR-$\gamma\delta^+$ cells. More than one year after transplantation 30% of the biopsy derived T cell cultures contained TCR-$\gamma\delta^+$ cells whereas the first year after HTx in only 8% of the cultures TCR-$\gamma\delta^+$ cells were found. Such an increase of TCR-$\gamma\delta^+$ cells was not observed in the peripheral blood of the patient.

In most biopsy derived cultures the $\gamma\delta$ cells were δ-TCS1$^+$.

No donor specific cytotoxic activity could be demonstrated for TCR-$\gamma\delta^+$ cells tested while non-MHC-restricted cytotoxicity was found in several cultures. The occurrence of non-alloreactive TCR-$\gamma\delta^+$ cells late after transplantation, when acute cellular rejection episodes are rare, suggests a role in the down regulation of the allo immune response.

7.1 Introduction

An alternative form of the TCR is composed of the products of rearranged γ and δ genes. In humans the TCR-$\gamma\delta$ heterodimer is found on a minor population (±5%) of thymocytes, spleen cells, lymph node cells, and of peripheral lymphocytes.[1,2] The majority of the TCR-$\gamma\delta^+$ cells are of the $CD4^-$ $CD8^-$ phenotype, although significant percentages $CD8^+$[1,2] cells can be found and $CD4^+$ $\gamma\delta$ cells occur at a very low frequency.[1]

Several mAbs specific for various segments of the $\gamma\delta$ chains have been described. Anti TCR-γ/δ-1[3] recognizes a Cγ-encoded epitope, and anti TCR-δ1 reacts with a Cδ-encoded determinant [4] on all TCR-$\gamma\delta^+$ cells. Until now, human $\gamma\delta$ lymphocytes can be divided in two major subsets, which are mutually exclusive.[4,5,6,7] The Vδ1 subset recognized by the δTCS1 mAb and the Vδ2 subset recognized by the BB3 and TiγA mAb. In most individuals tested the TiγA determinant is expressed on at least 70% of the human TCR-$\gamma\delta^+$ PBL.[8] Anti- δTCS1 detects the remaining 30%. It is not clear whether these monoclonals define functionally distinct lineages. It has been suggested that $Ti\gamma A^+$ $\gamma\delta$ cells exhibit a higher degree of non-MHC-restricted cytotoxicity than the $\delta TCS1^+$ T lymphocytes.[9,10] Differences in growth characteristics and morphology have also been reported.[11]

The biological role of lymphocytes bearing the TCR-$\gamma\delta$ is poorly understood.[12] After culture in IL-2 containing medium TCR-$\gamma\delta^+$ cells have been found to display non-MHC-restricted cytotoxicity.[9,10,13,14] Also alloreactive TCR-$\gamma\delta^+$ clones have been described.[12,15,16,17,18] However, naive TCR-$\gamma\delta^+$ cells seem unable to mount an alloreactive response in vivo, as was demonstrated in nude mice. The fact that alloreactive TCR-$\gamma\delta^+$ clones can be found only after in vitro restimulation in a MLR[12,15-18] suggests that the frequency of alloreactive cells *in vivo* is low. However, prolonged contact with alloantigens in vivo may upregulate the frequency of alloreactive TCR-$\gamma\delta^+$ cells.[15,19]

Since such a situation is encountered after organ transplantation, we looked for the presence of TCR-$\gamma\delta^+$ cells in EMB obtained from heart transplantation patients at several intervals after transplantation. In the present study we describe that TCR-$\gamma\delta^+$ cells occur more frequently in EMB after one year post transplantation than in the first year. Most $\gamma\delta$ cells were of the δ-TCS1 phenotype. No TCR-$\gamma\delta^+$ bulk cultures or clones showed lytic activity against donor antigens, whereas non-MHC-restricted cytotoxicity was found in some cultures.

7.2 Materials and Methods

7.2.1 Patients

All 118 heart transplant recipients studied had received preoperative blood transfusions and were under cyclosporin A in a dosage according to specific plasma trough levels (50-100 ng/ml) and low dose steroids. Mean number of HLA-A, HLA-B and HLA-DR mismatches was 1.3, 1.6 and 1.4 respectively. Actuarial 3 years graft survival was 89%.

Rejection was monitored by means of histological examination of EMB, the histological rejection grade was assessed according to Billingham's criteria.[20] Grade 0: no evidence of rejection; Grade 1: mild rejection, diffuse perivascular and endocardial infiltration with pyroninophylic lymphocytes, endocardial and interstitial edema; Grade 2: moderate rejection, more dense perivascular, endocardial and interstitial infiltrates, and focal myocytolysis (necrosis); Grade 3: severe rejection. Vessel wall and myocyte necrosis with interstitial bleeding. Interstitial infiltrates with polymorphonuclear cells and pyroninophylic lymphocytes. (This rejection grade was not observed in our study). Rejection therapy was only instituted in case of biopsy proved rejection, i.e. grade 2 according to Billinghams criteria. Serial biopsies were obtained at weekly intervals in the early post transplant period. Later EMB were taken at a lower frequency, declining to once every four months after one year. mAb. The mAb anti-TCR-γ/δ-1,[3] specific for human TCR γ-chains, was a gift of Dr. J. Borst (Dutch Cancer Institute, Amsterdam, Netherlands). The antibody TCR-δ1, specific for all human TCR δ-chains[4] and

δTCS1, specific for a Vδ1Jδ1 and Vδ1Jδ2 encoded epitope on the δ-chain of human TCR-γδ [7] were obtained from T Cell Sciences (Cambridge, MA). The mAb anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8) and WT31, reactive with the human TCR-αß chain, were purchased from Becton Dickinson, (Mountain View, CA). TCR-γ/δ-1 was used as an unconjugated ascites. The other mAb were conjugated to FITC or PE.

7.2.2 Generation of T cell lines from EMB

During right ventricular catheterisation four biopsy specimens were obtained. Three were used for histological examination, one was cut in 1 mm fragments and placed in 2 or more wells of a 96 well round bottom tissue microtiter plates (Costar) with 200 μl culture medium in the presence of 10^5 irradiated (40 Gy) autologous PBMC. Culture medium (CM) consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% human serum, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. As exogenous source of IL-2 10% v/v lectin free Lymphocult (Biotest, Dreieich, Germany) was added. Biopsy cultures were grown at 37°C in a humidified 5% CO_2 incubator. Half the culture medium was changed every 2-3 days. When growth was observed, cells from several wells were pooled and transferred into 2 or more wells. At sufficient cell density (10^5 - 10^6 cells/ml), cells were transferred into additional wells. Most cultures were assayed for phenotypic expression and cytotoxicity within 4 weeks after initiation of the culture.

7.2.3 Target cells

To determine allospecific cytotoxicity PHA-blasts and/or EBV transformed B lymphoblast cell lines (B-LCL) from donor origin were used. Third party B-LCL or PHA-blasts served as negative controls. As target for non-MHC-restricted cytotoxicity, the proerythroblastic tumour cell line K562 was used.

7.2.3.1 Generation of PHA-blasts

10^7 nucleated donor spleen cells/ml were stimulated for 3 days with 1% PHA-M (DIFCO, Detroit, MI) in CM and then cultured for at least 3 more days in CM supplemented with 5% Lymphocult (Biotest) as a source of IL-2.
Generation of B-LCL. Lymphocytes were isolated from donor spleen or peripheral blood from panel members by Ficoll-isopaque density gradient centrifugation. Cells were washed twice, pelleted, and resuspended at 10^7 cells/ml supernatant culture medium of the marmoset cell line B95-8[21] that had been filtered through a 0.45-μm filter. The cells were incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2. The cells were pelleted again, washed and 2 x 10^6 cells were resuspended in 2 ml CM containing 1% PHA and 5% heat-inactivated FCS in a 24 well culture plate (Costar). The cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 and fed weekly by replacement of half the supernatant.

7.2.4 Generation of TCR-γδ clones

Cloning was done by limiting dilution at 1 cell in every 3 wells in 96 well microtiterplates (U-bottom) in 0.2 ml CM in the presence of a feeder cell mixture consisting of 5 x 10^4 irradiated (40 Gy) fresh PBMC from random donors and 5 x 10^3 irradiated (50 Gy) third party B-LCL in CM containing 10% Lymphocult and 1% PHA. Growing cultures were expanded in 24 well culture plates (Costar) by restimulation with the feeder cell mixture in the presence of 10% lymphocult and PHA.

7.2.5 Cytotoxicity assay

2.5 x 10^3 ^{51}Cr labelled target cells [PHA-blasts or B-LCL] were mixed with effector cells (effector : target ratio 20:1 or higher) in 0.2 ml of culture medium in 96 well U-bottom microtiterplates (Costar). The plates were centrifuged (60g, 1 min.) and incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2.
Supernatants were collected with a Skatron harvesting system (Skatron-AS, Norway). The

Days after HTx	No. of EMB	No. of culture Phenotyped	% cultures with[a] γδ	αß
0- 90	509	281	5	99
91-180	208	103	10	97
181-365	193	89	14	98
>365	323	138	30	92

[a] Expressed as % from phenotyped cultures

Table 7.1 *Culture results, and the presence of TCR-γδ⁺ and TCR-αß⁺ cells in relation to time after transplantation.*

Billingham's Rejection grade	No. of EMB	No. of cultures Phenotyped	% Cultures with[a] γδ	αß
0	421	150	20	98
1	721	395	11	97
2	91	66	8	99

[a] Expressed as percentage from phenotyped cultures

Table 7.2 *Culture results, and the presence of TCR-γδ⁺ and TCR-αß⁺ cells in relation to histological rejection grade.*

percentage of specific lysis was calculated according to the formula:
Maximal release was determined in six-fold

$$\%S = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\%$$

from a Triton X100 (5% v/v solution in 0.01 M TRIS-buffer) lysate of the target cells.
Spontaneous release was determined in six-fold, by incubation of target cells in CM only.

7.2.6 Immunofluorescence analysis

PBMC, T cell lines and clones were analyzed by two colour flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA) for the expression of cell surface differentiation antigens. Most antibodies were available as fluorochrome conjugates and were used as direct staining reagents, whereas anti-TCR-γ/δ-1 was used in combination with goat anti-mouse IgG-FITC (Becton Dickinson) for indirect immunofluorescence analysis.
Initial screening was performed with the combinations CD3 PE - WT31 FITC, CD8 PE - CD4 FITC, and CD56 PE - CD16 FITC. When CD3⁺ WT31⁻

Antibody	No. of cultures positive
WT31 (αß)	0
CD3	80
TCR-δ1	80
TCR-γ/δ-1	80
δ-TCS1	54[b]
CD8	
Strong (MFI 158)	21[c]
Weak (MFI 90)	25
Negative (MFI 24)	30[c]
CD4	0

[a] Six cultures were not tested with δ-TCS1 and four not with CD8

[b] Included are six cultures containing δ-TCS1 negative and δ-TCS1 positive TCR-γδ cells.

[c] Included are five cultures containing CD8 positive and CD8 negative TCR-γδ cells.

Table 7.3 *Surface phenotype of the TCR-γδ cells isolated from EMBa*

cells were found cultures were staining with TCR-γ/δ-1.Less than 5% WT31$^-$ cells were usually not recovered when stained with TCR-γ/δ-1.

7.2.7 Statistical analysis

The X^2 tests were performed to evaluate TCR-γδ incidence in relation to rejection. The Mann-Whitney U test was used to evaluate the occurrence of TCR-γδ in the PBL before one year, and more than one year after transplantation.

7.3 Results

7.3.1 Presence of TCR-γδ$^+$ cells in EMB and PBL

1233 EMB obtained from 118 patients at several time intervals after heart transplantation (*Table 7.1*) were cultured in IL-2 enriched medium on autologous feeder cells. In the first half year after transplantation 64 - 69 % of the EMB cultures yielded growing cells declining to 54 % thereafter. Within 3 weeks 80 % of the growing cultures contained sufficient cell numbers for phenotype determination. In 80 cultures more than 5% of the cells were CD3$^+$ WT31$^-$ TCR-γ/δ - 1$^+$, indicating that these cells could carry the TCR-γδ heterodimer.

After HTx a significant (4x2 X^2 table, $p<0.001$) and gradual increase could be observed in the incidence of cultures with TCR-γδ cells. In the first 90 days after HTx only 5 % of the phenotyped cultures contained TCR-γδ positive cells (*Table 7.1*). In the period from 91 - 180 days this was 10 % , and in the period between 181 and 365 this was 14 %. The highest incidence was found more than one year after transplantation when 30% of the phenotyped cultures contained TCR-γδ$^+$ cells.

Not all cultures contained the same percentage of anti TCR-γ/δ-1 positive cells: a range from 6 - 100%, median 35% was observed. This proved to be independent of the time post HTx at which the EMB were taken.

Only 5 of the 80 TCR-γδ$^+$ cell containing cultures were obtained from a biopsy with histopathological signs of rejection (Billingham criteria grade 2). This was in strong contrast with the occurrence of TCR-αß cells that could be cultured from nearly all grade 2 biopsies (*Table 7.2*).

In 20% of the phenotyped cultures propagated from EMB with rejection grade 0 TCR-$\gamma\delta^+$ cells were detected. A significantly lower incidence of TCR-$\gamma\delta$ positive cultures was found for EMB with rejection grade 1 (11%) ($p < 0.025$) and rejection grade 2 (7%) ($p < 0.05$).
Since most TCR-$\gamma\delta^+$ cells were found in a period when patients start to develop signs of chronic rejection, (> 1 year post HTx) fifty patients were screened for the presence of vascular lesions typical for chronic rejection. In 12 of 20 patients with such lesions at least one EMB yielded TCR-$\gamma\delta^+$ cells. However, there was no significant difference with patients without signs of chronic rejection since 1 or more EMBs from 16 of the 30 patients without lesions also contained TCR-$\gamma\delta^+$ cells.
As controls, biopsies of the original diseased heart of two patients and from 3 patients biopsies taken 30 min after transplantation were cultured. From none of these lymphoid cells could be propagated in IL-2 containing CM .
Phenotypic analysis of PBL from 30 patients drawn at the same day as the biopsy that yielded TCR-$\gamma\delta^+$ cells, revealed that between 1% and 13% (mean 3.5%) of the PBL were TCR-γ/δ-$^{1+}$ cells. No difference was observed between the percentage TCR-γ/δ^+ cells present in PBL samples taken more than one year after HTx (Mean 5.7 $\pm$ SD 3.6%, n = 14) and the percentage found in the PBL samples taken in the first year after HTx (5.7 $\pm$ 3.9%, n = 16). In addition we did not find a relation between the percentages of TCR-γ/δ cells present in the EMB and the PBL samples taken concomitantly.

