The involvement of donor specific CTL in cardiac transplant rejection: relevance of avidity

De rol van donorspecifieke CTL bij afstoting van het getransplanteerde hart: het belang van aviditeit

Nancy van Emmerik

promotiecommissie

promotor:	Prof. Dr. W. Weimar
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	Dr. R. Marquet

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Nancy Elisabeth Maria van Emmerik

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the department of Internal Medicine I,
Erasmus University Rotterdam,
Dr Molewaterplein 50,
3015 GD Rotterdam,
The Netherlands.

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Contents

Abbreviations

Ag	antigen
ß2m	ß2-microglobin
B-LCL	B-lymphoblastoid cell line
CDR	complementarity-determining region
CML	cell mediated lympholysis
CTL	cytotoxic T lymphocyte
DC	dendritic cells
EMB	endomyocardial biopsy
GIL	graft infiltrating lymphocytes
HLA	human leucocyte antigens
HTL	helper T lymphocyte
ITAM	immunoreceptor tyrosine-based activation motif
LDA	limiting dilution analysis
mAb	monoclonal antibody
MHC	major histocompatibility complex
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
TCR	T cell receptor

Chapter 1

1.1. General introduction

When a micro-organism penetrates the body, defensive mechanisms of the non-specific innate immune system come into play, such as soluble chemical factors (bactericidal lysozyme, acute phase proteins, interferons), extracellular killing (NK cells, eosinophils), and phagocytosis (macrophages, polymorphonuclear neutrophils). This innate immune system is supported by the specific acquired immune system which comprises of B- and T-lymphocytes. These lymphocytes are educated to distinguish between self (host) and non-self (foreign) molecules by means of specific receptors on their cell surface and are further endowed with specific memory. B lymphocytes, called so because they differentiate in the bone marrow, participate in the immune response by producing antibodies that fix to antigen by their specific recognition site. These antibodies can mediate different functions, including opsonization, complement activation, antibody-dependent cell mediated cytolysis and neutrophil, basophil, or eosinophil activation. T cells, designated thus because they differentiate in the thymus, control the activation of cells of the innate and acquired immune system by producing lymphokines and play a role in killing aberrant cells such as virally infected cells and some tumour cells.

Although we generally benefit from the actions of the immune system, there is an other side of the coin. When a diseased organ is replaced by a healthy transplant of another individual (not being the recipients identical twin-brother or sister), the cells of this transplant are regarded as non-self by the immune system of the recipient. As a result, the recipient's immune system will bring all its power into play to get rid of this "intruder".

To prevent allograft rejection, the immunity of the transplant recipient is reduced with immunosuppressive drugs which must be taken throughout the patient's life. The cumulative

effects of such immunosuppression, however, include opportunistic infections, cancers, and drug specific cytotoxicity. Therefore, a long term goal of transplant immunology is the development of immunotherapies that target only those cells that are involved in the rejection process. To achieve this goal, an extensive knowledge of the immune response towards the allograft is essential.

Data obtained over the past three decades conclusively demonstrated that T-lymphocytes are crucial for allograft rejection. The critical role of T cells in allograft rejection was first established by the failure of SCID, neonatally thymectomized and congenitally athymic nude rodents to effect rejection (Miller, 1962; Corley and Kindred, 1977; Shelton et al, 1992). Reconstitution experiments showed that T lymphocytes adoptively transferred to above rodents or lethally irradiated hosts were sufficient to restore rejection (Dorsch and Roser, 1974; Hall et al, 1978A; Shelton et al, 1992). Furthermore, composite grafts in which allogeneic cells were mixed with syngeneic cells before they were engrafted into host animals showed that only allogeneic cells were destroyed despite the close proximity of syngeneic target cells (reviewed by Rosenberg, 1993). Such exquisite antigen-specificity ruled out the involvement of non-specific effector cells such as macrophages, NK cells, or eosinophils and implicated that rejection involved lymphocytes. The observation that antibodies were not required to destroy the allograft in these studies excluded the requirement for B-cells leaving only T cells as potential effector cells. Also supporting the involvement of T lymphocytes is the fact that accelerated rejection occurred when a second graft was transplanted into recipients that had already rejected a primary graft of the same type. Such accelerated rejection points towards the establishment of memory which is provided by lymphocytes (Hall et al, 1977,1978B).

Peripheral T-lymphocytes can roughly be divided into two subclasses: helper T lymphocytes (HTL) that, upon recognition of antigen-bearing cells, start producing lymphokines which induce the proliferation and differentiation of various cells of the innate and acquired immune system; and cytotoxic T lymphocytes (CTL) that, upon recognition of antigen-bearing cells, bind tightly to these cells. Such interaction triggers the secretion of granules containing soluble lytic proteins (perforin and granzymes) leading to the lysis and nuclear degradation of the attached target cell.

Although the relevance of both T cell populations has been examined extensively, obtained data have been far from conclusive. What has remained an open and much debated question is the extent to which graft destruction can be ascribed to CTL, whose direct cytotoxic function appears most suitable, and to what extent this function is due to HTL whose production of lymphokines can activate and recruit CTL but also additional effector cells such as macrophages and NK cells.

1.2. Cytotoxic T lymphocytes

1.2.1. Involvement in transplant rejection.

The dispute on the requirement for CTL in allograft rejection can be ascribed to the large variety in experimental settings such as the type of allograft transplanted (Hao et al, 1987; Krieger et al, 1996) and the use of primed versus naive T cell subsets (reviewed by Hall, 1991) but also to the incorrect assumption that HTL express CD4 molecules whereas CTL express CD8 molecules. With respect to the latter point, rejection of grafts that only differ at a MHC class II locus with the recipient (Simpson, 1993) and transplant rejection by CD8-depleted animals or by $CD4^+$T cell transfused nude/SCID animals have generally been regarded as evidence against an important role of CTL in transplant rejection. However, $CD4^+$T cells themselves can be cytolytic (chapter 3; Dallman and Mason, 1983; Miceli et al, 1988; Erb et al, 1990), although the extent of their activity seems to be somewhat lower than that of $CD8^+$T cells (chapter 6). Moreover, in most studies, animals were depleted for $CD8^+$T cells by treatment with specific mAb. Such treatment may not completely have eliminated $CD8^+$T cells (reviewed by Steinmuller, 1985 and Hall, 1991). As has recently been shown, a unique subpopulation of $CD8^+$T cells remained in hosts treated with CD8-depleting mAb and these cells rejected MHC class I disparate skin grafts (Rosenberg et al,1991). Consequently, conclusions drawn from such studies must be reconsidered in the light of the discovery that cell surface markers did not then and do not now define functional distinct T cell populations and that the immunoincompetent hosts in these studies may have harboured CTL. Therefore, in the transplant models discussed below, the function of T cells has been taken into account to analyze the requirement for CTL.

The contention that CTL are proximal mediators of graft rejection is generally based on the exquisite antigen-specificity of the rejection response (Sutton et al, 1989; Martz, 1993; Rosenberg, 1993). For example, in a mixed population of target cells in which some cells express MHC molecules allogeneic to the CTL and others express MHC molecules syngeneic to the CTL, only those expressing allogeneic MHC are destroyed. Additionally, CTL clones obtained from rejecting grafts specifically lysed donor target cells in vitro and mediated graft destruction in vivo when injected into appropriate hosts (Snider et al, 1986). More substantial evidence comes from studies in which the number of graft infiltrating CTL was estimated and compared with the rejection status of the allograft and from studies in which the expression of genes restricted principally to CTL were assessed. In this regard, graft destruction coincided with an accumulation of donor specific CTL in the allograft (Suitters et al, 1990; Bishop et al, 1992; Chapter 2). Likewise, mRNA for perforin and granzymes, molecules that may be critical to the "lethal hit" delivered by CTL, appeared to correlate with rejection of allogeneic grafts (Griffiths et al, 1991; Chen et al, 1993; Lipman et al, 1992,1994). Also the observation that CTL are the preponderant lymphocytes in rejecting allografts supports the involvement of this subset in transplant rejection. For example, Moreau et al. (1985) reported