7.3.2 Surface marker expression of the TCR-$\gamma\delta^+$ cells

As shown in Table 7.3, further flow-cytometric analysis of the surface phenotypes of the TCR-$\gamma\delta^+$ cells from EMB revealed that in 48 cultures all $\gamma\delta$ cells were δ-TCS1$^+$, in 20 cultures all $\gamma\delta$ cells were δ-TCS1$^-$, and in 6 cultures both δ-TCS1$^+$ and δ-TCS1$^-$ $\gamma\delta$ cells were found.
The expression of the CD8 antigen was variable (*Table 7.3*). In 16 cultures all TCR-$\gamma\delta^+$ cells expressed the CD8 antigen at higher density (MFI:158), 5 cultures contained bright as well as CD8 negative TCR-$\gamma\delta^+$ cells, whereas in 25 cultures the $\gamma\delta$ cells stained only weakly with CD8 (MFI:90). The expression of the CD8 antigen on the bright (MFI:158) TCR-$\gamma\delta$ cells however was always lower than on TCR-$\alpha\beta^+$ T cells (MFI: 206) in the same sample. In 25 cultures only CD8$^-$ (MFI:24) TCR-$\gamma\delta$ cells were present .
No relation was found between the phenotype of the outgrowing TCR-γ/δ cells (δTCS1$^+$ versus δTCS1$^-$, CD8$^+$ versus CD8$^-$ or that the level of CD8 expression) and the time after transplant the biopsies were obtained. Nor did such phenotypes in any way correlate with the rejection grade. CD4 positive TCR-$\gamma\delta^+$ cells were never observed.

7.3.3 Cell-mediated cytotoxicity

Not all TCR-$\gamma\delta^+$ cell containing lymphoid cultures could be tested in a CML assay before restimulation. In nearly 50 % of the cultures that contained TCR-$\gamma\delta^+$ cells, growth was slowing down before an adequate number cells were available to perform a CML assay. These cultures had to be restimulated, with donor B-LCLs or spleen cells. TCR-$\gamma\delta^+$ cells then often disappeared from the cultures because they were overgrown by faster proliferating TCR-$\alpha\beta^+$ cells.
From the 40 cultures that were tested in the CML 8 were restimulated with irradiated donor B-LCL or donor spleen cells. The 11 cultures (1 restimulated) that contained 90-100% TCR-γ/δ^+ cells did not lyse donor PHA-blasts or B-LCL, nor panel cells that shared HLA-specificities with the donor *(Table 7.3)*. Sixteen bulk cultures (4 restimulated) that contained both TCR-$\gamma\delta^+$ and TCR-$\alpha\beta^+$ cells did lyse donor cells. After cloning 7 of these bulk cultures, none of the TCR-$\gamma\delta^+$ clones were cytotoxic for donor antigen bearing cells (*Table 7.3*) whereas the TCR-$\alpha\beta$ positive clones killed donor derived target cells and/or panel cells that shared HLA-antigens with the donor (data not shown).
The NK sensitive cell line K562 was lysed by cells from 15 bulk cultures (*Table 7.3*), by all the clones generated from 4 bulk cultures (Pa,Wi,Sa,B) and by 3 of the clones obtained

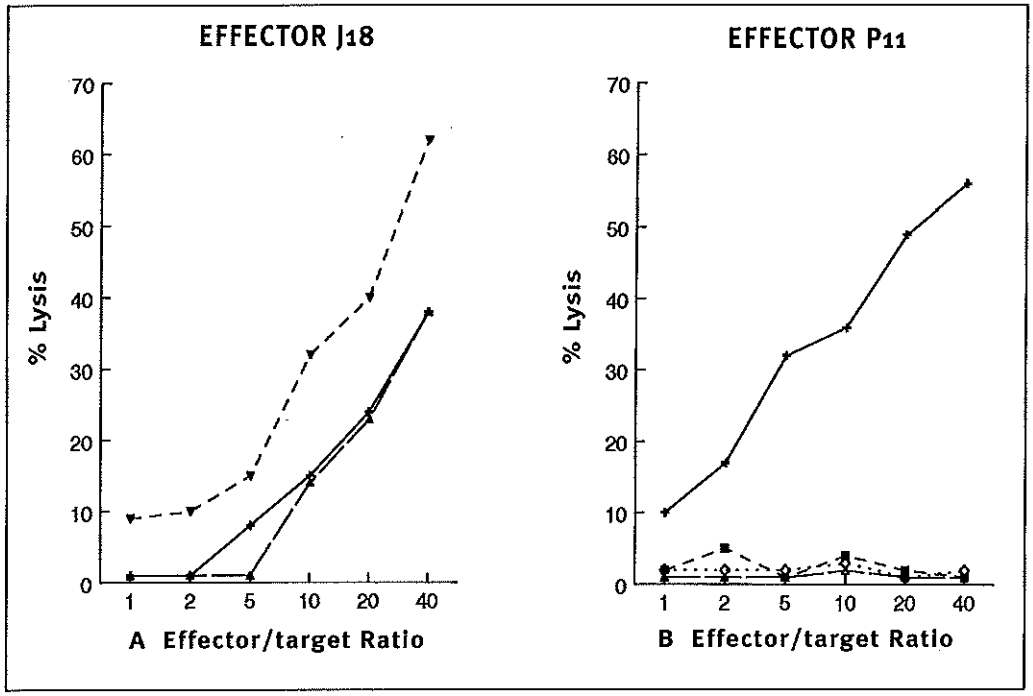

Figure 7.1 *Cytolytic reactivity of two EMB derived bulk-cultures with >90% TCR-γ/δ⁺ cells. A: LAK-like killing pattern: lysis of B-LCL without donor-specific HLA-antigens (▼ E4778 and ▲ EHolst) as well as of K562 (+). B: NK-like killing pattern: lysis of K562 (+) only and not of PHA-blasts from donor origin (■ T28) or B-LCL with (◇ E78) or without (△ E5) donor-specific HLA-antigens.*

from bulk culture Be. K562 was not lysed by 25 of the 40 bulk cultures tested and most clones obtained from bulk culture Be. With respect to the expression of δTCS1 or CD8-antigen, no significant difference was found between the group of cultures that killed K562 and the non cytotoxic group.

Three of the cultures that killed K562 also lysed all B-LCL, a characteristic example is showed in Figure 7.1. These three cultures contained both $\delta TCS1^+$ and $\delta TCS1^-$ cells. In one culture TCR-γ/δ^+ cells did not express the CD8-antigen. Another culture contained 51% TCR-α/β cells.

7.4 Discussion

Culturing endomyocardial biopsies in IL-2 containing medium in the presence of irradiated autologous feeder cells, we were able to propagate T lymphoblasts that express TCR-αß or TCR-γδ chains on their surface *(Table 7.1)*. This culture system is thought not to promote growth of resting lymphocytes but only of in vivo activated T cells. [22,23] As shown previously[24,25] most TCR-$\alpha\beta^+$ T cells propagated were donor reactive. The cultures that contained more than 90% TCR-$\gamma\delta^+$ cells and the TCR-$\gamma\delta^+$ clones never showed lytic activity against target cells with donor type HLA-antigens *(Table 7.3)*. The low incidence of TCR-$\gamma\delta^+$ cells *(Table 7.1)* in the first three months after transplantation, when the majority of the patients encountered one or more acute rejection episodes, suggests that TCR-$\gamma\delta^+$ cells do not play a role in the destruction process that causes myocyte damage. The observation that cultures with TCR-$\gamma\delta^+$ cells were significantly less frequent in the EMB group with myocytolysis *(Table 7.2)* than grade 0 biopsies supports this view. Involvement in the pathogenesis of

vascular lesions typical for chronic rejection is also unlikely because there was no significant difference in the possibility to grow TCR-$\gamma\delta$ cells from EMB of patients with or without such lesions.

Based on the generation of donor reactive TCR-$\gamma\delta^+$ clones from PBL of a kidney recipient 9 years after transplantation, Vandekerckhove and coworkers [19] suggested that the frequency of alloreactive TCR-$\gamma\delta$ cells might be upregulated by prolonged contact with alloantigens in vivo. Although 7 of our cultures with TCR-$\gamma\delta^+$ cells were obtained from EMB taken between 3-4 years after transplantation we never found donor specific reactivity. Stimulation of freshly isolated TCR-$\gamma\delta^+$ cells in a MLR with cells that express allogenic MHC antigens at high density might be necessary to obtain alloreactivity. This type of culture system was used by all investigators who have reported alloreactive TCR-$\gamma\delta$ cells [12,15-19]. In our system TCR-$\gamma\delta^+$ cells did not encounter donor cells in the first three weeks of culture.

FACScan analysis of PBL from 30 patients, concurrently obtained with biopsies from which TCR-$\gamma\delta^+$ cells were grown, revealed a percentage of TCR-$\gamma\delta^+$ cells within the same range as reported for normal individuals by Groh and coworkers[1] and Moissec et al.[26] And as also no difference in percentage of TCR-$\gamma\delta^+$ cells was observed between PBL obtained during the first year and PBL obtained more than one year after transplantation we conclude that (long term) immunostimulation does not result in an increase of the total TCR-$\gamma\delta^+$pool in the periphery.

Analysis of the cultures using the δ-TCS1 antibody specific for the Vδ1 gene products, revealed that most TCR-$\gamma\delta^+$ cells (*Table 7.3*) were of the Vδ1 subset. In the PBL however V$\delta1^+$ cells form the minor TCR-$\gamma\delta^+$ subpopulation.[8,26] This suggests a preferential homing pattern of the V$\delta1^+$ cells for the graft or a preferential retention and/or proliferation after a non specific homing.

The mechanism by which the number of TCR-$\gamma\delta$ cells in the biopsies increases in time is not clear. CsA might influence the differentiation or proliferation of the TCR-$\gamma\delta$ cells as patients receive a higher dose of CsA (6 - 8 mg/kg, plasma levels of $\pm$ 125 ng) during the first year than in the second year and thereafter (4 - 5 mg/kg, plasma levels $\pm$ 75 ng). Recently J. Allison [in Haas, et al.[27]] and Heeg et al.[28] however reported that in mice CsA had no effect on the generation of TCR-$\gamma\delta$ cells in the fetal thymus. For the human situation the influence of CsA is not known yet. If human adult thymic $\gamma\delta$ cells are, like mouse fetal $\gamma\delta$ cells, not affected by CsA there may be a different effect on the 2 different $\gamma\delta$ sub populations in the PBL as well. In adult thymus Vδ1 is the predominant $\gamma\delta$ subpopulation whereas in PBL Vδ2 cells predominate.[29] Since the latter cell type is thought to be more activated[29] it might be more CsA-sensitive. Wether the Vδ2 sub population in the PBL indeed is affected by CsA treatment is not known and is now subject of investigation.

It has been reported that Thy-1^+, asialo-gm1^+,Ia$^-$,sIg$^-$ dendritic epidermal cells (DEC) in skin of mice which are mainly TCR-$\gamma\delta^+$ T cells ,[30,31] can deliver down-regulatory signals in contact hypersensivity reactions.[32] Therefore one can speculate that $\gamma\delta$ cells in the skin are able to activate suppressor circuits. The occurrence of TCR-$\gamma\delta^+$ cells in our study late after transplantation, when acute cellular rejection episodes were rare, suggests that the cellular immune processes in the graft might be down regulated by TCR-$\gamma\delta^+$ cells. A direct relation between $\gamma\delta$ cells and suppression of the allo-immune response however remains to be elucidated.

NK-like activity with high lytic effects on K562 *(Figure 7.1)* was found in only 15 bulk cultures with TCR-$\gamma\delta^+$ cells (*Table 7.4*). In most[13] cultures this was not due to LAK activity since none of the panel B-LCL lines were killed. These characteristics are in contrast with previously described TCR-$\gamma\delta^+$ lines and clones with broader "nonspecific" (LAK-Like) target ranges. [3,13,32] Our clones either exhibited LAK-like reactivity or showed only NK-like activity (data not shown). When clones were generated in IL-

Type of culture	Number of Cultures		
	Tested	With donor reactivity	With K562 reactivity
90-100 % γδ cells	11	0	3
γδ and αß T cells	29	16[a]	12
clones of PA	47	0	47
clones of Wi	30	0	30
clones of Be	56	0	3
clones of SA	21	0	21
clones of B	45	0	45
clones of Dr	26	0	0[b]
clones of KU	13	0	0[b]

[a] Cytolysis is thougt to be mediated by TCR-αß$^+$ cells.

[b] Clones were generated in rIL-4 containing medium.

Table 7.4 *CML results from 40 bulk cultures with TCR-γδ cells and TCR-γδ clones generated from 7 mixed bulk cultures.*

4 containing culture medium LAK- and NK-like cytotoxicity were never seen. Some authors consider LAK- and NK-like cytotoxicity as a culture artefact induced by high levels of IL-2.[34] T cells expressing TCR-γδ are usually CD8$^-$CD4$^-$ [1,3,8,13] although a consistent proportion of the TCR-γδ$^+$ cells in the human peripheral blood and lymphoid organs has been reported to be positive for CD8.[1,2] In this study the expression of the CD8 antigen was variable. On TCR-γδ cells that expressed CD8 antigen at a rather high density (*Table 7.3*) this expression however was always lower than on TCR-αß$^+$ cells. Whether the CD8-antigen, expressed at this level on TCR-γδ$^+$ cells, has the same function as on TCR-αß$^+$ cells i.e. accessory molecule in class I restricted immune responses, is as yet unknown. In part of the cultures low levels of CD8-antigen were detected on TCR-γδ$^+$ cells (table III) as reported by others.[7,12] Since we used a supernatant obtained from lectin stimulated T cells that contained IL-4 in addition to IL-2, expression of low amounts of CD8 antigen might be induced by IL-4 as demonstrated by Paliard et al.[34]

In summary, the low incidence of TCR-γδ$^+$ cells in relation to rejection suggest that TCR-γδ$^+$ cells are not involved in graft destruction. Both TCR-γδ$^+$ clones as well as TCR-γδ$^+$ cells containing bulk cultures lack any donor specific cytotoxic activity. Biopsy derived cultures with TCR-γδ$^+$ cells mostly were reactive with the δ-TCS1 mAb, which suggest a preferential homing of Vδ1 cells to the graft or retention in the graft. The predominant occurrence of TCR-γδ$^+$ cells late after transplantation when acute cellular rejection episodes are rare, suggests that they might be involved in the down regulation of the cellular immune response.

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CHAPTER 8

INVERTED Vδ1/Vδ2 RATIO WITHIN THE TCR-γδ T CELL POPULATION IN THE PERIPHERAL BLOOD OF HEART TRANSPLANT RECIPIENTS

Abstract

We investigated the levels of TCR-$\gamma\delta$ T cells and their subpopulations Vδ1 and Vδ2 in the peripheral blood lymphocytes (PBL) of 28 heart transplant (HTx) patients. Patients (n=10) receiving cyclosporin A (CsA) for treatment of a nephrotic syndrome (NS) and 10 healthy individuals served as controls. There was no difference in the levels of TCR-$\gamma\delta$ T cells between the different groups. However an elevated proportion of V$\delta1^+$ $\gamma\delta$ T cells was found in the PBL of HTx patients, especially when these cells were present in their graft infiltrating lymphocyte (GIL) cultures. V$\delta1^+$ $\gamma\delta$ T cells of HTx patients showed normal expression of CD45RO and lacked the activation markers CD25 and HLA-DR. After expanding in IL-2 containing medium, PBL cultures of HTx patients more often were dominated by Vδ1 cells than PBL cultures of controls, in which Vδ2 cells were predominantly grown. The aberrant composition of the TCR-$\gamma\delta$ population in HTx patients was not a result of immunosuppressive medication since the proportion V$\delta1^+$ $\gamma\delta$ T cells was normal in the PBL of the NS patients receiving a similar dose of CsA. It is postulated that long term antigenic stimulation by the graft, at low level, might be responsible for the altered composition of the $\gamma\delta$ pool in the HTx patients. Since no donor HLA specific $\gamma\delta$ T cells have been detected, other ligands, like heat shock proteins may be involved.

8.1 Introduction

The T cell receptor (TCR) γδ is expressed in 0.5 - 10% of the human CD3 positive PBL.[1,2,3] They can be divided into two major, mutually exclusive, subsets. One expresses a TCR composed of a Vδ9-JδP-Cδ1 positive γ-chain in association with a Vδ2 positive δ-chain and can be identified by the mAbs TiγA, recognizing the Vδ9 gene product, and by BB3 or 15D recognizing the Vδ2 product.[4,5,6,7,8] The second subset of γδ cells carries a TCR using Vδ1 gene products rearranged to Jδ1 in association with members of the Vγ1 gene family, to be identified by the mAb δTCS-1.[5,9]

In the postnatal thymus approximately 15% of all γδ cells are of the Vδ2 ($Vδ9^+/Vδ2^+$) subset and 80% of the Vδ1 ($Vδ9^-/Vδ1^+$) type. These proportions remain constant throughout adult life.[10,11] In the peripheral blood however, the $Vδ2^+$ population gradually expands with age from approximately 20% in cord blood to 70% in the peripheral blood of most adults. Consequently the proportion $Vδ1^+$ cells decreases from about 45% in cord blood to 20% in the blood of adults.[10,11] The increase of the proportion $Vδ2^+$ cells is paralleled by the acquisition of CD45RO by these cells. In cord blood 10% of both Vδ1 and Vδ2 cells are strongly positive for CD45RO.[10] During adult life about 60% of the Vδ2 cells express CD45RO in a high intensity, whereas maximal 20 % of the Vδ1 cells are brightly stained for this antigen,[10,12,13] which is thought to be a marker for activated T cells.[14] The expansion of the CD45RO positive Vδ2 cells might be driven by antigens of intestinal flora or of opportunistic pathogens.[10]

The physiological and pathological role of γδ cells is poorly understood. Several reports show that Vδ2 cells are increased in the PB of patients with infectious diseases such as malaria,[15] toxoplasmosis,[16] infectious mononucleosis.[17] Vδ1 cells might be of pathogenic significance since they are found in inflamed tissues in autoimmune diseases such as rheumatoid arthritis (RA),[18-22] systemic lupus erythematosus (SLE),[23] coeliac disease,[24] multiple sclerosis,[25,26] autoimmune chronic liver disease[27] as well as in infectious diseases like HIV.[28] We reported the presence of Vδ1 cells within cardiac transplants especially more than one year after transplantation,[29] which recently has been confirmed by others.[30] Now we report an aberrant ratio between the two γδ subsets, and a different proliferation pattern in response to IL-2 in the PBL of these heart transplant patients compared to healthy individuals.

8.2 Patients and Methods

8.2.1 Heart transplant patients and controls

Peripheral blood from 28 HTx patients was taken at the time of endomyocardial biopsy (EMB) procedures. All HTx recipients studied had received preoperative blood transfusions and were under CsA medication in a dosage according to keep plasma 12h specific trough levels between 50 - 120 ng/ml) and low dose prednisolone (10 mg/day), none of the patients received azathioprine. Mean number of HLA-A, HLA-B and HLA-DR mismatches was 1.3, 1.6 and 1.4 respectively. The reason for transplantation was ischemic heart disease (n=13), and myocardiopathy (n=13) and valvular heart disease (n=2). None of them was transplanted because of autoimmune disease.

We had 15 PBL samples available of 15 HTx patients who had γδ cells in graft infiltrating lymphocyte (GIL) cultures propagated from the EMB concurrently taken with the PBL sample. Another 13 PBL samples were derived from HTx patients who had no γδ cells in GIL cultures propagated from the concurrently taken EMB. The timepoint post HTx when the PBL samples were taken did not differ significantly between the two HTx subgroups. For the patients with γδ cells in the graft this was 336 days, (median, range 28 - 1321) and for the other subgroup this was 257 days post HTx (median, range 8 - 1460, p=0.5 Mann-Whitney U test). As control groups served 10 patients with an idiopathic nephrotic syndrome (NS), receiving comparable doses CsA as HTx patients, but no

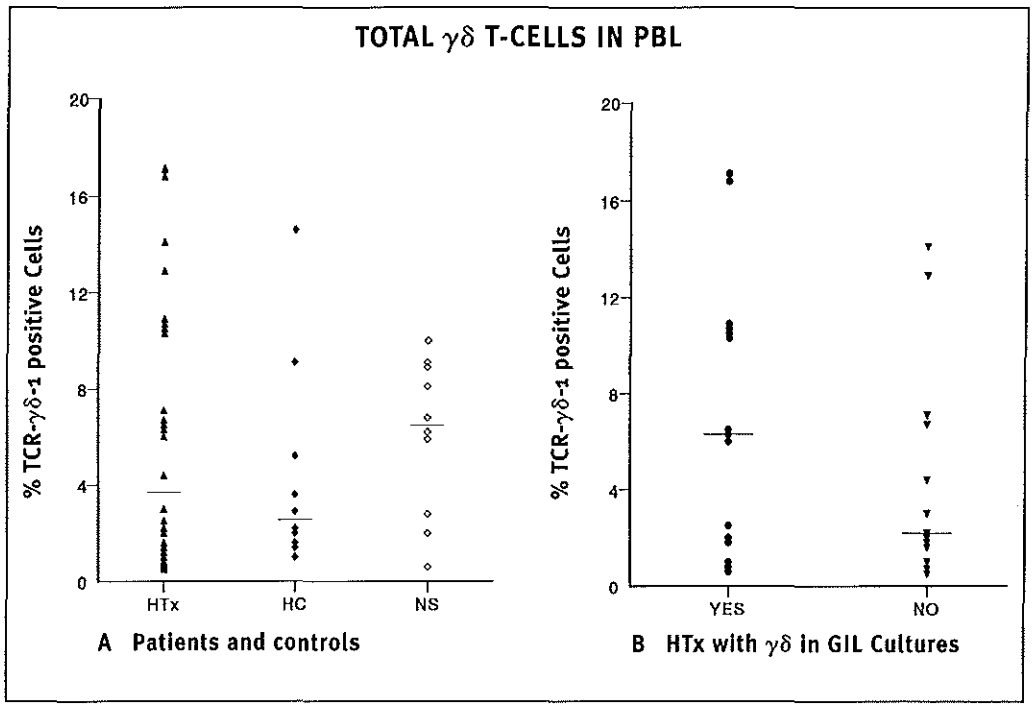

Figure 8.1. *The proportion TCR-γ/δ positive T cells expressed as percentage of all CD3 positive (T) cells in PBL samples of all heart transplant patients (HTx), healthy controls (HC) and patients who received CsA because of a nephrotic syndrome (NS) are compared in panel A. In panel B HTx patients are separated in a group with (yes) and without (no) γδ T cells in their EMB derived GIL cultures at the time the PBL sample was taken. No significant differences were found between the various groups.*

prednisolone and 10 healthy individuals. There were no significant differences in age between the 4 groups (p=0.1501, Kruskal-Wallis ANOVA), for HTx with γδ in GIL; median 48.5, range 15 - 57 year, HTx without γδ in GIL; median 50, range 19 - 50 year, Healthy controls (HC); median 37, range 26 - 52 year, and NS patients; median 37,5 range 24 - 69 year . There was also no difference in distribution of gender (male/female) between the groups. HTx group (21/7), versus HC, (6/4) (p=0.4318 Fisher's Exact test). HTx group versus NS patients (6/4)(p=0.4318). HTx group with γδ in GIL (10/5) versus HTx group without γδ in GIL (11/2) (p=0.3955)

8.2.2 Cell preparations

Mononuclear cells (PBMC) were prepared from heparinized blood by centrifugation over a Ficoll-Hypaque (Pharmacia, Netherlands) density gradient. From HTx patients samples were cryopreserved and kept in liquid nitrogen until analysis. Frozen samples were rapidly thawed, washed in HBSS, resuspended in RPMI-1640 and counted. Fresh samples were resuspended in RPMI-1640 directly after isolation and counted. For phenotype assessment 3.10^6 cells were taken into staining medium (HBSS supplemented with 1% BSA and 0.1 % Sodium azide) The remaining cells were resuspended in culture medium (CM) which consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10 % human

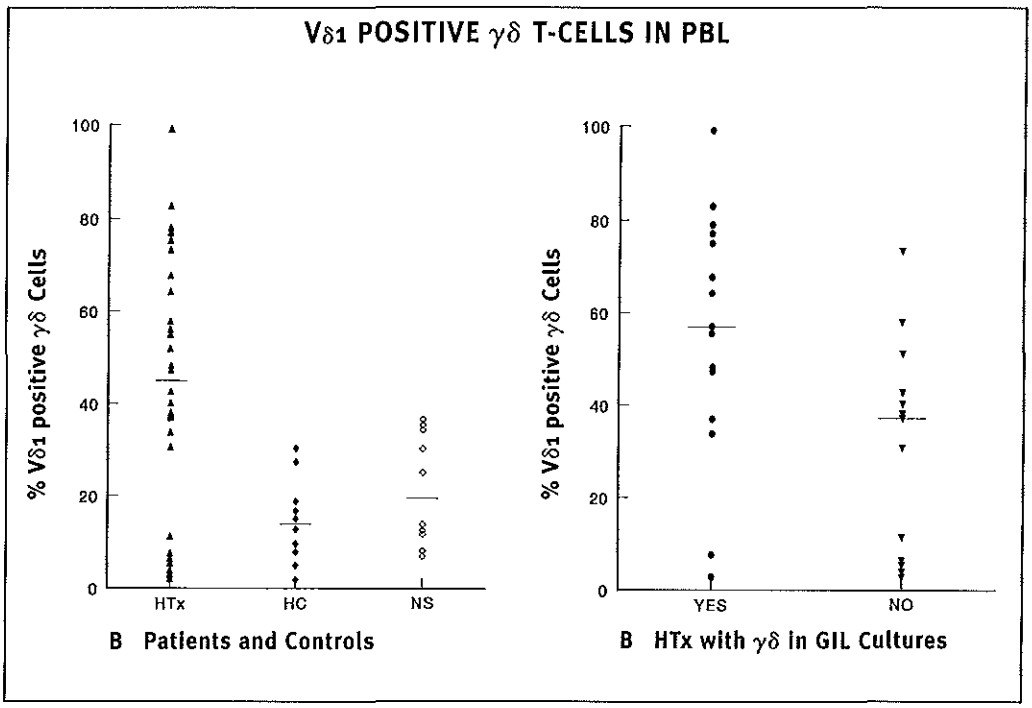

Figure 8.2. *The proportion Vδ1 positive T cells expressed as percentage of all TCR-γ/δ-1 positive cells in PBL samples of all heart transplant patients (HTx), healthy controls (HC) and patients who received CsA because of a nephrotic syndrome (NS) are compared in panel A. In PBL of HTx a significant higher proportion Vδ1$^+$ γδ cells were present than in HC (p=0.023) and NS (p=0.019).There was no difference between HC and NS.*
In panel B, HTx patients with (yes) and without (no) γδ T cells in their EMB derived GIL cultures at the time the PBL sample was taken are seperately depicted. In PBL from HTx patients with γδ cells in the GIL cultures a significant higher proportion of the γδ cells were Vδ1$^+$, than in the other HTx patients (p=0.012).

serum, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycine.

8.2.3 Monoclonal Antibodies

The mAb anti-TCR-γ/δ-1 (clone 11F2), specific for human TCR γ-chains,[31] anti-Leu-4 (CD3) and WT31, reactive with the human TCR-αß chain , were purchased from Becton Dickinson, (San Jose, CA) the mAb δTCS1, specific for a Vδ1Jδ1 encoded epitope on the δ-chain of human TCR-γδ [9] and the mAb 15D against the Vδ2 gene product[8] were obtained from T Cell Sciences (Cambridge, MA). Anti-CD45RO (clone UCHL1) and isotype control mAb were obtained from Immunotech (Marseille, France). All mAb were directly conjugated to FITC or Phycoerythrin (PE).