that 13 of 16 T cell clones established from rejected human kidney allografts were cytotoxic to donor cells and Mayer et al. (1985) reported similar findings.
In contrast with the previous data, Bishop and co-workers showed that CTL are not essential for cardiac transplant rejection (Bishop et al, 1992; Bishop, 1993). According to their results the rejection response is characterized by 3 events; I. expansion of donor specific CTL precursors (pCTL) in the spleen and mesenteric lymph nodes; II. vascular alterations in the cardiac allograft; III. sequential infiltration of the graft by primed donor specific HTL (cHTL) followed by large numbers of primed donor specific CTL (cCTL). This large influx of cCTL was associated with loss of graft function. Treatment of cardiac transplant recipients with CD4 depleting mAb abrogated all 3 events and resulted in graft acceptance in the majority of mice. This observation supports a pivotal role of $CD4^+$T cells in the initiation of transplant rejection and in the expansion and maturation of donor specific CTL. Unlike treatment with CD4 depleting mAb, depletion of cardiac transplant recipients for $CD8^+$T cells had no effect on graft survival although it totally abolished CTL reactivity. In such modified mice, eosinophils seemed responsible for allograft rejection (Chan et al, 1995). Also the finding that ß2M-knockout mice which lack $CD8^+$T cells and can not generate CTL, are competent to lyse allogeneic skin grafts supports the concept that graft rejection does not fully depend on CTL.
More insight into the relevance of CTL was obtained using rodents that lack T cells. Unmodified SCID mice that are genetically deficient of both T- and B-lymphocytes accept allogeneic skin grafts indefinitely but reject the allograft after transfer of either $CD4^+$ or $CD8^+$naive T cells. This observation suggests that skin transplants can be rejected by both $CD4^+$ and $CD8^+$T lymphocytes. Subsequent limiting dilution analysis (LDA), however, revealed that only recipients of $CD8^+$T lymphocytes had detectable numbers of donor specific CTL (Shelton et al, 1992). In the light of the previous study, these data suggest that allograft destruction after $CD4^+$T lymphocyte transfer may involve the activation of alternative effector cells with cytolytic potential. Additionally, as the transfused T lymphocytes were naive T cells, the data show that $CD8^+$T cells can differentiate into functional CTL without the requirement for any helper/inducer activity from $CD4^+$T cells. Such CTL which produce IL-2 themselves, have also been reported by others (Sprent and Schaefer, 1986; Cai and Sprent, 1993; Heath et al, 1993). A comparable model was described by vanBuskirk et al. (1996). $CD4^+$T cells can be modulated by removal or addition of extracellular IL-4 to differentiate into cells with direct cytolytic capacity ($CD4^+$CTL) or non-cytolytic IL-4 producing cells, respectively. Using this approach, the authors showed that adoptive transfer of either of these $CD4^+$T cell subsets into SCID mice led to cardiac transplant rejection.
The occurrence of transplant rejection in animals depleted for CTL is often regarded as evidence against the involvement of CTL in transplant rejection. Above studies demonstrated that other cells besides CTL can function as terminal effector cells. However, as the immune response can readily be rerouted when pathways for important biological functions are blocked, such alternative effector cells may only become involved when the immune system

is aberrant. In line with this assumption, eosinophil infiltration appeared not to be a prominent feature in the unmodified murine cardiac transplant model reported by Bishop (1993). The existence of alternative routes for the effector and induction phase of transplant rejection is supported by studies on knockout mice. $CD4^-$ and $CD8^-$knockout mice (Simpson, 1993) as well as $CD4^-CD8^-$double knockout mice (Schilham, 1993) are all able to reject allogeneic skin grafts. In the latter mice, rejection was ascribed to $CD4^-CD8^-TCR\alpha\beta^+$ T cells, a T cell population that is not found in significant numbers in conventional mice. Another example is the rejection of solely MHC class I disparate grafts by CD8-depleted rodents. Such rejection is ascribed to B cells whose production of class I specific alloantibodies seems to be induced by $CD4^+$HTL that recognized allo-MHC class I presented by self-APC (Gracie et al, 1990; Clements et al, 1996).
Another argument that is often held against the involvement of CTL in transplant rejection, is the fact that donor specific CTL can be recovered from well functioning allografts. In experiments where rat renal allografts are protected by donor specific blood transfusions it has proved possible to recover donor specific $CD8^+$CTL from the allograft (Dallman et al, 1987; Armstrong et al, 1987; Ruiz et al, 1988). Likewise, studies on heart transplant patients demonstrated that donor specific CTL can be recovered from rejecting grafts but also from stable grafts (Ouwehand et al, 1993; Vaessen et al, 1994, Chapter 2,3). Presuming that CTL are involved in transplant rejection, the question now arises why the presence of donor specific CTL not automatically results in graft rejection. One explanation might be that CTL present within stable allografts are prevented from mediating their cytotoxic function by cytokines or suppressor cells. Alternatively, donor specific CTL present within the allograft during rejection and during stable engraftment may be quantitatively or qualitatively distinct. Taken together, several pathways can lead to transplant rejection. The specificity of the rejection response as observed in unmodified animals, however, implies the involvement of CTL. These CTL may depend on HTL for their activation (as is the case in the cardiac transplant model) or may induce their own activation (as is the case in the skin transplant model).

1.2.2. Recognition of target cells.

The major histocompatibility complex. T cells recognize foreign peptides only when these peptides are presented to them by molecules of the major histocompatibility complex (MHC) of which the human equivalent is HLA (human leucocyte antigens). The requirement for such co-recognition is called MHC restricted antigen recognition. MHC molecules can be divided in MHC class I and MHC class II molecules. MHC class I molecules (figure 1) are generally expressed on the surface of all nucleated cells and are composed of a heavy peptide chain of 44 kD which is non-covalently associated with a smaller 12 kD peptide called ß2-microglobin (ß2m). The class I heavy chain contains an extracellular, transmembrane, and cytoplasmic region. The extracellular region is divided into 3 domains named $\alpha 1$, $\alpha 2$, and $\alpha 3$. The $\alpha 3$

and ß2m domains are conserved while the α1 and α2 domains are highly variable and consist each of four parallel ß-strands and an α-helix. When the α1 and α2 domains interact, a single eight-stranded ß-sheet is formed which is covered by the two α-helices. The cleft between these two α-helices forms the peptide binding side for antigen. Almost all polymorphic residues of the MHC class I molecules are positioned along the cleft and are thought to determine the peptide binding specificity. Other functional residues are located outside the cleft or point away from the peptide binding side and are thought to be involved in the interaction of the MHC molecule with the TCR. Peptides bound in the cleft of MHC class I molecules are 8-9 amino acids long.

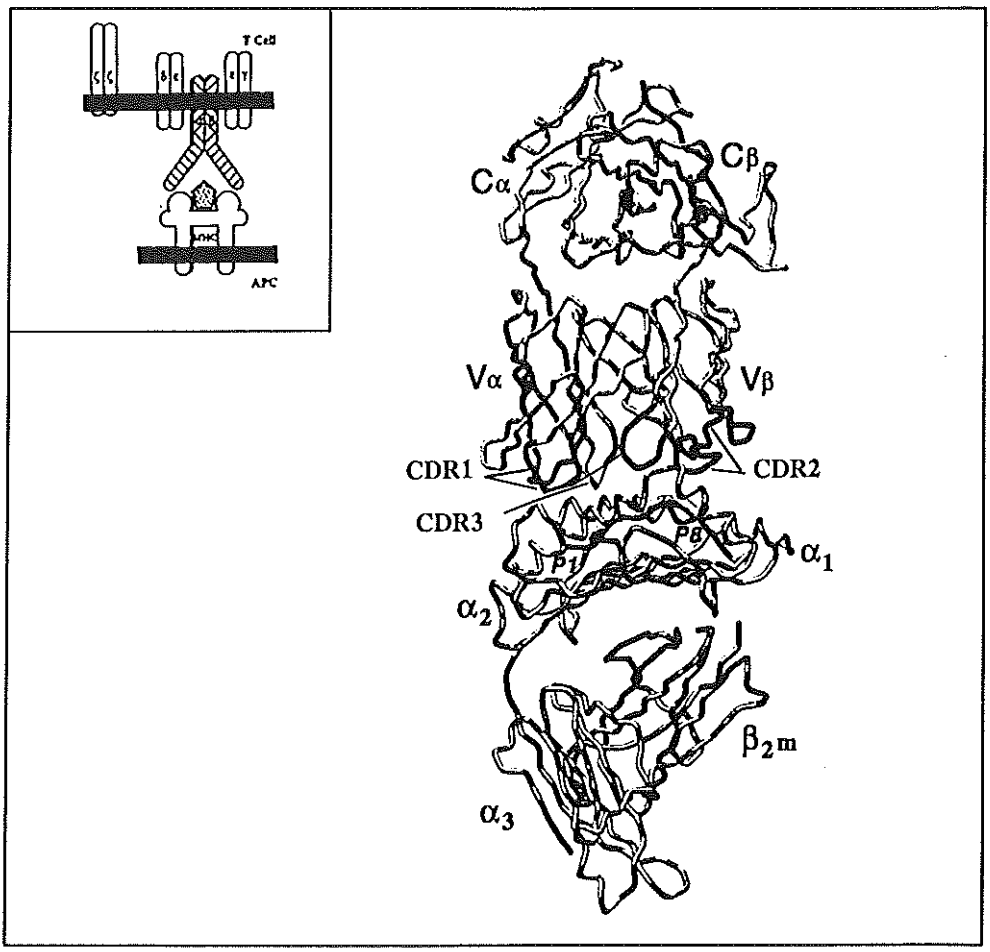

Figure 1. Three-dimensional model for the interaction between TCR and MHC/peptide (adapted from Garcia et al. 1996). The CDR1 and CDR2 regions of the TCR interact with the α-helices of the MHC molecule while its CDR3 regions interact with the peptide (P1 - P8) in the groove of the MHC molecule.