8.2.4 Immunofluorescence analysis

PBMC, T cell lines and clones were analysed for the expression of cell surface antigens with the mAbs defined above. 5.10^5 cells were incubated with fluoresceinated mAb for 30 min at Room temperature. Cells were subsequently washed in PBS and from each PBL sample at least ten thousand cells were analyzed in a lymphocyte

	GIL culture		PBL sample	
ID+	γδ T cells*	Vδ1#	γδ T cells*	Vδ1#
RO	41	100	0.8	48.2
LU	8	50	0.6	77.9
GI	30	90	1.0	99.9
BN	8	100	10.3	7.6
SC	34	85	10.3	82.7
OO	21	90	1.8	37.0
DR	12	92	10.9	55.5
BE	33	66	10.3	75.3
BA	92	100	17.1	64.2
JU	49	100	6.3	55.9
VU	83	100	6.5	33.8
ZE	35	86	6.0	77.1
VS	81	93	2.5	47.3
GR	53	70	16.8	2.8
MO	78	100	11.1	67.7

+patient identification; *$\gamma\delta^+$ T cells as % of $CD3^+$ cells; #$V\delta1^+$ T cells as % of all γδ cells.

Table 8.1 *Percentage $\gamma\delta^+$ T cells and $V\delta1^+$ subpopulation in peripheral blood and graft infiltrating lymphocyte culture propagated from EMB concurrently taken from heart transplant patients.*

gate on a FACScan (Becton Dickinson). Lymphocyte gate was set on Forward (FSC and Sideward (SSC) light scatter, while cell debris was gated out by a threshold on FSC. For the expression of the activation markers CD45RO, CD25 or HLA-DR on the Vδ1 and Vδ2 population, cells were stained with anti-CD45RO, -CD25, -HLA-DR respectively, conjugated to PE, in combination with δTCS1 or 15D conjugated to FITC. FITC negative cells were gated out electronically by a threshold on FITC channel number 250 . In this way all Vδ1 or Vδ2 negative cells were gated out and we were able to accumulate at least 500 Vδ1 or Vδ2 positive cells, and analyzed them for the expression of PE stained activation markers. Limits for 'non activated' cells were set by PE-labelled isotype control monoclonal antibodies.

8.2.5 Generation of T cell lines in IL-2

PBL were cultured in CM supplemented with 30-50 U rIL-2 (Biotest, Dreieich, Germany). Cultures were grown in 24 well plates (Costar) in 2 ml culture medium at a concentration of 10^6 cells/ml at 37°C in a humidified 5 % CO_2 incubator. Half of the culture medium was changed 3 times a week. When a well was grown confluently, the cells were resuspended and divided over two or three wells. Cultures were analyzed for phenotype expression at the start of the culture and thereafter once a week during a period of 2-3 weeks.

8.2.6 Statistical analysis

Differences in γδ and Vδ1 levels between the groups were tested for significance with the Kruskal-Wallis Nonparametric one-way analysis of variance (ANOVA) test, corrected for ties. If significant differences were found, this was narrowed down using two by two comparisons in the Mann-Whitney U test. Fischer' Exact test with Yates' correction was used to test for differences in gender, and to test for differences between patients groups for the number of PBL

cultures with expanding Vδ1 or Vδ2 T cells. All calculations were performed with InStat software (GraphPad Software,Inc San Diego, CA).

8.3 Results

8.3.1 Levels of γδ T cells in HTx patients and controls

The total level of γδ positive T cells in PBL was determined with the mAb 11F2 (anti-TCR-γ/δ-1) and expressed as percentage of $CD3^+$ T cells (*Figure 8.1*). The levels of $\gamma\delta^+$ T cells in HTx patients (median 5.2%, range 0.5 - 17.1%) were not different from those of the healthy control (HC) group (median 2.6%, range 1.0 - 14.6%) or CsA treated NS patients (median 6.5%, range 0.6 - 10%). No differences were found for total levels of γδ cells between the two HTx subgroups, HC and NS patients (*Figure8.1B*) (p=0.4856, Kruskal-Wallis ANOVA). Since absolute numbers may give more information about differences in $\gamma\delta^+$ T cell population between the several groups we determined the amount γδ cells/μL. In the HTx group 29.5 γδ /μL (median, range 4 - 281) were present. This was neither significant different from HC, median 28, range 9 - 122 γδ/μL, nor from NS patients, median 63, range 9 -161 γδ/μL, (p= 0.5594 as tested with Kruskal-Wallis ANOVA). There were also no differences between the two HTx subgroups, HC and NS patients (p=0.4712 Kruskal-Wallis). HTx patients with γδ in GIL had 46 γδ/μL (median), range 4 - 261, and HTx patients without γδ in GIL had 22, range 6 -281 γδ/μL in their PBL. The levels of $V\delta1^+$ γδ T cells ($\delta TCS1^+$) were expressed as percentage of all $\gamma\delta^+$ T cells (*Figure 8.2A*). In PBL of HTx patients significantly (p=0.023) more $V\delta1^+$ γδ cells (48%, 2.8 - 99.9) were present compared to HC (21.7%, 6.0 - 38.0).

The proportion of $V\delta1^+$ γδ T cells in NS patients was compatible to HC (median 19.5%, range 6.0 - 36.5%). In PBL samples of all HC and all NS patients Vδ2 cells were the predominant subpopulation, resulting in a Vδ1/ Vδ2 ratio < 1.0. In PBL samples from 11/28 HTx patients, Vδ1/ Vδ2 ratio was >1.0. Nine of these 11 PBL samples were from patients that had γδ cells in the GIL cultures propagated from the EMB taken at the same time as the PBL sample. Comparison of the two subgroups of HTx patients, HC and NS patients with Kruskal-Wallis ANOVA test, revealed a significant difference between the groups (p=0.0024). Subsequently the groups were compared two by two in a Mann-Whitney U test, which showed that indeed in HTx patients with TCR-$\gamma\delta^+$ cells in their GIL cultures, the proportion of Vδ1 cells in PBL was significantly higher than in PBL of HTx patients without γδ cells in their GIL cultures (p=0.012). In the HTx group with γδ cells in the GIL cultures 56% (median, range 2.8 - 99.9%) of the γδ cells in PBL were Vδ1 positive (Table 8.1), while in the HTx group, without γδ cells in their GIL cultures, 37.0% (median, range 2.8 - 73.2%) of the γδ cells in the PBL were Vδ1 positive *(Figure 8.2B)*. The latter was comparable to the percentages found in HC (p=0.4) and NS patients (p=0.3). We also have tried to correlate the existence of an inverted Vδ1/Vδ2 ratio with clinical features such as transplant coronary artery disease, acute rejection and the occurrence of infectious diseases. A correlation with these clinical features however, was never found. (data not shown)

8.3.2 Expression of activation markers on Vδ1 and Vδ2 T cells

The Vδ1 and Vδ2 cells were analyzed for expressing CD45RO strongly (bright), weakly (dim), or not at all (neg)*(Table 8.1)*. No difference was found between the various groups with respect to the proportion of $V\delta1^+$ and $V\delta2^+$ γδ cells expressing CD45RO. In HC the proportion, [median (range)], CD45RO (bright and dim) positive $V\delta1^+$ cells was 42%(28 - 56%), for Vδ2 positivity this was 92.5%(87 - 100%). In NS patients 41%(29 - 50)% for $V\delta1^+$ cells and 93%(87 - 97%) for $V\delta2^+$ cells. In HTx patient with γδ cells in the GIL cultures 31.6% (13 - 78%) of the c cells and 83.4%(12.8 - 99.2%) of the $V\delta2^+$ cells were CD45RO positive, in HTx patients without γδ in the GIL cultures this was

	Vδ1			Vδ2		
	bright	dim	neg	bright	dim	neg
HTx with γδ in GIL	10@ (0.8-53)#	26.1 (3.9-38)	67.7 (22-86)	45.5 (8-84)	19.1 (5-48)	15.7 (0-87)
HTx without γδ in GIL	31.1 (8-44)	18.4 (10-32)	54.1 (23-75)	55 (31-70)	32.9 (20-47)	9.4 (7-19)
Patients with nephrotic syndrome	11 (6-17)	28.5 (14-42)	59 (52-71)	75 (56-85)	18 (10-37)	7 (2-13)
Healthy controls	11 (6-14)	29.5 (19-42)	60.5 (44-72)	64 (32-87)	27.5 (8-62)	8 (0-13)

Percentage Vδ1 positive and Vδ2 positive cells expressing CD45RO in PBL of HTx patients, healthy controls and patients recieving CsA because of nephrotic syndrome. @Median percentage, #range.

Table 8.2 *CD45RO expression on Vδ1 and Vδ2 cells*

43.6%(24.8 - 77.9) and 90%(79 - 90.9) respectively. There was also no significant difference in the intensity of CD45RO expression, neither on the $V\delta1^+$ cells, nor on the $V\delta2^+$ cells. *(Table 8.2).*

The percentage of $V\delta1^+$ and $V\delta2^+$ cells in the PBL, bearing HLA-DR was very low, only weakly positive, in all patient groups tested. For Vδ1 this was 7.5,(1 - 16% dim) [median,(range)], 9%,(7 - 13) and 8%,(5 - 15) for HTx with γδ, HTx without γδ and HC respectively. Comparable results were obtained for the Vδ2 population. The IL-2 receptor (CD25) was neither detectable on $V\delta1^+$ nor on $V\delta2^+$ cells except for one patient, in which 20% of the $V\delta1^+$ PBL co expressed CD25 on their surface at low intensity.

8.3.3 Proliferation after stimulation with IL-2

We also tested whether γδ cells in PBL proliferated in response to 30-50 units riL-2, the results are summarized in table 8.3. Cultures were scored as expanders when the proportion of γδ T cells had increased 5 times or more after 3 weeks in culture. Compared to HC, more PBL samples obtained from HTx patients showed $V\delta1^+$ cells as the major expanding γδ population after stimulation with IL-2 (p=0.011). In nearly all HTx patients with γδ T cells in the graft, PBL samples that showed expansion of the γδ cells, the $V\delta1^+$ population dominated, whereas in all PBL samples with expanding γδ cells taken from HC, $V\delta2^+$ cells became predominant. This difference was statistically significant (p=0.015). There was no significant difference between the two HTx groups. Results in the CsA treated NS patients were inconclusive. However in contrast to the HC, some Vδ1 cell dominated PBL cultures were found after expansion in IL-2 *(Table 8.3)*. The percentage CD25 positive γδ cells in the expanded populations was variable but not different in the groups tested. For HTx patients the median percentage was 54.8, range 34.9 - 88.8%. For the HC this was 56.7%, range 44.9 - 93.6% after three weeks of culture.

	n@	Vd1#	Vd2#
HTx with γδ in GIL	14	6	1
HTx without γδ in GIL	12	3	1
Patients with nephrotic syndrome	10	2	3
Healthy controls	10	0	4

@ Number of PBL samples cultured. # Number of cultures in which predominantly Vδ1 or Vδ2 cells expanded. Expansion was defined as an increase of 5 times or more from the particular γδ population during the culture period of three weeks in 30-50 units IL-2.

Table 8.3 *Proliferation of PBL γδ T cells in IL-2*

8.4 Discussion

In PBL of heart transplant patients Vδ1$^+$ γδ cells were more often the predominant γδ subpopulation as compared to PBL of HC. Especially in HTx patients from whose endomyocardial biopsy γδ$^+$ T cells could be propagated, PBL contained significant more Vδ1$^+$ γδ T cells than PBL of HTx patients in whose biopsy derived GIL cultures no γδ cells were present. This resulted in a reversed Vδ1/Vδ2 ratio compared to HC, since the mAbs δTCS1 and 15D identify two distinct, non- overlapping subpopulations of γδ T cells and represent approximately 95% of the γδ cells in the peripheral blood of healthy individuals.[6,8,10] In the PBL of the HC the Vδ2 (15D positive) T cells predominate, comprising 65% or more of the γδ T cells. While the remaining γδ cells were nearly all of the Vδ1 (δTCS1$^+$) subpopulation. This distribution in PBL of HC was similar to that reported by several other investigators.[6,10,11] As far as we know, no study has determined the ratio between Vδ1 and Vδ2 in the PBL of transplant patients. PBL of patients with autoimmune disease are studied more intensively in this respect. Like in GIL cultures propagated from the EMB, particularly in those taken more than one year after HTx,[29,30] Vδ1$^+$ cells are the predominant γδ T cells in the rheumatoid joints,[18,19,20,21] in autoimmune chronic active hepatitis,[27] in the affected muscles of patients with polymyositis [32] Several studies in these patients showed a similar, reversed, Vδ1/Vδ2 ratio as we now report for PBL of HTx patients [20,21,23,27]

The total level of γδ T cells in the PBL of both HTx groups had not changed compared to healthy controls, and were concordant with results published by others for large series of healthy individuals.[1,10] In patients with autoimmune chronic active hepatitis and primary sclerosing cholangitis, showing reversed Vδ1/Vδ2 ratios, Wen et all found elevated levels of total γδ T cells.[27] For patients with rheumatoid arthritis (RA) conflicting results have been reported, both decreased percentages of total circulating γδ cells [18,19,20] as well as increased [33,34] or normal levels [34,35] were found. The reason for these discrepancies might be due to marked variations in median levels of the control and patient groups, because of small sample sizes. In most studies the given data for patients are within the range for normal individuals.

Normal expression of CD45RO, and the lack of activation markers CD25 and HLA-DR on the Vδ1$^+$ PBL in the HTx patients of our study, in combination with a normal level of total γδ T cells argue against peripheral expansion of the Vδ1 population due to activation. Nevertheless,

the γδ population in the HTx patients seems to be distinct from that in HC, showing different in vitro expansion patterns. When cultured in 30-50 units IL2, a significant difference became apparent between HC and HTx patients. In the HTx patients with Vδ1$^+$ γδ T cells in their GIL cultures, predominantly the Vδ1$^+$ γδ T cells expanded, in contrast to the HC were only the Vδ2$^+$ γδ T cells expanded. This finding in HC was in harmony with the results of Orsini et all,[36] who found in 12/30 HC selectively the Vδ2$^+$ γδ T cells expanded in IL2, whereas in their other 18 cultures γδ T cells did not expand. The NS patients seemed to be in a more intermediate position. This group of 10 patients with nephrotic syndrome received a comparable dose of CsA as the HTx patients. Since Vδ2$^+$ cells are thought to be activated γδ cells[10,11] CsA might have reduced this population and in this way Vδ1/Vδ2 ratio might have been reversed. However the results showed no significant difference between HC and NS group, neither in the percentage CD45RO$^+$ Vδ2 cells nor in the level of CD45RO expression on Vδ2 cells. CD45RO expression on Vδ2 and on Vδ1 cells was the same in all groups and very similar to the results of Parker et all for normal adults. [10] Obviously CsA did not alter the Vδ1/Vδ2 ratio by inhibiting the Vδ2$^+$ γδ T cell population, but might have diminished the capacity of Vδ2 cells to proliferate in response to IL2. However, definitive proof for this assumption has to be given. Kjeldsen-Kragt et all [38] gave evidence for the expansion of resting γδ T cells, only expressing the intermediate affinity IL-2Rß chain. Orsini et all [36] however postulated that especially γδ cells previously activated by a, hitherto unknown ligand expand in IL-2.