MHC class II molecules have a more restricted expression than MHC class I molecules. These molecules are expressed on B cells, cells of the macrophage-monocyte lineage, endothelial cells, and activated T cells. MHC class II consist of an α and a ß polypeptide chain of 33 and 28 kD, respectively. Each polypeptide chain has two extracellular domains (α1 and α2; ß1 and ß2), as also a transmembrane and a cytoplasmic region. The most polymorphic domains are the α1 and ß1 domains whereas the α2 and ß2 domains are more conserved. Recently the crystal structure of MHC class II has been solved and it shows an almost identical core structure as MHC class I, the major difference being the longer length of the bound peptide (13-26 amino acids). In case of MHC class II, the sides of the peptide binding groove are formed by two α-helical structures, one of the α1-chain and one of the ß1-chain, while the floor is formed by eight anti-parallel ß strands.
After synthesis in the cell, MHC class I and class II molecules follow different intracellular routes and bind peptide at different moments during their route to the cell membrane. This results in a dichotomy of peptide presentation to T cells. MHC class I molecules present peptides predominantly of intracellular origin (endogenous peptides) while MHC class II molecules present peptide predominantly from extracellular origin (exogenous peptides). There is also a dichotomy in the T cells that react with antigen presented by MHC class I or class II molecules. As CD8 molecules bind to α3 domains whereas CD4 molecules bind to ß2 domains, $CD8^+$T cells recognize (endogenous) Antigen presented by MHC class I molecules while $CD4^+$T cells recognize (exogenous) antigen presented by MHC class II molecules.

The T cell receptor. Co-recognition of MHC plus antigen by T lymphocytes is accomplished by their T cell receptor (TCR). This TCR is composed of a polymorphic part involved in antigen recognition, and a non-polymorphic part (CD3) involved in signal transduction. The CD3 molecule consists of two non-covalently associated heterodimers of the ϵ- and δ-chains or the ϵ- and γ-chains and a single disulphide-linked dimer containing either two ζ-chains or one ζ-chain and one η-chain. About 90% of the CD3 molecules examined to date incorporate the $\zeta\zeta$-homodimer. Upon TCR ligation, specific motifs, named ITAMs (immunoreceptor tyrosine-based activation motifs), that are expressed in the cytoplasmic domains of the various CD3-chains become phosphorylated. When phosphorylated, these motifs serve as binding templates for several kinases involved in signalling pathways (see section on signalling).
The polymorphic part of the TCR consists of two disulphide-linked chains, either an α- and a ß-chain or a γ- and a δ-chain which are expressed in a mutually exclusive way on 95% and 2-5% of the T lymphocytes, respectively. The following description will focus on the TCRαß. The αß-chains are clonally variable and are encoded by families of variable (V), diversity (D), joining (J), and constant (C) gene segments which assemble by gene rearrangements to form an antigen specific TCR chain (Davis and Bjorkman, 1988). The formation of a functional α-chain requires the rearrangement of a V-region gene segment to

a J-region gene segment, whereas a functional ß-chain is formed by rearrangements of V, D, and J-region gene segments. The rearranged V-J and V-D-J regions are then attached to their respective C regions to assemble the mature α and ß-chains. Gene rearrangement allows many combinations of V(D)J with C. This results in the generation of TCR diversity. The number of possible combinations is further enhanced by random addition of nucleotides (also called N-region addition) at the V-(D)-J junctions.
The recently determined crystal structure of the TCR (Garcia et al, 1996; Bentley, 1996) supports the view that the secondary and tertiary structure of the TCR resembles that of Fab fragments (Chothia et al, 1988). Four regions of hypervariable amino acid sequence are found on both the α- and the ß-chain, three of which are analogous to the antibody complementarity-determining regions (CDR) which serve as the primary contact points between antibody and ligand. Two CDR regions on both the α- and the ß-chain (CDR1α and CDR2α, CDR1ß and CDR2ß) are encoded by the V genes and interact with the α-helices of the MHC molecule. The third CDR region is encoded by the V-J junction in the α-chain (CDR3α) and the V-D-J junction in the ß-chain (CDR3ß) and interacts with the peptide in the groove of the MHC molecule. The impact of these CDR regions in TCR-MHC/peptide interactions has been supported by a number of studies involving mutagenesis of the TCR (Engel and Hedrick, 1988; Nalefski et al, 1992). A three-dimensional model for the interaction between TCR and MHC/peptide is shown in figure 1. This model provides a solution to the puzzle of how the TCR simultaneously recognizes both peptide and MHC determinants.

Direct and indirect allorecognition. MHC molecules are highly polymorph and are expressed on all nucleated cells throughout the body. In transplant rejection, the immune response is mainly directed against the MHC molecules on a transplant as these generally differ from the MHC molecules expressed on cells of the recipient. Normally, T cells are educated in the thymus to recognise nominal antigens as processed peptides presented by self-MHC molecules. The question now arises how T cells taught to recognise foreign peptides in a self-MHC restricted way, can recognise allo-MHC expressed on the transplant.
According to the available data, two pathways of allorecognition exist. In the so-called "indirect pathway", allo-MHC or other antigens shed from the allograft are taken up by recipient APC, processed, and presented as allo-peptides to T cells in the context of self-MHC molecules (reviewed by Bradley et al, 1992; Shoskes and Wood, 1994; Sayegh et al, 1994; Bradley, 1996). This pathway resembles the physiological induction of an immune response to nominal antigen.
In the "direct pathway", T cells recognize intact allo-MHC molecules on the surface of donor cells (reviewed by Lechler et al, 1990; Sayegh et al, 1994). $CD8^+$T cell recognize allo-MHC class I while $CD4^+$T cells recognize allo-MHC class II. The frequency of T cells engaged in this form of allorecognition is about 100-fold higher than that of T cells participating in indirect recognition. Depending on the similarities and differences between responder and

donor MHC types, two models have been put forward for the direct recognition pathway of alloantigen. In case the TCR binding region (i.e. the upper face of the α-helices) of allo-MHC molecules resembles the TCR binding region of self-MHC molecules, allo-MHC molecules are regarded as self-MHC molecules by the TCR on T cells. The alloresponse in such cases would depend on the peptide carried by the allo-MHC molecule. These peptides may be foreign (donor) peptides which have not previously been encountered by the responder T cells or, alternatively, may be non-immunogenic self-peptides. In both cases, binding of these peptides to allo-MHC may induce structural changes in the MHC molecule. Due to such conformational changes, allo-MHC molecules may mimic self-MHC molecules that have been altered due to their interaction with alloantigen. In support of this hypothesis are experimental data showing that many antigen-specific T cell clones also display alloreactivity.

When the TCR binding region of allo-MHC molecules differs from that of self-MHC molecules, the actual ligand for the alloreactive T cells may be the MHC molecule itself. In such cases the peptide bound to allo-MHC may not be relevant. In support of this concept, alloreactive CTL that recognize empty MHC molecules have been reported. To what extent the TCR recognizes the endogenous peptide bound in the groove of allo-MHC and to what extent it recognizes epitopes of the allogeneic MHC molecule adjacent to the peptide binding groove is still unclear.

Although the existence of indirect and direct pathways of antigen recognition has been demonstrated in various experiments, their relative contribution to transplant rejection is less well defined. The general consensus is that $CD4^+$HTL orchestrate and amplify allograft induced immune responses. Early after transplantation, $CD4^+$HTL become activated by the direct recognition of donor dendritic cells (DC) present within the graft. In time after transplantation, DC migrate out of the graft and are replaced by APC of recipient origin. In such circumstances, activation of $CD4^+$HTL would involve indirect recognition of alloantigen. As rejection is most frequently experienced early after transplantation, graft rejection is usually considered as the result of direct recognition of allogeneic MHC molecules by effector T cells. Recent data, however, showed that this view may be an oversimplification and that the indirect pathway of allorecognition plays a more central role than expected. $CD4^+$HTL responding to indirectly presented allogeneic class I molecules were found to initiate graft rejection independent of $CD8^+$T cells by eliciting an alloantibody response (Gracie et al, 1990; Clements et al, 1996). Sherwood and colleques (1986) reported that injection of APC obtained from the spleens of mice immunized with allogeneic cells sensitized secondary recipients to reject skin grafts in an accelerated and alloantigen-specific fashion. This observation suggests that indirect allorecognition may enhance the rejection response. Alternatively, the indirect pathway might be relevant for the induction of transplant unresponsiveness as preoperative blood transfusions in which the blood donor and recipient shared at least one MHC class II locus were found to improve clinical renal graft survival (Lagaaij et al, 1989).

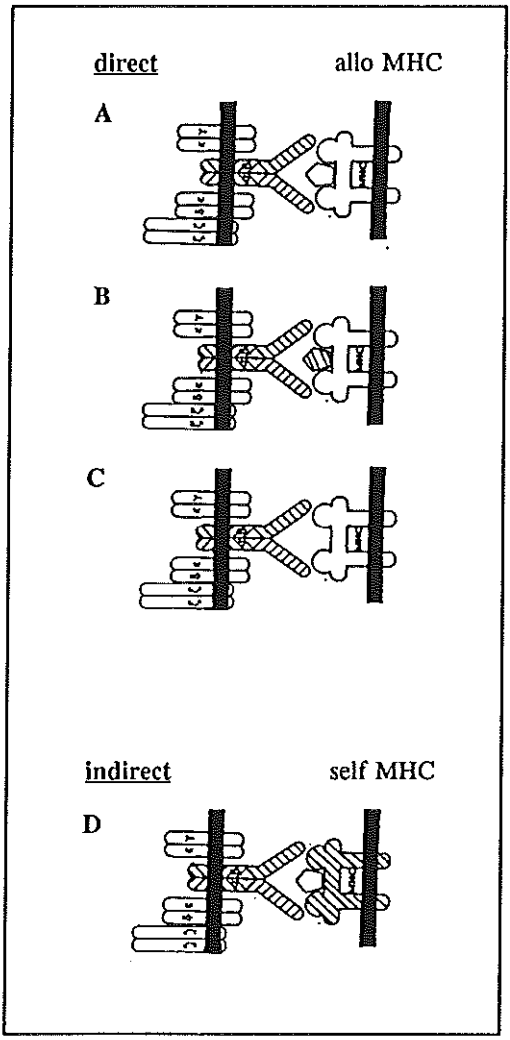

Figure 2. Direct and indirect allorecognition. The TCR on a T lymphocyte either recognizes an allopeptide (A) or a self peptide (B) in the context of an allo-MHC molecule on a donor cell or an 'empty' allo-MHC molecule (C). Alternatively, the TCR recognizes allopeptide processed and presented by MHC molecules of recipient APC (D).

1.2.3. Assessory molecules involved in CTL activation.

When effector CTL recognize peptide antigen bound to MHC molecules on the surface of an allogeneic target cell, they bind tightly to form a cell-cell conjugate, their cytolytic machinery becomes activated, and the target cell is killed. In addition to the antigen specific TCR, numerous other receptors on the CTL and ligands on the target cell have been implicated in CTL adhesion and activation. Such receptor/ligand pairs include CD8/MHC class I or CD4/MHC class II, LFA-1/ICAM-(1, 2, 3), VLA-5/fibronectin, and CD2/LFA-3. To study the relative contribution of each of these receptors, the reductionist method has been employed. For this approach, the antigen-bearing cell is replaced by an artificial cell

membrane onto which the ligand(s) of interest is (are) incorporated at a particular density. The ability of this construct to stimulate CTL adhesion and activation is then measured.
Using this approach, Kane et al, (1988, 1989) demonstrated that incorporation of purified antigen-MHC class I complexes at a density seen for intact cells resulted in degranulation of allogeneic CTL lines. As the release of perforin and granzymes by degranulation is an important mechanism by which $CD8^+$CTL kill their target cells (Isaaz et al, 1995; Griffiths, 1995), this observation suggests that antigen complexed to allogeneic MHC class I molecules is sufficient to trigger the effector function of CTL. The MHC class I molecule is a ligand not only for TCR but also for CD8. Accordingly, both these molecules might be relevant for CTL adhesion and activation.
To unravel the contribution of TCR and CD8 separately, an extensive study was started by the same group (reviewed by Mescher, 1995). To analyse the contribution of CD8 alone, the response of $CD8^+$CTL lines towards immobilized non-antigen MHC class I molecules (i.e. molecules for which the TCR on these CTL lines is not specific) was analyzed. To examine the contribution of TCR alone, CTL were incubated with fluid-phase anti-TCR mAb. Both conditions did not result in CTL degranulation. Incubation of CTL with non-antigen MHC class I immobilized onto artificial cell membranes did not even result in CTL adhesion. However, when CTL were treated with anti-TCR mAb and subsequently incubated with immobilized MHC class I, both CTL adhesion and degranulation occurred. To explain these data it was proposed that TCR occupation, in itself, is insufficient to trigger CTL degranulation but provides a signal which activates adhesion of CD8 to MHC class I. Further data revealed that, in case of sufficient CD8/MHC class I interactions, CD8 then generates a co-stimulatory signal resulting in degranulation (Kane and Mescher, 1993). The relevance of CD8 in this testsystem was supported by the observation that antibodies against CD8 blocked CTL degranulation.
Although above data suggest a critical role of CD8 in CTL activation, we should bear in mind that these data were obtained using artificial structures as target. It has repeatedly been demonstrated that some CTL can lyse whole target cells in the presence of CD8-blocking mAb suggesting that these CTL do not require CD8 to display their function. When such CD8-independent CTL were tested for their response against immobilized MHC class I (where only TCR and CD8 can contribute) they became very sensitive to CD8 blocking. This observation indicates that, under physiological circumstances, other receptor/ligand pairs can compensate for CD8/MHC class I interactions (Kane et al, 1989). Accordingly, CD8 might not be the only assessory molecule that cooperates with the TCR to initiate functional CTL responses.
Incubation of cloned CTL with fluid-phase anti-TCR mAb and purified fibronectin immobilized onto artificial cell membranes resulted in CTL adhesion and degranulation provided a second antibody to further cross-link the TCR was added (O'Rourke et al, 1990). This observation implies that also the VLA-5/fibronectin interaction might deliver a signal that is co-stimulatory for degranulation. However, as stimulation by fluid-phase anti-TCR

mAb plus second antibody alone also resulted in a low but significant degranulation of CTL, the contribution of the VLA-5/fibronectin interaction might be restricted to situations in which the TCR alone has already partly activated the signalling cascade.
Another assessory molecule that has been considered to play an important role in T lymphocyte activation is CD28 (Linsley and Ledbetter, 1993; June et al, 1994; Bluestone, 1995). Upon binding of B7-1 or B7-2 molecules on APC, CD28 molecules are thought to transduce an unique signal into the T cell which stimulates its production of growth-promoting cytokines such as IL-2. To test the involvement of CD28 on CTL activation, RMA-S T lymphoma cells that lack B7-1 and B7-2 were pulsed with antigen and analyzed for their ability to act as APC for $CD8^+$2C T cells (Cai and Sprent, 1996). The response was strongly immunogenic and led to extensive specific killing of antigen-pulsed RMA-S. This experiment indicated that the cytolytic response of 2C cells to antigen-pulsed RMA-S cells does not require CD28-B7 interactions. Additional experiments, demonstrated that the proliferative response of $CD8^+$2C cells heavily dependent upon CD28-B7 interactions but that the requirement for these interactions could be overcome by increasing the antigen density on target cells. Likewise, Gervois et al. (1996) demonstrated that melanoma cells that lack B7 could efficiently be lysed by CTL clones but that an increase in the antigen density on B7$^-$melanoma cells was required to activate cytokine production and proliferation by these CTL clones. In another study, Bachman et al. (1996) investigated the requirement for CD28 co-stimulation using TCR transgenic (TCR-Tg) cells from CD28 deficient mice. This study demonstrated that the strength of TCR-MHC/peptide interactions determined the requirements for CD28-mediated co-stimulatory signals both in vitro and in vivo. T cells required CD28 to proliferate and to produce IL-2 upon weak TCR-MHC/peptide interactions but not upon strong TCR-MHC/peptide interactions. In contrast, the cytotoxic capacity of these T cells did not dependent on CD28 (Mak, 1994; Bachman et al, 1996). According to these experiments, the CD28-B7 interaction functions to increase the strength with which a CTL interacts with its target cell. Such increase is not required to induce the cytotoxic machinery of CTL but seems to be essential for the induction of proliferation and cytokine release by CTL.

1.2.4. Signalling events involved in CTL activation.

Proliferation, cytokine production, and degranulation are all induced in a TCR specific manner. This has led to the assumption that similar signalling events may initiate the occurrence of these effector responses. Although a total understanding of the signalling events leading to T cell activation and the precise temporal order in which they occur are still far from complete, it is generally accepted that TCR engagement is successively coupled to protein tyrosine kinase (PTK) and phospholipase C (PLC) activation. According to one model (reviewed by Weiss, 1993; Ravichandran et al, Thome et al, 1996), an encounter of the TCR with MHC/antigen activates PTK, most likely Lck, which then mediates tyrosine phosphorylation of ITAMs expressed in the cytoplasmic domain of the CD3-chains. This, on

its turn, causes the recruitment of ZAP-70 and Syk tyrosine kinase to this side as their SH2 domains bind to the phosphorylated ITAMs of CD3ζ. Subsequent phosphorylation of ZAP-70 allows its association with the SH2 domain of Lck that is noncovalently associated with the cytoplasmic domain of CD4 and CD8 (Veillette et al, 1988). Interaction of ZAP-70 with CD8/CD4 associated Lck then results in the recruitment of CD8 or CD4 to the TCR (Thome et al, 1995, 1996) allowing both the TCR and its co-receptor to interact with the same antigen-MHC complex to enhance the stability of the interaction. Lck, ZAP-70, Syk, or additional PTK that have become activated due to their interaction with remaining phosphorylated ITAMs then activate PLC which on its turn initiates phosphatidylinositol-4,5-biphosphate (PIP_2) hydrolysis and ca^{2+}mobilisation, a pathway leading to degranulation and protein kinase C (PKC) activation. Aside from degranulation, PLC and PKC are thought to participate in other signalling pathways leading to cytokine gene transcription and the activation of raf-1, MAP-2 kinase, and Ras (June, 1991).