The cause for the reversed Vδ1/Vδ2 ratio in PBL of heart transplant patients remains an open question. It is possible that Vδ1 cells slowly proliferate in response to chronic antigenic stimulation. However the responsible ligand must be another than donor HLA-antigen since in previous studies no donor reactive Vδ1 cells were found in the grafts of these patients.[29,30] A group of ligands responsible for this activation might be formed by heat shock proteins (HSP). HSP, also called stress proteins, can be expressed in the graft due to inflammation processes, since injurious stimuli to cells induce an increased production and subsequent expression of HSP on the cell surface (reviewed by Harboe and Quayle[39] Moliterno et all [30] showed that both TCR α/ß and TCR γ/δ cells responsive for HSP could be propagated from EMB. The γδ T cells were predominantly of the Vδ1 subpopulation and they could be propagated more often from long term transplant biopsies, and in line with our results, they did not find a relation with acute rejection. These HSP may be transported to the lymphoid organs and activate γδ T cells which subsequently migrate and home in the graft. The lack of activation markers on the Vδ1 cells in PBL represents a problem in this concept. However these Vδ1 cells may express the intermediate-affinity IL2 receptor, and may slowly expand in the graft due to IL-2 produced by activated αß T cells as suggested by Kjeldsen - Kragt et all in their report on the expansion of γδ T cells in rheumatoid arthritis lesions.[37] IL2 producing αß cells are indeed present in the graft in the period between 90 and 1770 days after HTx cells [40] when γδ cells can be cultured most frequently.[29,30] Finally, since we did not find a relation with acute and chronic rejection and no known donor specific ligand is involved, Vδ1$^+$ cells might play a role in a more aspecific down regulation of the immune response as we postulated before.[29]

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CHAPTER 9

FREQUENCIES OF T HELPER CELLS AND PRECURSORS OF CYTOTOXIC T CELLS WITH HIGH AVIDITY FOR DONOR ANTIGENS IN PERIPHERAL BLOOD CORRELATE WITH ACUTE REJECTION

9.1 Introduction

For monitoring of rejection, heart transplantation (HTx) patients have to undergo frequent transjugular endomyocardial biopsy (EMB) procedures. This method is invasive, time consuming, and expensive. Consequently, several attempts have been made to monitor rejection based on immunological parameters in peripheral blood. Changes in T cell subpopulations [1,2] T cell activation markers such as CD25, HLA-DR [1,3-5], specific morphology (Cyto-Immunological Monitoring) [6-8], and levels of soluble IL-2 receptor (sIL2R) [9,10] were studied. The results have been variable and therefore inconclusive. Also more donor specific parameters such as the frequency of donor directed cytotoxic T lymphocyte precursors (pCTL), and the frequency of IL-2 producing helper T lymphocytes (HTL) with specificity for donor antigens were subject of investigation. One study in HTx patients reported that acute rejection episodes were accompanied by an increase of the HTL frequency in the blood.[11] The results obtained with pCTL frequency measurements were variable. Some investigators found a correlation between elevated pCTL levels and rejection, others did not.[12,13] In this communication we present preliminary results on the investigation of the avidity for donor antigens of pCTL and donor specific HTL frequencies in the peripheral blood at the time of rejection.

9.2 Materials and Methods

9.2.1 Patients

We studied PBL samples, and graft infiltrating lymphocyte (GIL) cultures propagated from endomyocardial biopsies (EMB) of heart transplant recipients taken during and prior to rejection episodes in the first 2 months after transplantation. All patients had received preoperative blood transfusions. Cyclosporin (CsA) and low dose prednisone were used as maintenance immunosuppression. The mean number of HLA-mismatches between donor and recipient for the A, B, and DR-antigens was respectively 1.25, 1.50 and 1.37.

Detection of acute rejection was performed by histological examination (according to ISHLT grading [16] of EMB taken from the right ventricular septum. During each biopsy procedure, four fragments of endomyocardium were obtained. Three were used for histological evaluation, and one was placed in IL-2 conditioned medium for culture of GIL. Clinically relevant rejection was diagnosed when coexistence of multifocal mononuclear cell infiltrates and myocyte damage was found (ISHLT $\geq$ 3A). In these cases, anti-rejection treatment was instituted.

9.2.2 Peripheral blood lymphocytes

PBL were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation.

9.2.3 Graft Infiltrating Lymphocyte cultures

GIL cultures from EMB were established in culture medium containing 30 units IL-2, and irradiated (30 Gy) autologous PBL as feeder cells as described in detail in chapter 3.

9.2.4 Allogenic target cells

T cell blasts (T-BL) were obtained by culturing donor spleen cells for 4-5 days in the presence of 1% Phytohaemagglutinine-M (PHA) (Difco, Detroit, MI) in RPMI-1640 containing 5% lymphocult-T (Biotest GmbH, Dreieich, FRG).

9.2.5 Limiting dilution analysis

9.2.5.1. CTL frequencies and avidity

LDA cultures were stimulated with autologous (irradiated 30 Gy) PBL to measure cCTL frequencies, or with donor spleen cells to assay total CTL (tCTL = cCTL + pCTL) as described in detail in chapter 6. Replicates of graded numbers of responder cells (PBL or GIL) were titrated down in 8 fold doubling dilutions starting from 20.000 cells/well. After 7 days at 37°C and 5% CO_2, LDA cultures were split in two, anti-CD8 mAb was added to one of the daughter series to determine the avidity of the CTL. Each well was then individually tested for cytolytic activity against donor T-BL.

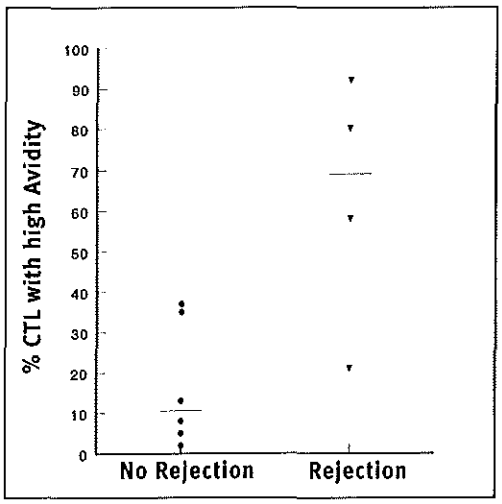

Figure 9.1. *The percentage of donor specific pCTL with high avidity in PBL and its relation with EMB diagnosed rejection episodes within the first 2 months after HTx in 10 patients. Significantly (p=0.03) more high avidity pCTL were present in PBL during rejection (ISHLT grade 3) than during a period without rejection (ISHLT grade 0 and 1). (ISHLT grade 2 rejection were not diagnosed).*

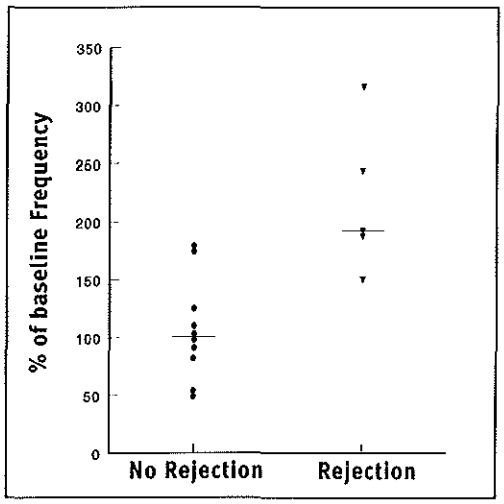

Figure 9.2. *Donor specific HTL in PBL and relation with EMB diagnosed rejection during the first 2 months after HTx in 6 patients. During rejection (ISHLT grade ≥ 3) HTLf increased significantly (p< 0.005) more above baseline frequency than in periods without rejection (ISHLT grade 0 and 1). (ISHLT grade 2 rejections were not diagnosed).*

9.2.5.2 HTL frequency estimates

24 replicates of graded numbers of PBL responder cells were titrated in 7 fold double dilution starting from 20,000 cells per well down to 312 cells per well in V bottom plates (Greiner, Alphen a/d Rijn, the Netherlands). As stimulator cells we used 5,000 irradiated (60 Gy) extensively washed B-LCL per well in a total volume of 0.2 ml RPMI 1640 DM culture medium supplemented with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% heat inactivated pooled human serum. 24 wells containing irradiated stimulator cells alone served as a background control. After 3 days of culture at 37°C in a humidified atmosphere containing 5% CO_2, plates were centrifuged (3 min, at 200 g), and from each well 100 μl culture supernatant was transferred to U bottom plates to assay for IL-2 in a bioassay using the IL-2 dependent CTLL-2 cell line as indicator system.

CTLL-2 cells cultured in IL-2 (10 - 20 Units) containing CM were extensively washed and resuspended at a concentration of 5×10^4 cells per ml.

The IL-2 produced by the LDA cultures was assessed by adding 100 μl of this CTLL-2 suspension to each well. After 24 hours of incubation at 37°C in a humidified atmosphere containing 5% CO_2, including 4 hours pulse with 0,5 μCi ^{3}[H]-thymidine/well (Amersham, U.K.; spec. activity 5mCi/ml) the plates were harvested. For each assay a control curve was constructed by adding 100 μl cell suspension to 100 μl serially diluted, recombinant IL-2 (30 - 0.015 units). The cells were harvested onto glassfibre filter mats (LKB-Wallace, Turku, Finland). The ^{3}[H]-thymidine incorporation into DNA was measured by liquid scintillation spectrophotometry (Betaplate 1205 LKB-Wallace).

9.2.6 Statistics.

CTL and HTL frequencies and their 95% confidence limits were determined by the jack-knife procedure for maximum likelihood as proposed by Strijbosch et al [18] using their computer program. The Fisher's exact test was used to compare for significant differences between the groups, and the Mann-Whitney U test for differences in median frequencies between the two groups.

9.3 Results and Discussion

tCTL with donor specificity were found during a period with rejection in all 6 PBL samples tested. Their frequency (median 58, range 6 - 340/10^6 cells) however, was not significantly different from the tCTL frequencies (tCTLf) found in 36 PBL samples taken during periods without rejection (median 7, range 1-420 tCTL/10^6 cells). These results are consistent with those reported by Herzog et al. for kidney transplant patients.[13] In chapter 5 it is shown that donor specific cCTL were only present in 1 out of 12 PBL samples taken during a rejection period and in 1 of 28 PBL samples taken some time before or after rejection.[14] So it is obvious that cCTL do not circulate and that tCTL found in PBL represent pCTL. In contrast, cCTL were abundantly present in the graft at the time of rejection, as shown in 8/8 GIL cultures propagated from EMBs taken during a rejection period[15] (chapter 6). Bishop et al [19] came to the same conclusion based on experiments with skin allografts and alloantigen loaded sponge grafts in mice. They found cCTL in the grafts of these animals but not in PBL, while pCTL were present both in the graft and PBL. We found in the graft (8 GIL cultures) that the pCTLf during rejection periods was significantly ($p<0.005$) higher (median 11,065, range 4869 - 28,095/10^6) than in 10 GIL cultures obtained when no rejection was diagnosed (323, range 0 - 13744 pCTL/10^6). Moreover, pCTL propagated from the graft during rejection differentiated into CTL with high avidity (resistant to inhibition with CD8 Mab) for donor antigens (see chapter 6 for details). It is conceivable that these high avidity pCTL are the activated stage of the pCTL, the poised CTL (poCTL) in the terminology of Bach et al.[20] Since cCTL do not circulate in measurable amounts in the peripheral blood, and high avidity pCTL were present in EMB with myocyte damage, we reasoned that the presence of activated pCTL in PBL might correlate with rejection. We investigated this in a pilot study. The analysis revealed that during rejection significantly more high avidity pCTL were present in the blood than when no rejection was diagnosed ($p=0.03$) *(Figure 9.1)*. Another cell population relevant for rejection, [21] and accessible in the peripheral blood, is the HTL population. LDA of IL2 producing HTL in PBL samples from 6 HTx patients showed considerable patient to patient variation for the level of HTL frequencies (HTLf). Before transplantation this ranged from 96 to 746 HTL/ 10^6 cells, in the first week after HTx this varied from 97 to 282 HTL/ 10^6 cells. To master this inter patient variation, HTL frequencies were evaluated as percentage of the individual baseline frequency. This approach was also followed by DeBruyne et al.[11] As individual baseline frequency served the HTL frequency measured in the first or second week after transplantation when maintenance immunosuppression was instituted and no signs of rejection were diagnosed. In figure 9.2 the results are summarized for 6 patients. The relative HTLf of one patient who did not have a rejection episode varied between 117 - 143 percent of the baseline frequency. This patient is in figure 9.2 represented by one value (the mean percentage 131). Five patients had a rejection episode, during that period the median of the percentage of baseline frequency was significantly higher ($p<0.005$ Mann-Whitney) than in periods without rejection. When a HTL frequency of 150 % of baseline or higher was considered as a risk factor for rejection, 2/10 non rejection periods would be scored as false positive. However despite the limited test group, also with this "threshold" value, the difference between the two groups was significant ($p=0.007$) as tested with the Fisher exact test.

Although the analysis of high avidity pCTL and HTL frequencies in PBL presented herein were performed in a relative small cohort of patients the results promise usefulness of these parameters for rejection monitoring. In a larger series of patients, time-line studies must give definitive proof of the clinical value of both HTLf and high avidity pCTLf in peripheral blood in replacing (at least a part) of the biopsy procedures.