According to another model suggested for $CD8^+$T cells, TCR occupation activates binding of CD8 to MHC class I most likely by induction of a conformational change in CD8 that increases its affinity for MHC class I (Casabo et al, 1994). Interaction of CD8 with MHC class I then successively results in sequestration of CD8 towards the TCR-CD3 complex, phosphorylation (i.e. activation) of CD8-associated Lck, phosphorylation of the CD3ζ-chain by CD8-associated Lck, and recruitment of several kinases such as ZAP-70 to this side. When activated, these kinases initiate degranulation by activating PLC (O'Rourke and Mescher, 1993; Mescher, 1995).

As stated in both models, Lck is generally considered to be one of the earliest tyrosine kinase involved in T cell signalling. This assumption is based on the observation that tyrosine phosphorylation of intracellular substrates does not occur in cells lacking Lck kinase activity (Karnitz et al, 1992). The impact of Lck for T cell activation is further supported by the observation that introduction of Lck into cells that have lost expression of endogenous Lck restores their ability to respond to signalling through the TCR and exhibit cytolytic activity (Karnitz et al, 1992; Abraham, 1991; Straus and Weiss, 1992). According to the data of Xu and Littman on $CD4^+$T lymphocytes (1993), however, expression of a chimeric receptor containing CD4 extracellular and transmembrane domains fused to a kinase-deleted Lck resulted in similar IL-2 production as when a kinase functional form of Lck was fused to CD4. Similar data were reported by Collins and Burakoff, (1993). In contrast, mutations in the SH2 domain of CD4-associated Lck considerably affected the production of Il-2 by these cells (Xu and Littman, 1993). This observation suggests that the SH2 domain rather than the kinase domain of CD4-associated Lck is essential for T cell activation. Although these data suggest that CD4 (and perhaps CD8)-associated Lck kinase activity is dispensable they do not exclude the possibility that the T cell response observed was initiated by endogenous free-Lck present within the cells. As so, the former model in which free-Lck could have initiated T cell signalling seems more plausible than the second model in which CD8/CD4-associated Lck kinase activity initiates the signalling cascade by phosphorylating ITAMs.

Nevertheless, Feito et al. (1996) showed that tyrosine phosphorylation of CD3-chains in $CD4^+$T cells was restricted to those physically associated with CD4. This observation implies that CD4-associated Lck initiates CD3-chain phosphorylation.

The above models portray how TCR occupation leads to different T cell responses. In these models, we focused on degranulation as the secretion of vesicles containing perforin and granzymes is an important mechanism by which $CD8^+$CTL deliver the "kiss of death" to apposing cells (Immunological Reviews, no 146, 1995). Aside from degranulation, however, T cell-mediated cytotoxicity can be accounted for by a second mechanism. This second mechanism, which is the main lytic mechanism of $CD4^+$CTL but can also be executed by $CD8^+$CTL depending on the target cell (Lancki et al, 1995), requires the expression of Fas on the target cell and of Fas ligand (FasL) on the CTL. Engagement of FasL with Fas causes the activation of death genes in the target cell which results in apoptosis (Immunological Reviews, no 146, 1995).
The signalling pathways resulting in degranulation are speculative. Less is even known about the events leading to FasL expression on CTL. According to the available data, the ability of CTL to kill via the FasL pathway is tightly controlled by the TCR. Herbimycin A, an PTK inhibitor specific for src-like PTK (i.e. Fyn and Lck) has been reported to abrogate tyrosine phosphorylation, ca^{2+}mobilisation and inositol phosphate turnover induced by TCR occupation. Analysis of its effect on FasL induced cell kill, demonstrated that it totally abrogated FasL expression induced through TCR engagement but not through activation of PMA plus ionomycin (Anel et al, 1994). PMA triggers the activation of PKC while ionomycin triggers ca^{2+}mobilization. These findings suggest that src-like PTK and ca^{2+}mobilization are implicated in the induction of FasL expression and that the activation of src-like PTK precedes PKC activation. Oyaizu et al. (1995) recently addressed the question whether Lck was involved in TCR mediated FasL induction. The induction of FasL mRNA by TCR triggering was analyzed both in wild type (Jurkat) and Lck-deficient (JCaM) cells. Jurkat cells were able to upregulate FasL mRNA after TCR stimulation while JCaM cells failed to do so. However, both cell types were able to express FasL mRNA following treatment with PMA plus ionomycin. Collectively these data suggest that FasL expression is initiated by similar events as degranulation.

1.3. Impact of the avidity of TCR-MHC/peptide interactions on the T cell response

As suggested above, the CD8 (or CD4) molecules on T cells play an important role in the T cell response to target cells. Both CD8 and CD4 bind to the same ligand as the TCR. CD8 binds to the $\alpha 3$ domain of MHC class I (Salter et al, 1989; Connolly et al, 1990; Shepherd

et al, 1992) while CD4 binds to the ß2 domain of MHC class II (Cammarota et al, 1992; Nag et al, 1993). Therefore, it is generally believed that these molecules serve to strengthen the interaction between the TCR and MHC/peptide. Direct prove for this concept was only recently provided by Garcia et al. (1996B). Additionally, both CD8 and CD4 are believed to play a role in TCR signalling by focusing Lck in the vicinity of the TCR-CD3 complex. Several studies, however, have demonstrated that some T cells can exhibit their function in the presence of anti-CD4 or anti-CD8 mAb. In this section, the significance of such T lymphocytes that obviously do not require their co-receptors to display their function is discussed.

1.3.1. The requirement for CD8/CD4 varies according to the avidity of TCR-MHC/peptide interactions.

Over the past years, numerous groups have reported a correlation between the MHC/peptide density on target cells and the susceptibility of $CD8^+$CTL to blocking by anti-CD8 mAb. In 1983, Gromkowski et al. demonstrated that a reduction in H-2 density by papain treatment of target cells was accompanied by an increase in the susceptibility of CTL to CD8-blocking mAb. In 1985, Shimonkevitz et al. compared the ability of CTL clones to lyse target cells with a low MHC class I surface expression with their ability to lyse the same target cells after IFN-γ treatment had increased their MHC class I surface expression. Lysis of IFN-γ treated cells appeared to be less susceptible to CD8 blocking. Using class I bearing artificial membranes, Goldstein and Mescher (1987) showed a reciprocal relationship between the number of immobilized class I molecules and the susceptibility of T cells to blocking with anti-CD8 mAb. Consistent with these findings, Dembic and colleagues (1986, 1987) showed that anti-CD8 mAb blocked the lytic activity of a $CD8^+$T cell clone against target cells with a low antigen expression but not against target cells with a high antigen expression. Alexander et al. (1991) and Al-Ramadi et al. (1995) reported that CD8-independent CTL clones became CD8-dependent when the antigen density on target cells was reduced. A similar correlation between the ligand density on antigen-bearing cells and the requirement for CD4 in the activation of $CD4^+$T cells has been described (Marrack et al, 1983; Biddison et al, 1984; Greenstein et al, 1985; Gougeon et al, 1985; Feito et al, 1996).

Also the density of the TCR might influence the cell's requirement for CD4 or CD8 co-receptors. In this prospect, Greenstein et al. (1985) reported that a $CD4^+$murine hybridoma which had a 3-fold higher expression of TCR than the parental cell line could be activated independently of CD4-MHC class II interactions. Combining this finding with the previous data it can be stated that the requirement for CD8 or CD4 co-receptors corresponds inversibly with the number of TCR and MHC/peptide complexes that can associate.

Additional studies in this field indicated that this variable was not the only factor. Under conditions where the antigen complexed to MHC was the only variable, it was shown that CD8-independent T cells became heavily dependent on CD8 when a mutated variant of this

antigen was bound to MHC (Maryanski et al, 1988). Likewise, Cai and Sprent (1994) demonstrated that CD8-independent CTL became CD8-dependent when TCR contact with antigen was impaired by anti-TCR mAb. In that same year, Auphan et al. (1994) proved that the T cell's dependency on CD8 could be transferred by the TCR. In this study, mice transgenic for the TCR isolated from two $CD8^+$CTL clones that were, respectively, highly dependent and independent of CD8 for their stimulation by cells expressing the $H\text{-}2K^b$ alloantigen, were generated. Analysis of $CD8^+$T cells derived from these mice demonstrated that these cells had the same characteristics of sensitivity to inhibition by anti-CD8 mAb as the original CTL clone.

Recently, soluble TCRs became available which allowed a direct estimation of TCR affinity. Al-Ramadi and co-workers (1995) analyzed the ability of soluble 2C TCRs to bind p2Ca complexed to soluble $H\text{-}2L^d$ and compared it with their ability to bind p2Ca-analogs complexed to soluble $H\text{-}2L^d$. Some p2Ca-analog/$H\text{-}2L^d$ complexes were as efficiently bound as p2Ca/$H\text{-}2L^d$ while others were less firmly bound or were not detectably bound by soluble 2C TCRs. Despite the apparent differences in TCR affinity, 2C CTL could generally lyse $H\text{-}2^d$ target cells expressing various peptide-analogs with comparable efficiency. This disparity could not be ascribed to differences in peptide affinity for $H\text{-}2L^d$. Additional data, however, showed a clear link between the susceptibility of 2C CTL mediated lysis to inhibition by anti-CD8 mAb and the affinity of the 2C TCR. Whereas anti-CD8 mAb abrogated lysis of $H\text{-}2^d$ target cells pulsed with p2Ca-analogs for which the 2C TCR had a low affinity, it could not inhibit lysis of $H\text{-}2^d$ target cells for which the 2C TCR had a high affinity.

The above data conclusively demonstrate that variations in either the affinity of the TCR-ligand interaction or the number of TCR and MHC/peptide that can interact inversibly corresponds with the T cell's requirement for CD8/CD4. Along with the observation that CD8 and CD4 molecules bind to the same ligand as the TCR, the concept arose that simultaneous adhesion of CD8/CD4 co-receptors enhances the avidity (i.e. strength) of TCR-MHC/peptide interactions. According to this concept, T cells require CD8 or CD4 molecules when the avidity of TCR-MHC/ligand interactions is suboptimal and can function without these molecules in case the avidity of TCR-MHC/peptide interactions is sufficiently high.

1.3.2. Signalling events in absence of the CD8/CD4 co-receptor: a model.

As the cytoplasmic tail of the CD8 and CD4 co-receptor is non-covalently associated with Lck, it is generally believed that the co-receptor is involved in the intracellular signalling. Addition of anti-CD8 and anti-CD4 mAb prevents the sequestration of Lck to its ligand, the TCR/CD3 complex. Despite this, some T cells are perfectly able to display their function in the presence of anti-CD8 or anti-CD4 mAb. The question now arises how signalling occurs in these CD8/CD4-independent T cells. At present, we can only speculate on this subject. As a constitutive active form of Lck was found to substitute for the CD4 co-receptor in

enhancing the T cell response (Abraham et al, 1991), we suspect that differences in the relative level of Lck activation might account for the differences in CD8/CD4 dependency. One possibility might be that the avidity of TCR-ligand interactions determines the number of Lck that becomes activated. In the absence of CD8/CD4 enhanced binding, a low avidity interaction between the TCR and MHC/peptide complex will result in accelerated dissociation of TCR-ligand complexes, which implies shorter TCR ligation times (Luescher et al,1995). The resulting low Lck activation level may now only give rise to monophosphorylation of CD3ζ-chains which is insufficient to bind and activate ZAP-70 (Madrenas et al, 1995). On top of this, anti-CD8/CD4 mAb would prevent the co-receptor from focusing Lck in the vicinity of the TCR-CD3 complex to initiate further phosphorylation of CD3ζ-chains. In case of high avidity TCR-MHC/peptide interactions, the TCR ligation times will be sufficiently long to allow the phosphorylation of both ζ-chains even without the CD8/CD4 enhancement.

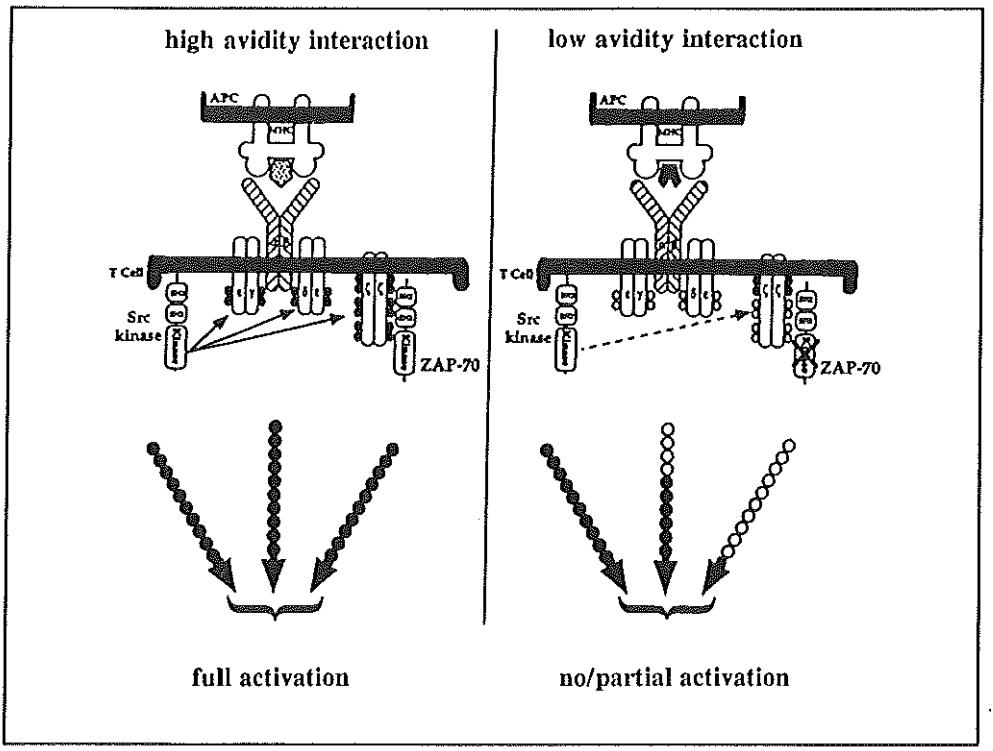

Figure 3. Intracellular signalling in case of a high (left panel) or a low (right panel) avidity TCR-MHC/peptide interaction (adapted from Sloan-Lancaster and Allen, 1997). Whereas a high avidity (co-receptor independent) interaction leads to saturated phosphorylation of all CD3 ITAMs (filled circles) and full T cell activation, a low avidity (co-receptor dependent) interaction causes incomplete phosphorylation of CD3 ITAMs which results in partial T cell activation or no T cell activation at all.

This model might also explain why impaired thymic development of $CD4^+$ or $CD8^+$ single-positive T cells due to altered co-receptors that are unable to associate with Lck can be restored by overexpression of these mutant co-receptors (Killeen and Littman, 1993; Chan et al, 1993) and why transfection of Lck in cells lacking CD4 is sufficient to recover their response to similar levels found for the $CD4^+$parental line (Straus and Weiss, 1992).
In addition to the above model, the resulting shorter TCR ligation time may also affect the phosphorylation of additional CD3-chains. A recent study on the involvement of individual CD3-subunits revealed that CD3ζ alone could induce the tyrosine phosphorylation of various cellular substrates including ZAP-70, Fyn, and PLC but was incapable of generating inositol triphosphate (IP3) and to mobilize intracellular free Ca^{2+} (Jensen et al, 1997). The CD3ϵ-chain, on the contrary, could drive IP3 production and Ca^{2+} mobilization. Along with the observation that CD8-independent CTL can lyse their target cells without inducing PIP_2 hydrolysis whereas CD8-dependent CTL can not (Knall et al, 1995), these data might indicate that CD8-independent signalling can occur via CD3ζ alone whereas additional CD3-chains such as CD3ϵ are required for the activation of low avidity CTL. In support of this hypothesis, altered peptide ligands which are discussed in the subsequent section did not stimulate tyrosine phosphorylation of CD3ϵ (Madrenas et al, 1995).

1.3.3. The avidity of the TCR-MHC/peptide interaction determines the fate of the T cell.
TCR triggering is generally believed to occur when the total avidity of individual TCR-peptide-MHC interactions exceeds a particular threshold. The extent of this interaction depends on I. the number of MHC/peptide complexes on the target cell, II. the number of TCR and CD8/CD4 molecules on the T cell, and III. the affinity of the TCR for the MHC/peptide complex. For a normal T cell repertoire where the TCR and CD8/CD4 densities show little variation, avidity is controlled by the affinity of the TCR for MHC/peptide complexes and the number of these complexes available. The delicate balance between these latter two parameters is best reflected when we examine the consequence of varying one parameter. In such cases, the effect of altering one parameter can be compensated for by making a reciprocal quantitative change in the other component (Sykulev et al, 1994B; Kim et al, 1996). The present section will discuss the impact of the avidity of TCR-MHC/peptide interactions in the light of CD8/CD4-requirement, ligand density, and TCR affinity.