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CHAPTER 10

SUMMARY AND CONCLUSIONS

Transplantation has become an accepted modality for patients with an endstage kidney, heart or liver disease. Generally donor organs have a different antigenic make up than the recipient, which leads to rejection processes in the graft. Such an allograft rejection is the result of a complicated sequence of interactions between the patients immune system and the grafted tissue. In chapter 1 an introduction is given in the molecular and cellular processes leading to allograft rejection. Since cytotoxic T cells (CTLs) are the main study object of this thesis it is also discussed why these cells probably are the most important effector cells causing graft damage.

The introduction of the immunosuppressive agent cyclosporin A some 15 years ago significantly improved the graft survival. Despite good graft survival only ±20% of the heart transplant patients remain free from rejection and all patients have to take immuno-suppressive agents live-long.
Studying the function of CTL and HTL present in the graft and the peripheral blood during rejection, and at the time of stable engraftment may learn us to recognize patients in which immunosuppression may be tapered or even stopped.
The clinical heart transplant program provided us with an excellent opportunity to study this, since endomyocardial biopsies are taken at regular intervals to monitor histologically for acute rejection.
In chapter 2 a brief overview is given of the literature considering the culture and characterization of graft infiltrating cells propagated from transplanted hearts, livers and kidneys, including the major drawbacks of studies investigating the correlation between graft function and immunological parameters measured in the peripheral blood.

In chapter 3 the results are presented of our studies on 283 cell cultures propagated from endomyocardial biopsies (EMB) taken from 87 heart transplant recipients. A good correlation was found between rejection grade and propagation results. Eighty percent of EMB with signs of myocytolysis gave cultures with growing T cells that were mostly cytotoxic for donor cells. In the patients that experienced one or more acute rejections in first three months after transplantation 57% of the cultures were cytotoxic for both mismatched MHC class I as well as Class II antigens. This incidence decreased to 33% more than three months after transplantation when in most patients stable engraftment was observed. This was comparable with the incidence of cultures with cytotoxicity for both class I and class II antigens found in the patients that never had an acute rejection.
We concluded that propagation results reflected very well the rejection status of the graft, and that patients whose grafts are infiltrated with activated CTL reactive with both mismatched donor MHC class I as well as class II antigens are at high risk for rejection.
Propagation kinetics performed with all biopsies taken from 2 patients in the first 4 months after transplantation revealed that donor reactive CTL are generally not released from the biopsy before the 4TH day of culture. The cells present in the cultures established in the first four days of culture may represent precursors cells with specificity for donor or third party antigens. The presence of precursor cells with reactivity for third party antigens were also demonstrated in graft infiltrating cell cultures obtained from sponge grafts loaded with alloantigens [1] and in the cultures propagated under standard conditions with IL-2.[2] Third party reactive precursors may be attracted by the cytokines produced by donor reactive cells and donor tissue, activated as a result of the inflammatory process or may be innocent passengers present in the capillaries.
From these observations it is obvious that care should be taken with the interpretation of cultures stimulated *in vitro* with donor antigens in the early phase of propagation. *In vitro de novo* activation of precursor T cells may have been occurred. When graft infiltrating cells are pro-

pagated from kidney and liver biopsies, that contain more peripheral blood than cardiac grafts, the cells exudated from the biopsies during the first 2 days of culture should be discarded, since they are dominated by precursor CTL. Those are sensitive to activation by IL-2 and may develop into lymphokine activated killer (LAK) cells able to kill many targets in an aspecific way *(see chapter 5)*.

In chapter 4 it is shown that graft infiltrating lymphocytes (GIL) propagated from biopsies,taken at different time points from one cardiac transplant patient, express only a limited number of TCRAV and TCRBV genes while in peripheral blood lymphocytes all families were present.
This restricted TCRBV gene usage was also seen in T cells infiltrating rejecting human lung allografts.[3,4] The results of studies of Hall et al [5] in kidney transplant patients, showed the usage of many TCRBV gene families when analyzing GIL cultures propagated from core-needle biopsies. Those cultures were analyzed within 2 days after start of the culture. When the cultures were analyzed for TCRBV gene usage later after onset of the culture a more restricted pattern was found. The authors stated that such a restricted TCRBV gene pattern was due to selection of some clones during culture and therefore may be an culture artefact. However, the broad range found in the early cultures may not reflect activated GIL with specificity for donor antigens only, but also TCRBV used by other peripheral blood lymphocytes present in cultures as discussed above in relation to chapter 3. The more restricted pattern found in cultures grown for a longer period may reflect the expression in the real graft infiltrating lymphocytes.
Some of the V genes were shared by several GIL lines, which indicates a restricted usage. In one patient, functional analysis of T cell clones and sequence analysis of the TCRBV used, revealed that CTL that were able to lyse donor cells with high avidity were only present in the rejection biopsy with damaged myocytes. The cells with this particular TCRBV gene usage and donor directed specificity were not found in earlier biopsies. This suggests that these CTL have been recently recruited into the graft.
Of particular interest is also the observation that CTL clones with shared specificities, established from GIL lines propagated from sequentially taken rejection biopsies, displayed usage of different TCRAV and TCRBV genes. This suggests that different clones can exert effector functions leading to tissue damage and a particular clone may be unique for only a certain timepoint after transplantation. These findings discourage the idee that monoclonal antibodies against TCRV-gene family products can be used for specific rejection treatment or prophylaxis.

In chapter 5 we studied the possible association between acute rejection, and the presence of activated, donor specific CTL (cCTL) in peripheral blood and the graft. Based on the protocol used to culture activated CTL from EMB, we also tried to expand activated, donor specific CTL from the peripheral blood.
T cell blasts of donor origin were killed by only one of the PBL lines tested, indicating that activated, MHC class I reactive, CTL were hardly present in the peripheral blood. The measurement of CTL activity against MHC class II antigens was hampered by the LAK activity that many PBL cultures displayed, which is in contrast to the GIL cultures that hardly ever showed LAK-activity. Several PBL derived lines killed the LAK sensitive target cells K563 and Daudi, as well as third party EBV transformed B cell lines. This LAK activity is caused by the 30 units IL-2 in which the cells were cultured. This frustrated the interpretation of the results of the anti class II reactivity. B-LCL of donor origin have to be used as target to measure the reactivity against class II antigens, because T cell blasts, activated with PHA, are not suitable as target to measure anti class II reactivity.
To detect donor specific MHC class II directed CTL activity, cold target inhibition studies with K562 were performed with ^{51}Cr labelled (hot)

B-LCL as donor specific targets. The LAK activity was inhibited by a 10 fold excess cold target (K562). In this way in only one culture of one patient donor specific reactivity was found. From this study we concluded that an acute rejection episode histologically diagnosed in an endomyocardial biopsy is not associated with the presence of activated donor specific CTL in the peripheral. Recently we confirmed this finding in an other cohort of heart transplant patients.[6]

In chapter 3 is described that activated, donor class I reactive CTL were found in the graft during rejection as well as at the time of stable engraftment. In chapter 6 we studied these cCTL and their precursors (pCTL) in more detail. Therefore cCTL and pCTL in GIL cultures propagated from EMB with signs of acute rejection (damaged myocytes) were compared with those in GIL cultures propagated from EMB taken at the same time after HTx from patients that never had an acute rejection. Limiting dilution assays (LDA) in combination with inhibition studies with a monoclonal anti-CD8 antibody showed that in EMB with myocyte damage the majority of the cCTL and pCTL had high avidity for donor class I antigens. The effector function of these high avidity CTL cannot be inhibited with anti-CD8 mAb. In GIL cultures of patients that never experienced a rejection episode most cCTL and pCTL could be inhibited with anti-CD8. Those CTL have low avidity for their antigen [7,9]. The studies in chapter 4 and recent studies [10] have provided 'circumstantial' evidence that accumulation of those high avidity cells resulted in myocyte damage typical for an acute rejection episode. After successful treatment with rATG the graft is repopulated with low avidity CTL.[10,11]
In a study of Roelen et al [12] rejection of cornea transplants was also associated with the presence of CTL with high avidity for donor antigens. Stable engraftment was associated primarily with the presence of CTL with low avidity.
The studies described in chapter 6 also showed that pCTL propagated from both EMB with and EMB without myocyte damage (rejection) are sensitive to inhibition with cyclosporin A (CsA). Addition of CsA to the LDA cultures blocked the differentiation from non functional pCTL into a functional cCTL. The function of fully maturated cCTL derived from rejection EMB could not be inhibited by the addition of CsA. The function of a substantial part of the cCTL in GIL cultures obtained from EMB from non rejectors was inhibited when CsA was added to the LDA cultures. Similar results were communicated by others for CTL functions [13,14] and for the proliferation of primed lymphocytes propagated from EMB of rejecting and non rejecting heart transplant patients.[15]
From these results it is evident that an increase of the CsA dose can not inhibit the rejection process when myocytolysis already occurs.

In chapter 7 and 8 TCR-$\gamma\delta^+$ cells, a T lymphocyte population of which the role in the immune response is still under much debate, are studied in heart transplant patients. As shown in chapter 3 TCR-$\gamma\delta$ T cells were regularly found in the GIL cultures propagated from EMB. Since intact rather than processed polypeptides appear to be recognized by TCR-$\gamma\delta$ T lymphocytes, and while in transplantation it is thought that direct recognition of intact allo-antigens on donor type antigen presenting cells (MO and DC) initiate the immune-response, it was hypothized that if allo-reactive TCR-$\gamma\delta$ cells exist, there is a good chance to find them in GIL cultures. The reality was different, it turned out to be very difficult to elicit a response of TCR-$\gamma\delta$ T cells against MHC class I and II antigens. In chapter 7 we describe 11 pure TCR-$\gamma\delta$ T cell cultures from 9 patients and 238 TCR-$\gamma\delta$ T cell clones established from GIL cultures propagated of EMB from 7 other patients. None of these TCR-$\gamma\delta$ T cell clones and cultures displayed allo-specific cytotoxicity. Most clones and cultures showed only LAK activity. Also Kirk et al [16] reported TCR-$\gamma\delta$ T cells, propagated from kidney allografts, displaying only LAK activity (lysis of K562 and Daudi), that did not kill tar-

gets expressing relevant alloantigen.
This contrasts with the situation for TCR-αß T cells, where allo-reactivity is rather frequently encountered. (*chapter 3, 4 and 6*).
The incidence of GIL cultures with TCR-γδ T cells was significantly higher when they were propagated from EMB taken more than 1 year after transplantation. We found no positive relation between acute rejection and the presence TCR-γδ T cells and concluded that TCR-γδ T cells are not involved in acute allograft rejection. More recently comparable results were published by Moliterno et al.[17] Kirk et al [16] found TCR-γδ T cells in 33% of the GIL cultures propagated from biopsies of rejecting kidneys. These TCR-γδ T cells were able to lyse kidney epithelial cells *in vitro* in a LAK-like way, which prompted them to assign a role for these cells in the rejection of kidney grafts. However, in their discussion they suggested that infiltration of TCR-γδ T cells may be the result of an established acute rejection. Such an established rejection (damaged function) is hardly ever encountered in heart transplantation where myocyte damage is already treated before deterioration of the function is expected. The accumulation of TCR-γδ T cells in cardiac transplants, in our study, more than one year after transplantation might be due a prolonged expression of heath shock proteins as discussed in chapter 8.
Another finding of interest, reported in chapter 7, was the predominance of the Vδ1 subset within the infiltrating TCR-γδ T cells. In the peripheral blood and peripheral lymphoid organs of normal individuals this population forms the minor TCR-γδ T cell subset, while 70% of the TCR-γδ T cells are normally of the Vδ2 type.

In chapter 8 we investigated the inverted frequency of TCR-γδ populations in more detail. Peripheral blood samples of 28 heart transplants patients were studied for the levels of TCR-γδ T cells and their subpopulations Vδ1 and Vδ2. An elevated proportion of $V\delta1^+$ γδ T cells was found in the PBL of transplant patients, particularly in those who had Vδ1 cells in the GIL cultures propagated from their graft. The total TCR-γδ T cells levels in the peripheral blood was not different from that in normal individuals. The aberrant composition of the TCR-γδ T cell population was not the result of the cyclosporin A medication. Patients that received a comparable dose of CsA because of a nephrotic syndrome, had a Vδ1-Vδ2 distribution comparable to normal individuals. The Vδ1 cells in the PBL of the HTx patients showed no features of activation since they lacked the markers CD25 (high affinity IL-2 receptor), HLA-DR and CD45RO. Despite the lack of CD25, Vδ1 cells from HTx patients with TCR-γδ T cells in their graft could be expanded *in vitro* in IL-2. This may be the result of CD122 expression (intermediate affinity IL-2 receptor) by these cells.
The reversed Vδ1/Vδ2 ratio we found in the PBL of HTx patients was also reported for PBL of patients with auto-immune disease. The cause of this reversion remains an open question, as a possibility is discussed that Vδ1 cell slowly proliferate in response to chronic antigenic stimulation by heat shock proteins (hsp). Hsp can be expressed in the graft due to inflammation, since any injurious stimulus to cells induce an increased production and subsequent expression of hsp on the cell surface.

In chapter 9 the results are described of a pilot study in which we investigated whether levels of pCTL with high avidity for donor antigens and IL-2 producing helper T lymphocytes (HTL) can be used for non invasive rejection monitoring. Since the most relevant cells for rejection, donor reactive cCTL, were not detectable) In the peripheral blood and no association was found between rejection episodes and peripheral blood levels of all pCTL with specificity for donor antigens, (shown in chapter 9 and in refs 18-20) these two cell populations might be good alternatives.
Indeed, both cell types turned out to be good candidates for monitoring purpose. During rejection the fraction of pCTL with high avidity was significant higher than in PBL of patients

that never experienced an acute rejection episode. A recently finished study showed that also in sequentially taken PBL samples a forthcoming episode with acute rejection can be seen based on the increase of pCTL with high avidity [6] However for practical use in daily monitoring routine this LDA method has a major drawback, it takes 7 to 10 days before an answer is reached. Therefore this method seems more suitable for monitoring transplant patients longer after transplantation, not in an attempt to diagnose rejection but to predict non responsiveness
Measurement of HTL frequency with LDA in PBL samples of six 6 HTx patients showed considerable patient-to-patient variation. To master this inter-patient variation HTL frequencies were expressed as percentage of the individual baseline frequency. During a rejection episode this relative frequency was significant higher than at periods without rejection. At its present form also this LDA takes to long (4 days) before an answer is reached. Studies are currently in progress to shorten the assay time to 48 hours. Based on recently gathered data with this assay, again the impression evolved that these analysis will be more helpful to study non responsiveness than to diagnose acute rejection.