Although numerous studies have demonstrated the existence of T cells whose function could not be blocked by addition of anti-CD8 or anti-CD4 mAb, hardly any attention has been paid to the immunological significance of these cells. Our own data in this field refer to donor specific CTL propagated from graft biopsies obtained from heart transplant patients at different time-intervals after transplantation (chapter 2,3). As reported by others, T cell cultures grown from rejecting or stable transplants were both able to specifically lyse donor

B-LCL in vitro indicating that donor specific CTL are present within the graft irrespective of its rejection status (Dallman et al, 1987; Armstrong et al, 1987; Ruiz et al, 1988). Subsequent data, however, demonstrated that donor specific CTL propagated from graft samples obtained immediately preceding or during clinical rejection were generally not susceptible to inhibition by anti-CD8 or anti-CD4 mAb whereas those propagated from "non-rejecting" graft samples were highly sensitive to CD8 or CD4 blocking. Comparable data were found with respect to peripheral donor specific $CD8^+$CTL of cardiac transplant (chapter 4) and corneal transplant (Roelen et al, 1996) patients. In both studies, CD8-independent CTL were far more prominent within blood samples taken during a rejection episode than in blood samples taken during "non-rejection". Despite the fact that both CD8/CD4-dependent and -independent CTL could lyse donor target cells in vitro, the selective presence of CD8/CD4-independent CTL during a rejection episode prompted us to believe that only this subset is functional in vivo. Several data have shown that CD8/CD4-independent CTL can lyse target cells expressing low levels of antigen whereas an increase in surface antigen density is required for the activation of CD8/CD4-dependent CTL (Shimonkevitz et al, 1985; Mentzer et al, 1990). As target cells used for in vitro assays are generally considered to express substantial higher antigen levels than target cells encountered in vivo, the latter target cells may only effectively be killed by CD8/CD4-independent CTL. In support of this concept are the data of Alexander-Miller and co-workers (1996). They compared the in vitro and in vivo efficiency of virus specific CTL lines that differed in their antigen density requirements for optimal target cell lysis. Although all CTL lines effectively killed virally infected cells in vitro, only those that required low antigen determinant density for their activation could clear virally infected cells in vivo. These cells were also the less susceptible to CD8 blocking. Likewise, Auphan et al. (1994) showed that $CD8^+$T cells of mice transgenic for a CD8-dependent TCR were unable to lyse target cells expressing antigen levels found in vivo.

Also supporting the impact of CD8/CD4-independent CTL in transplant rejection was the observation that these CTL produced cytokines that promote graft rejection. CD8/CD4-independent CTL secreted IFN-γ when they encountered donor cells whereas CD8/CD4-dependent CTL did not. However, both subsets could produce IFN-γ after stimulation by immobilized anti-CD3 mAb, a stimulus which bypasses the requirement for TCR-MHC/peptide association (chapter 5). Likewise, CD8-independent CTL were able to produce IL-2 while CD8-dependent CTL could not (Cai and Sprent, 1993; Heath et al, 1993). These latter findings add to the growing body of literature showing that the avidity of TCR-MHC/peptide interactions not only determines whether or not a T cell will respond but also determines the quality of the response. Most studies performed in this field refer to the influence of ligand density and TCR affinity on the differentiation of naive HTL into Th1 or Th2 effector cells (reviewed by Romagnani, 1996; Constant and Bottomly, 1997) or refer to the use of altered peptide ligands (APL) that bind with different affinity to either the TCR or the MHC molecule (reviewed by Sloan-Lancaster and Allen, 1996). Although these studies

generally do not consider the cytotoxic capacity of T cells, they do shed light on the impact of avidity in immune responses and are therefore discussed briefly in this thesis.

Several studies showed that varying the dose of antigen used during $CD4^+$T cell priming could shift the response to a Th1- or a Th2-type response. Priming of naive $CD4^+$T cells from a cytochrome c-specific TCR-transgenic mice with a low peptide dose led to their differentiation into Th2-like cells producing abundant IL-4, whereas a high dose of the same peptide led to their differentiation into Th1-like cells producing abundant IFN-γ (Constant et al, 1995). In another model of TCR-trangenic mice, priming naive T cells with extremely low doses of ovalbumin peptide generated a Th2 response (IL-4 > IFN-γ) whereas priming with intermediate antigen doses generated a Th1 response (IFN-γ > IL-4). However a further increase in the dose of antigen resulted in the disappearance of IFN-γ and the development of IL-4 producing cells (O'Garra and Murphy, 1995). An explanation for the induction of a Th2-type response after a further increase in antigen dosage may be sought in the differential susceptibility of Th1 and Th2 cells to apoptosis at high doses of antigen. With Th1 cells being more sensitive to dose-induced apoptosis than Th2 cells, the IL-4 response may be due to the selective outgrowth of Th2 effector cells (Constant and Bottomly, 1997). The previous experiments established that naive $CD4^+$T cells with a homogeneous TCR responded to different doses of peptide antigen by differentiating into distinct effector cell types. In light of these findings the prediction follows that the dose of antigen given had influenced the antigen density on APC and hence the extent of TCR ligation. By using a set of ligands with various class binding affinities but unchanged T cell specificity, it has indeed been shown that stimulation with the highest affinity ligand resulted in IFN-γ production, whereas ligands with relative lower MHC class II binding induced only IL-4 secretion. Pfeiffer et al. (1995) analyzed the response of I-A^s restricted naive $CD4^+$T cells primed in vivo with their natural ligand α2 or the α2-analogs, α2Glu and α2Ala, which bound less well to MHC than α2. $CD4^+$T cells primed with the α2-analogs expressed IL-4 but not IFN-γ mRNA while $CD4^+$T cells primed with α2 expressed IFN-γ but not IL-4 mRNA. Similar data were reported by Chaturvedi et al. (1996) using a comparable experimental setting. Also the cytokine pattern of already differentiated T cells can alter by varying the ligand density. Kumar et al. (1995) reported that an analog of myelin basic protein (MBP) that bound more efficiently to I-A^u than MBP induced the production of IFN-γ by a Th2 clone. Windhagen et al. (1995) analyzed the response of human autoreactive Th0 clones specific for MBP epitope 85-99 and compared it with the response to MBP analogs that bound MHC class II less well. While stimulation with native peptide induced proliferation and the production of IL-2, IL-4, IL-10, and IFN-γ, the peptide analogs solely induced TGF-ß1 production.
Further confirmation that weak/strong interactions between TCRs and MHC/peptide ligands can influence the quality of the response was obtained by the use of analogs of immunogenic peptides in which the TCR contact sites had been manipulated (APL; reviewed by Sloan-Lancaster and Allen, 1996). These peptide analogs were then tested for their capacity to

stimulate a variety of T cell responses in T cells specific for the wild-type ligand. Pfeiffer et al. (1995) compared the cytokine response of I-A^b restricted naive $CD4^+$T cells primed in vivo with their natural ligand α2 or with a similar dose of the α2-analog α2Ala, which bound equally well to I-A^b than α2 but had an 80-fold increased ability to activate IL-2 production. Priming with α2 led to IL-4 but not IFN-γ gene expression while priming the same T cells with α2Ala induced IFN-γ but not IL-4 gene expression. Comparing the response of cytochrome c specific TCR transgenic naive $CD4^+$T cells primed with the analog K99R with that induced by priming with wild-type peptide, revealed that K99R was less potent to stimulate proliferation. Moreover, priming with K99R led to the generation of effector cells producing IFN-γ and IL-4 while priming with the wild-type ligand led to the generation of effector cells producing IFNγ but no IL-4 (reviewed by Constant and Bottomly, 1997). Evavold and Allen (1991) found that a peptide differing from the wild-type peptide (murine haemoglobin peptide Hb(64-76)) in a single residue lost the capacity to induce proliferative responses by a Th2 clone while remaining able to evoke IL-4 production. With respect to Th1 cells specific for Hb(64-76), peptide analogs stimulated cytolytic activity but failed to induce proliferation and cytokine production (Evavold et al, 1993).

The preceding data lend credence to the notion that the total avidity of individual TCR-ligand interactions determines the biological behaviour of T cells. A low avidity interaction results in only partial activation of T cells and induces the differentiation of naive HTL into Th2 effector cells. A high avidity interaction results in complete T cell activation and induces the differentiation of naive HTL into Th1. How TCR triggering can generate such distinct profiles is unknown. A simple view of the mechanism accounting for the generation of distinct effector functions is that the extent of TCR occupation determines the extent of CD3-chain phosphorylation. In case of a low avidity interaction, the incomplete phosphorylation of CD3 ITAMs would result in a lack of binding templates for some SH2-domain containing proteins. Thus, some signalling pathways would remain inactive while others may be stimulated weakly or completely, depending on the tyrosine phosphorylation state of the ITAMs. Such a model has already been proposed above for CD8/CD4-dependent and -independent signalling.

Aside from a lower threshold that should be reached to induce a T cell response there may also be an upper limit for TCR-ligand avidity. In support of this concept, high doses of peptide antigen presented by APC induced apoptotic death in a high avidity $CD8^+$CTL line and this effect could be restored by addition of anti-CD8 mAb (Alexander-Miller et al, 1996B). The underlying mechanism for the induction of apoptosis is not known but may include the production of TNF-α as addition of anti-TNF-α restored proliferation of high avidity $CD8^+$CTL in this study. According to Cai et al. (1997) the T cell may protect itself from reaching this upper limit via TCR internalization. Internalization of TCR after contact with antigen on a target cell has been viewed as a device to enable a large number of TCR molecules to make contact with a limited number of antigenic epitopes on the target cell.