In conclusion

The experiments described in this thesis show that acute allograft rejection is strongly associated with the concurrent presence in the graft of both primed CTL reactive with HLA Class I as well as CTL reactive with Class II antigens mismatched between donor and recipient. For the primed CTL specific for HLA class I antigens of the donor, present in the graft during rejection it is shown that they are cyclosporin A resistant and have high avidity. In patients without rejection primed CTL were found with low avidity, partially inhibitable with CSA.
Analysis of TCRAV and TCRBV gene usage showed that the deleterious high avidity CTL clones infiltrate the graft just before myocyte damage is seen. Those analyses also revealed the usage of different TCRAV/TCRBV gene combinations at different rejection episodes in the same patient, even when the specificity of the clones were the same. This type of analysis also showed that oligoclonality existed within the graft infiltrating T cell populations.
For non-invasive rejection monitoring in peripheral blood, in an attempt to replace histological examination of endomyocardial biopsies or at least to decrease the biopsy frequency, the measurement of primed, high avidity CTL can not be used since those cells are not detectable in peripheral blood.
However, a predominance of pCTL with high avidity for donor antigens and elevated levels of helper T cells were found during rejection in the peripheral blood which may be suitable for monitoring purposes.
Taken together the results of the experiments reported in this thesis show that the assays used are relevant to study the difference between cell populations present in the graft and peripheral blood during acute rejection, and in the graft and blood of patients that never suffered from an acute rejection.
However, since the experiments described primarily show the difference between rejectors and non rejectors, further research is needed on the exact kinetics of primed CTL with high avidity and their precursors during a rejection episode, in the graft and for pCTL also in peripheral blood.
Of interest is also to find markers or methods to distinguish naive pCTL from the partly activated, but not fully maturated pre-effector cells: peCTL and poCTL *(Figure 10.1)*. The reason for this is to investigate whether the elevated levels of high avidity pCTL in PBL and the graft during rejection belong to the first or these latter cell types. This could give more insight in the exact locations and pathways of CTL activation and maturation during the rejection process.
To obtain a complete picture of the donor directed immune responses also the kinetics of high and low avidity CTL reactive to donor class II antigens and that of donor reactive T helper

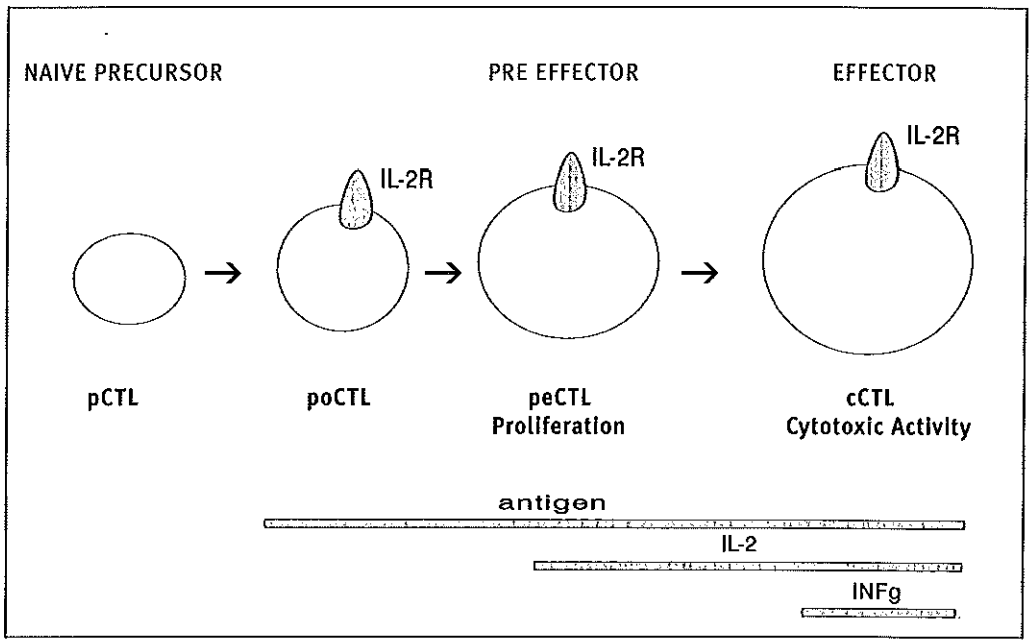

Figure 10.1 *Stages of cytotoxic T cell Activation.*
pCTL = precursor CTL, small circulating lymphocyte, negative for IL-2 receptor CD25.
poCTL = poised cell, small lymphocyte, possitive for IL-2 receptor, sensitive to the IL-2 proliferating signal.
peCTL = pre-effector cell, large proliferating "blast", lacks cytolytic activity.
cCTL = committed proliferating "effector" cytotoxic cell.
(adapted from Gromo et al. Nature 1987; 327:424.)

cells are of interest, both during acute rejection and stable engraftment.
Enumeration of pCTL with high avidity in the peripheral blood may not be the appropriate tool for immunological monitoring of PBL to predict an acute rejection episode to replace biopsy procedures. For practical purpose the culture time of at least seven days for the LDA assay is rather long. The elevated levels of donor reactive, IL-2 producing, T helper cells during acute rejection may be of more use. This assay is performed now in 4 days and can probably be shortened to 2 days. All assays however might be helpfull to select patients in who the immunosuppression can safely be tapered, based on the lack of high avidity pCTL and low HTL frequencies in the peripheral blood .

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SAMENVATTING

Het immuunsysteem beschermt tegen levensbedreigende infectieziekten doordat het reageert op binnendringende, lichaamsvreemde eiwitten waaronder micro-organismen zoals bacteriën, virussen en schimmels. Ditzelfde immuunsysteem frustreert echter ook de resultaten van orgaantransplantatie door het nieuwe, levenreddende orgaan als vreemd te beoordelen en vervolgens af te breken. Dit proces heet afstotingsreactie en is het eindresultaat van een serie interacties tussen het immuunsysteem van de patiënt en het getransplanteerde weefsel. In hoofdstuk 1 wordt een overzicht gegeven van de moleculaire en cellulaire processen die leiden tot afstoting.
Toch is transplantatie een algemeen geaccepteerde therapie geworden voor patiënten met een hart-, nier-, of leverziekte in eindstadium. Dit komt door de beschikbaarheid van middelen die de immuunreacties kunnen onderdrukken. Vooral de introductie van Cyclosporine A (CsA), ongeveer 15 jaar geleden, verbeterde de kans op lange transplantaatoverleving aanmerkelijk. Maar ondanks goede transplantaatoverleving blijft maar ± 20% van de patiënten die een harttransplantatie ondergingen vrij van afstotingsverschijnselen. En alle patinten moeten permanent immunosuppressieve middelen blijven gebruiken.
Het bestuderen van de functie van cytotoxische T lymfocyten (CTL) en helper T lymfocyten (HTL), aanwezig in het transplantaat en in het perifere bloed tijdens afstoting en ten tijde van goede transplantaatfunctie, moet ons leren hoe we patinten kunnen herkennen waarbij de immunosuppressie kan worden verminderd.
Het klinische harttransplantatieprogramma biedt de gelegenheid om dit te bestuderen. Bij harttransplantatie patiënten kan de diagnose afstoting namelijk alleen worden gesteld door middel van histologisch onderzoek van een endomyocard biopsie (EMB). Daartoe wordt volgens protocol regelmatig een EMB afgenomen en kan het infiltraat worden bestudeerd ten tijde van afstoting en wanneer er niets aan de hand is.

In hoofdstuk 2 wordt een overzicht gegeven van de literatuur die betrekking heeft op het kweken en karakteriseren van transplantaat infiltrerende cellen, afkomstig uit hart-, nier- en levertransplantaten. Ook wordt aandacht besteed aan de tegenstellingen tussen de studies die transplantaatfunctie vergelijken met immunologische parameters in het bloed. Tenslotte wordt het doel van de studies, beschreven in dit proefschrift, aangegeven.

In hoofdstuk 3 worden de resultaten gepresenteerd van een studie aan 283 celculturen die gekweekt zijn uit EMB afkomstig van 87 harttransplantatie patiënten. Goede correlatie werd gevonden tussen de mate van afstoting en de groeiresultaten. Tachtig procent van de EMB met beschadigingen aan hartspiercellen leverden culturen op met CTL die cytotoxiciteit vertoonden tegen donorcellen. In de patiëntengroep die een of meer perioden met afstoting doormaakten tijdens de eerste drie maanden na transplantatie, vertoonden 57% van de cultures cytotoxiciteit gericht tegen zowel HLA-klasse I antigenen (HLA-ABC) als HLA-klasse II (HLA-DR) antigenen van de donor. Dit daalde naar 33% voor biopsiën afgenomen gedurende de periode tussen drie maanden en een jaar na transplantatie. In deze periode hadden de meeste patiënten een stabiele transplantaatfunctie. Deze incidentie aan culturen met reactiviteit tegen zowel klasse I als klasse II antigenen komt overeen met die, gevonden in de patiëntengroep die nooit een acute afstoting doormaakte.
Geconcludeerd wordt dat de groeiresultaten goed correleren met de ernst van de histologisch geconstateerde afstoting en dat transplantaten die geïnfiltreerd raken met een populatie geactiveerde CTL, reactief met zowel HLA klasse I als II antigenen, verhoogd risico lopen afgestoten te worden.
Van 2 patiënten werden alle biopten ingezet om het tijdstip na te gaan waarop, de relevante lymfo-

cyten in de kweek verschenen. Deze studie liet zien dat de geactiveerde donor reactieve CTL's niet voor de 4de kweekdag uit het biopt te voorschijn komen. De cellen die eerder uit het biopsiestukje verschijnen zijn waarschijnlijk niet, of slechts gedeeltelijk geactiveerde cellen, de zogenaamde voorloper cellen (pCTL).

In hoofdstuk 4 is geanalyseerd welke T-cel receptor Vα- (TCRAV) en Vß-genen (TCRBV) worden gebruikt door de CTL's die aanwezig zijn in het transplantaat tijdens afstoting en vlak voor afstoting. Hieruit bleek dat slechts een beperkt deel van de 22 TCRAV- en 24 TCRBV-gen families wordt gebruikt. Dit in tegenstelling tot het perifere bloed waarin alle families konden worden aangetoond. Sommige van de V-genen werden gebruikt door verschillende transplantaat infiltrerende lymfocytenlijnen. Dit duidt ook op een beperkt gebruik van het mogelijke repertoire. Functionele analyse van T-cel clonen en sequentie analyse van de door deze clonen gebruikte TCRBV-genen lieten zien dat bepaalde CTL's met hoge aviditeit alleen aanwezig waren in het bioptie dat myocyt beschadigingen vertoonde. Deze T-cellen werden niet gevonden in eerdere biopsiën. Dit suggereert dat deze CTL's slechts zeer recent naar het transplantaat zijn gemigreerd.
Opmerkelijk is ook dat CTL-clonen gericht tegen dezelfde HLA-antigenen, gebruik maakten van verschillende TCRBV-genen. Deze clonen waren afkomstig van opeenvolgende afstotings biopsiën van dezelfde ontvanger. Dit suggereert dat verschillende clonen, gericht tegen dezelfde HLA-specificiteit, dezelfde schade kunnen aanrichten en dat iedere cloon uniek is voor een bepaald tijdstip na transplantatie. Deze laatste bevinding suggereert, dat het niet zinvol is gebruik te maken van monoclonale antilichamen, gericht tegen een bepaald TCRBV produkt om een specifieke anti T-celbehandeling te geven bij een afstoting ter vervanging van polyclonale middelen als ATG en OKT3 die het hele T-celrepertoire uitschakelen.

In hoofdstuk 5 worden experimenten beschreven waarin bekeken is of acute afstoting geassocieerd is met het optreden van een verhoogd aantal geactiveerde donor specifieke CTL's (cCTL) in het bloed. Met een protocol dat is gebaseerd op de methode die wordt gebruikt om cCTL uit hartbiopsiën te expanderen, is getracht deze cellen uit bloed te kweken.
T-celblasten van donor origine werden slechts door 1 van de 40 uit het bloed gekweekte T-cellijnen gelyseerd. Dit betekent dat geactiveerde CTL's, gericht tegen donor klasse I antigenen nauwelijks in de circulatie voorkomen, niet tijdens, maar ook niet voor of na een afstotingsfase. Het meten van CTL activiteit gericht tegen donor klasse II antigenen werd ernstig bemoeilijkt door de "Lymphokine Activated Killer" (LAK) activiteit die de lijnen vertoonden. Verschillende T-cellijnen die uit het bloed konden worden gekweekt lyseerden de LAK gevoelige cellijnen DAUDI en K562 en diverse EBV getransformeerde B cellijnen (B-LCL). Deze LAK-activiteit wordt veroorzaakt door de 30 units IL-2 waarin de cellen worden gekweekt. De activiteit gericht tegen HLA-klasse II antigenen van de donor kan alleen worden gemeten door donor B-LCL als targets te gebruiken. Door de LAK-activiteit werden zowel donor als derde partij B-LCL gelyseerd. Remmings experimenten met een 10 x overmaat ongelabelde "koude" K652 cellen in combinatie met gelabelde donor B-LCL toonden aan dat slechts één cellijn specifiek donor klasse II antigenen herkende.
Uit deze studies werd concludeerd dat in vivo geactiveerde cytotoxische T-cellen niet of nauwelijks in het perifere bloed voorkomen.

Geactiveerde CTL's, reactief met donor klasse I antigenen, bleken aanwezig tijdens perioden met afstoting maar ook tijdens perioden zonder afstoting (hoofdstuk 3). In Hoofdstuk 6 werden deze cCTL's en hun precursors (pCTL) aan een nader onderzoek onderworpen. cCTL's en pCTL's in T-cellijnen afkomstig uit EMB met histologische verschijnselen van afstoting werden vergeleken met die,

afkomstig uit EMB zonder afstoting. De frequenties van cCTL en pCTL werden zowel in aan- als afwezigheid van een monoclonaal antilichaam gericht tegen CD8 gemeten. CTL's die niet kunnen worden geremd met CD8 hebben een hoge aviditeit voor hun antigeen, terwijl CTL's die wel kunnen worden geremd een lage aviditeit hebben. De studies lieten zien dat de meeste CTL's die voorkomen tijdens afstoting een hoge aviditeit hebben voor donor klasse I antigenen. CTL's die aangetroffen werden in cultures afkomstig uit het transplantaat van patiënten die nooit een acute afstoting hadden doorgemaakt, bleken overwegend een lage aviditeit te hebben.
De studies beschreven in hoofdstuk 6 laten ook zien dat de differentiatie en rijping van pCTL naar cCTL geremd kan worden door cyclosporine A, ongeacht of deze pCTL afkomstig waren uit EMB afgenomen tijdens een afstotingsfase of uit EMB zonder afstotingsverschijnselen. De functie van cCTL's afkomstig uit EMB afgenomen tijdens een periode met acute afstoting kon nooit worden geremd met CsA, terwijl een deel van de cCTL's afkomstig uit patiënten zonder afstoting wel kon worden geremd met CsA. Deze resultaten geven aan dat verhogen van de CsA-dosis weinig zinvol is om het afstotingsproces te stoppen indien er reeds beschadiging van de hartspiercellen heeft plaats gevonden.

In de hoofdstukken 7 en 8 wordt de mogelijke rol van γδT-cellen bij het afstotingsproces nader onderzocht. γδ cellen vormen een betrekkelijk kleine populatie T-cellen waarvan de rol in het immuunsysteem nog onduidelijk is. Uit hoofdstuk 3 blijkt dat deze γδ T-cellen regelmatig aangetroffen worden in de cellijnen gekweekt uit EMB. In hoofdstuk 7 wordt beschreven wanneer deze cellen in het transplantaat voorkomen, of ze reageren met de (allo) antigenen van de donor en of ze mogelijk een rol spelen bij het afstotingsproces. De γδ T- cellen werden voornamelijk gevonden in EMB die meer dan een jaar na transplantatie waren afgenomen. In deze periode treden nog zelden acute afstotingen op. Wel werden in deze periode bij 20 tot 30 percent van de patiënten verschijnselen van chronische afstoting gezien. Geen relatie kon echter worden gevonden tussen het voorkomen van γδ cellen in het transplantaat en het optreden van vaatafwijkingen, karakteristiek voor chronisch afstoting.
Bij de functionele analyse van 238 γδ T-cel clonen afkomstig van 7 patiënten en van 11 cellijnen, die volledig uit γδ T-cellen bestonden en afkomstig waren van evenveel EMB van 9 harttransplantaat ontvangers, bleek er niet één reactiviteit te vertonen tegen donor (allo) antigenen. De meeste vertoonden LAK activiteit, ze lyseerden de LAK gevoelige K562 en Daudi cellen.
De γδ T-cellen die werden gevonden, behoorden meestal tot de Vδ1 populatie. Deze subpopulatie vormt in de periferie van normale controles de kleinste γδ populatie. De accumulatie van deze Vδ1 cellen in het transplantaat, kan hebben plaatsgevonden onder invloed langdurige stimulatie door "Heat Shock" eiwitten. Deze "Heat Shock" eiwitten kunnen in het transplantaat tot expressie komen als gevolg van ontstekingsreacties.

In hoofdstuk 8 wordt de analyse beschreven van de TCR-γδ subpopulaties Vδ1 en Vδ2 in het perifere bloed van de harttransplantatie patiënten. Hierbij bleek dat in het bloed van de patiënten die γδ cellen in het transplantaat hadden, een andere subpopulatieverdeling voorkwam dan bij gezonde controles. Bij de controles is de Vδ2 populatie de grootste γδ populatie. Bij de harttransplantatie patiënten bleek de Vδ1 populatie het meest voor te komen. De totale hoeveelheid aan γδ cellen in het bloed bleek niet veranderd.
De omgekeerde ratio bleek niet te worden veroorzaakt door de cyclosporine die transplantatie patiënten kregen. Patiënten die vanwege een nefrotisch syndroom eenzelfde dosis CsA kregen bleken niet af te wijken van het normale patroon. Op basis van oppervlakte eiwitten bleken de Vδ1 cellen niet geactiveerd te zijn. IL-2receptor (CD25), HLA-DR en CD45RO expressie werden niet waarge-

nomen op de Vδ1 cellen.
Toch konden de Vδ1 cellen in het bloed van harttransplantatie patiënten gemakkelijker geëxpandeerd worden door kweek in IL-2 dan Vδ1 cellen van de controles. De oorzaak van deze omgekeerde ratio is nog steeds een open vraag. Enkele mogelijkheden worden bediscussieerd

In hoofdstuk 9 worden de resultaten beschreven van een "pilot" studie. Hierin werd onderzocht of het meten van frequenties pCTL met hoge aviditeit voor donorantigenen en IL-2 producerende helper-T-lymfocyten (HTL) bruikbaar is om in het perifere bloed acute afstoting op te sporen.
Deze studie liet zien dat de fractie pCTL met hoge aviditeit voor donorantigenen tijdens afstoting in het perifere bloed inderdaad verhoogd is ten opzichte van patiënten die niet afstoten.
Ook de HTL frequentie bleek verhoogd tijdens afstoting. De tijdsduur nodig om een uitslag te krijgen is voor beide assays nogal lang, respectievelijk 7-10 dagen en 4 dagen, om in de dagelijkse, klinische praktijk toepasbaar te zijn. Voor de HTL onderzoeken we momenteel een kortere methode.
Voor de testen, zoals beschreven in hoofdstuk 9 geldt dat ze waarschijnlijk meer bruikbaar zijn voor het opsporen van patiënten met een verlaagde reactiviteit tegen donorantigenen later na transplantatie

Hoofdstuk 10 bevat de samenvatting en algemene discussie waarbij geconcludeerd wordt dat geactiveerde alloreactive CTL met hoge aviditeit een belangrijke rol spelen bij het afstotingsproces. En dat de hoog avide cellen vlak voordat de weefselschade wordt gezien als geactiveerde precursors het transplantaat binnen komen, waar ze ter plaatsen uitrijpen en in aantal toenemen. De volledig uitgerijpte, functionele, hoog avide CTL komen niet in het bloed voor.
Verder wordt geconcludeerd dat de rol van Vδ1 positieve γδ T cellen bij de transplantatiepatiënten nog steeds onduidelijk is omdat ze geen allo-antigenen herkennen en niet duidelijk is waarom ze de grootste subpopulatie zijn gaan vormen. Een rol bij het laten uitdoven van de immuunrespons wordt gesuggereerd.

List of publications

This thesis was based on:

AJ Ouwehand, LMB Vaessen, CC Baan, NHPM Jutte, AHMH Balk, CE Essed, E Bos, FHJ Claas and W Weimar. *Alloreactive lymphoid infiltrates in human heart transplants. Loss of class II-derected cytotoxicity more than 3 months after transplantation.*
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Dankwoord

De incubatietijd is lang geweest, bij velen rees er twijfel of het boekje er ooit nog zou komen. Nu het er dan eindelijk is, gaat mijn eerste dank uit naar mijn hooggewaardeerde promotores Prof Dr Willem Weimar en Prof Dr Frans Claas, die bleven aandringen om het boekje te schrijven.
Beste Willem, behalve dat je me steeds gestimuleerd hebt dit proefschrift te schrijven, heb je mij ook laten zien dat er niet alleen met ratten en muizen basaal transplantatie-immunologisch onderzoek kan worden gedaan, ook patientgebonden onderzoek bleek leuke gegevens op dit terrein op te leveren. Je leerde me ook hoe je deze gegevens moet ordenen en opschrijven om ze in goede bladen gepubliceerd te krijgen en je waakte ervoor dat ik niet teveel immunologische bla-bla, zoals je immunologische theorie-vorming pleegt te noemen, in de artikelen stopte.
Beste Frans, jij stond aan de basis van het aviditeits werk en stimuleerde me de $\gamma\delta$ cellen die we de in de infiltraat kweken vonden verder uit te zoeken. Tevens waren jij en diverse van je medewerkers voor mij een permanente bron van informatie en goederen voor studies aan humane lymfocyten. Vooral in de beginfase van het onderzoek was deze hulp onontbeerlijk omdat mensencellen duidelijk anders bleken te zijn dan rattencellen.
De leden van mijn promotiecommissie dank ik voor hun snelle beoordeling van het manuscript, ondanks het feit dat aanvankelijk de discussie en samenvatting ontbrak.
De hulp van Carla Baan was onontbeerlijk bij het uitvoeren van de vele limiting dilution assays en cytotoxiciteits testen. Beste Carla, dank je voor je inzet en hulp. Binnenkort ga jij ook promoveren, veel succes daarmee. Ik hoop dat we samen nog jaren het lab van Willem kunnen voortstuwen in de vaart der volkeren.
Alice Ouwehand, dank je voor de gezellige en dynamische samenwerking bij het opstarten van het biopten project. Zonder de samenwerking met Gert Datema en Peter van den Elzen van de afdeling Immunohematologie en Bloedbank van het Academisch Ziekenhuis Leiden was een deel van dit proefschrift nooit tot stand gekomen. Gert en Peter, hartelijk dank voor jullie bijdragen aan dit werkstuk en voor jullie steun in de moeilijke (kleine) uurtjes bij congressen en symposia.
Frank Schipper, Chris Knoop, Rene Daane en Lisette Loone, dank voor jullie bijdrage aan een aantal studies beschreven in dit proefschrift.
Verder wil ik alle collega's van het Transplantatie lab van de afdeling Inwendige Geneeskune I bedanken voor hun plezierige, soms heftige, maar bovenal chaotisch gezellige en vruchtbare samenwerking gedurende reeds zeer lange tijd: Cees Hesse en Fred Bonthuis; langere tijd: Nicolette Jutte (dank voor het kritisch doorlezen van mijn manuscripten), Carla Baan, René Daane, Chris Knoop; middellange tijd: Lisette Loonen, Nicolle van Besouw, Nancy van Emmerik, Francisca Hoekstra, Wendy Mol (nog dank voor de blauwe schenen); kortere tijd: Cecile Holweg, Ronella de Kuiper, Barbara van de Mast, Paula van Miert en zeer korte tijd: Frans Oei en Mary Schoenmaekers.
Zonder de thoraxchirurgen en klinische harttransplantie afdeling zou dit proefschrift niet mogelijk zijn geweest. Van de transplantatiecardiologen wil ik Dr Aggie Balk bedanken voor haar interesse in en medewerking aan het werk beschreven in dit proefschrift en het kritisch doorworstelen van mijn manuscripten. Van de thoraxchirurgen wil ik met name Bas Mochtar en Prof E Bos bedanken voor hun belangstelling in ons onderzoek en het beschikbaar stellen van financiën en menskracht in de vorm van AIO's waardoor we het bioptenwerk konden doen.
De internisten van de niergroep Teun van Gelder, Isa van Riemsdijk, Bob Zietse en René van Dorpel dank voor de sfeer op de labborrels, bij Dizzy en de polibesprekingen van de laatste jaren. De regelaar van de nierengroep, Willij Zuidema bedankt ik voor de meest uiteenlopende zaken.
Het regelen van financiën voor congressen, labweekeinden, after refereeravond diners; de sfeer bij diverse gelegenheden en het vinden van gaatjes in de agenda van de Prof.

Tot slot wil ik mijn levensmaatje Rieja van Aart bedanken voor de gouden inval waardoor dit boekje eindelijk klaar kwam.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 10 juli 1949 te Blerick in de gemeente Venlo. Na het behalen van het MULO A en B diploma in 1967 en een jaartje weg- en waterbouw op de HTS te Tilburg starte hij 1968 met de studie voor klinisch-chemisch analist aan de Brabantse Medische Analisten School te Breda. Hier werd in juni 1971 het analistendiploma HBO-A behaald. In september 1973 starte hij vervolgens aan het Dr Struycken Insituut te Breda, in deeltijd, de opleiding HBO-B Medische Biologie. Hier studeerde hij in januari 1976 af op het onderwerp "Het effect van sulfinpyrazone op de intensiteit van de vaatbeschadigingen en de overleving van een hart-allo-transplantaat bij de rat." Dit onderwerp werd bewerkt in het laboratorium van de afdeling Inwendige Geneeskunde I, van de Erasmus Universiteit/Academisch ziekenhuis Rotterdam-Dijkzigt (AZR) olv Prof Dr. L.D.F. Lameyer.

In dit laboratorium werkte hij sinds 6 december 1971 als analist, in dienst van de Erasmus Universiteit.

In september 1975 starte hij aan de Rijks Universiteit Utrecht (RUU) de deeltijd opleiding MO-biologie gevolgd door de docteraalfase biologie met als hoofdvakken: Algemene dierkunde - Prof. Dr. J.C. van de Kamer (onderwerp: "Indol mechanisme in de epifysis cerebri van de rat" olv Dr. M.G.M. Balemans) en Immunologie/Immunopathologie - Prof. Dr. R.E. Ballieux [Academisch Ziekenhuis Utrecht(AZU)/RUU] (onderwerpen: "Vroege B cel differentiatie in de rat" olv Dr. J. Rozing [Inwendige Geneeskunde I,AZR/EUR]; en "T cel differentiatie in de thymusloze Nude rat" olv Dr. J. Rozing, Dr. H-J. Schuurman [Immunopathologie, AZU] en Dr. J.G. Vos [Pathologie,RIVM] en het bijvak Systematische Plantkunde - Prof. Dr. A.L. Stoffers (onderwerp: "Inventarisatie van epilitische lichenen op betonnen palen" olv Dr. H. Sipman [RUU]).

Deze docteraalonderwerpen werden tussen 1981 en medio 1985 afgerond, de bijbehorende tentames werden niet afgelegd, de studie werd eind 1985 gestaakt.

Gedurende deze tijd was hij in dienst van de Erasmus Universiteit te Rotterdam en werkzaam bij de afdeling Inwendige Geneeskune I, eerst als analist A (tot 1976), vervolgens als laboratorium assistent (tot januari 1982), laboratorium assistent A (tot januari 1983) en tot heden als hoofdanalist. Achtereenvolgend olv Prof. Dr. L.D.F. Lameyer, Dr. J. Rozing en Prof. Dr. W. Weimar.

In 1990 werd olv Prof. Dr. W. Weimar en Prof. Dr. F.H.J. Claas, (Immunohematologie en Bloedbank, Academisch Ziekenhuis Leiden) gestart met het in dit proefschrift beschreven onderzoek.