This theory would explain the finding that T cell stimulation requires engagement of a considerable number of TCR but only few MHC/peptide complexes on the opposing cell (Valitutti et al, 1995; Viola and Lanzavecchia, 1996). According to the data of Cai et al. (1997), however, TCR internalization is not essential for T cell activation but rather is a byproduct of strong TCR ligation. This finding is supported by a study in which mice trangenic for the TCR isolated from two $CD8^+$CTL clones that were, respectively, highly dependent and independent of CD8 for their stimulation by cells expressing the $H\text{-}2K^b$ alloantigen were matched with $H\text{-}2^b$ mice (Auphan et al, 1994). Both types of $H\text{-}2^{k/b}$ mice had markedly reduced T cell numbers in their peripheral blood and the remaining T cells were aberrant. T cells present within the CD8-dependent TCRtg mice lacked CD8 surface expression whereas T cells present within CD8-independent TCRtg mice lacked both TCR and CD8 surface expression.

According to the above studies, the total avidity of TCR-ligand interactions determines the fate of the cell. Not all groups, however, support the impact of TCR affinity in T cell activation. In most studies, the affinity of a TCR-peptide interaction is assayed by the ability of this peptide to displace a TCR binding mAb or a reference peptide from the TCR. Using this approach Sykulev and colleques (1994) and Cai and Sprent (1996) confirmed a strict correlation between TCR affinity and the efficiency of specific T cell killing. Others, however, reported that the affinity of the TCR for its ligand was not crucial in determining T cell activation (Matsui et al, 1994; Yoon et al, 1994; Kessler et al, 1997). An explanation for this contradiction was provided by the data of Matsui et al. (1994). In their study, the reactivity of the 2B4 T cell hybridoma specific for PCC bound to the MHC class II molecule $I\text{-}E^k$ was compared with its response to variants of the PCC peptide. Although all peptides bound $I\text{-}E^k$ equivalently, the concentration of peptide required to achieve maximal IL-2 production varied enormously between PCC and its analogs. Consistent with the above concept, these differences in dose-response were ascribed to differences in TCR affinity. To investigate this, $I\text{-}E^k$ molecules loaded with PCC or with a PCC-analog were tested for their ability to displace a TCR binding mAb from the TCR. As both complexes were equally capable, it was assumed that the TCR had comparable binding affinities for PCC and its analog despite the fact that PCC was far more effective in inducing IL-2 production. The affinity of a TCR-MHC/peptide interaction is the result of the continuous association (Kon) and dissociation (Koff) of the interaction (Kd=Koff/Kon). Comparison of the Kon and Kof rates separately, however, revealed that $I\text{-}E^k$/PCC associated relatively slow with the TCR and dissociated relatively slow from it. The $I\text{-}E^k$/PCC-analog, on the contrary, associated somewhat faster with the TCR but dissociated almost immediately from it.
Along with data showing that TCR antagonists have a higher dissociation rate than TCR agonists (Lyons et al, 1996), a model for T cell activation was proposed in which the duration of TCR ligation is crucial. An increase in the dissociation rate could then result in an inability of the T cell to maintain an adequate local pool of engaged complexes, and TCR-

peptide-MHC association might not persist for the time needed for the full development of cytosolic multiprotein assemblies involved in signal transduction. The role of the CD8/CD4 co-receptor in this model is simply to stabilize the TCR-MHC/peptide interaction to prolong its duration. Likewise, an increase in the number of TCR-ligand interactions will add to the stability of TCR ligation. In support of this model it was shown that the immunogenicity of antigens was determined by their dissociation rate rather than by their affinity for MHC. In comparison to non-immunogenic peptides, immunogenic peptides have a low dissociation rate. This low dissociation rate would allow them to form stable MHC-peptide complexes which would persist for a time sufficient to activate T cells (van der Burg et al, 1996).
Another point worth discussing is the slow rate with which PCC/I-E^k associates with the 2B4 TCR. This slow association rate indicates that the binding of the TCR to its ligand is in some way intrinsically limited. One explanation is that a peptide must induce a conformational change in the TCR to elicit a T cell response. This theory was first launched by Janeway and co-workers (Janeway, 1995). In support of this theory, it was shown that not the affinity of an anti-TCR mAb determined whether a T cell clone became activated but rather the TCR epitope recognized by the antibody (Rojo and Janeway, 1988; Janeway et al, 1989).

1.3.4. Immuno-therapeutical strategies based on the CTL's avidity.

Several laboratories are currently investigating adoptive transfer of in vitro expanded autologous CTL for the treatment of a number of diseases including cytomegalovirus (Riddell, 1992A,B), human immunodeficiency virus type I (Lieberman, 1994), and cancer (Topalian and Rosenberg, 1991; Rubin and Lotze, 1993). The conventional approach to elicit an optimal CTL response in vivo is by transferring large numbers of in vitro activated antigen-specific CTL precursors. However, according to Alexander-Miller et al (1996A), the in vivo functionality of such reinfused CTL depends on the avidity with which their TCRs interact with MHC/peptide complexes. High avidity CTL lines efficiently cleared antigen-bearing cells in vivo whereas low avidity CTL lines, though effective in vitro, were incompetent. This observation is pivotal as it implies that adoptive transfer of high avidity CTL rather than of every potential CTL would benefit the efficacy of adoptive immunotherapy.
Numerous groups have demonstrated that high avidity CTL react to cells expressing low levels of antigen whereas an increase in surface antigen is required to induce the proliferation and activation of low avidity CTL (Marrack et al, 1983; Shimonkevitz et al, 1985; Gougeon et al, 1985; Mentzer et al, 1990). Accordingly, to generate high avidity CTL for adoptive immunotherapy, one should culture the immune cells of a patient with low doses of the specific antigen as under such conditions only high avidity CTL would expand.
In the case of allograft transplantation, we rather would like to undo the immune system of its high avidity donor specific CTL. A recent study showed that high avidity CTL were selectively eliminated from in vitro cultures after challenge with high doses of antigen. When

exposed to supraoptimal antigen doses, high avidity CTL became apoptotic while low avidity CTL proliferation was not affected (Alexander-Miller et al. 1996B). Administration of high antigen dosages has been shown to result in the selective deletion of antigen reactive T lymphocytes in different experimental systems. Virus specific T cells were selectively eliminated after challenge with high doses of virus (Moskophidis et al, 1993). Likewise, transfused MBP specific $CD4^+$T cells were depleted in recipient mice treated with high doses of MBP peptide (Critchfield et al, 1994). Accordingly, administration of high dosages of donor antigen might be of utmost importance for the prevention of transplant rejection as such treatment would cause the deletion of high avidity, donor specific CTL leaving only low avidity CTL inadequate to mediate donor tissue destruction.

For more than two decades, donor cell transfusions have been associated with prolonged survival of donor but not third-party allografts in both experimental and clinical studies (Foster et al, 1988; Westra et al, 1991; Brennan et al, 1995). Comparable results were obtained when membrane bound donor MHC molecules (Foster et al, 1992) or recipient cells transfected with donor MHC genes (Saitovitch et al, 1996) were administered demonstrating that donor antigens are responsible for inducing suppression of allograft rejection. Despite intense investigation, however, considerable controversy remains as to the underlying mechanism(s). Answers have been sought in various factors including the establishment of donor-recipient microchimerism (Starzl et al, 1992), anergy (Eynon and Parker, 1992; Freeman et al, 1993), altered cytokine profiles (Salom et al, 1993), and a reduction in antigen specific CTL precursors (Irschick et al, 1990). As the beneficial effect of donor specific transfusions appeared to depend on the number of cells injected (Foster et al, 1988; Heeg and Wagner, 1990), the destruction of high avidity donor specific CTL might be (part of) the underlying mechanism.

Although the body of literature that examines the induction of tolerance is rapidly growing, the impact of high avidity CTL in this process has generally been ignored. Only one group has analyzed the effect of donor specific transfusion on high avidity CTL (Heeg and Wagner, 1990). According to their results, donor specific high avidity CTL, while present within the peripheral blood of control mice, were undetectable in mice made tolerant to allogeneic skin grafts by donor specific transfusion. Additionally, high avidity donor specific CTL have been found to disappear from the peripheral blood of heart transplant patients long after transplantation when rejection episodes are seldomly experienced (Chapter 4). Though both studies demonstrate that high avidity CTL are absent in case of stable engraftment or transplant tolerance, a causal relationship has yet to be proven. For this, the effect of reconstitution of high avidity donor specific CTL into tolerant animals should be examined. In case such a treatment abrogates the tolerant state, transfusion of accurate amounts of donor antigen might extend the benefits of current transfusion protocols towards indefinite transplant survival even in the clinical setting.

Finally we would like to mention a newly discovered membrane molecule, p38 (Valiante and Trinchieri, 1993). As shown in chapter 6 of this thesis, high avidity $CD4^+$CTL can be

delineated from naive- and non-cytotoxic CD4-expressing lymphocytes as well as from low avidity $CD4^+$CTL by their surface expression of p38. Within the $CD8^+$T cell population, however, p38 is not selectively expressed by high avidity CTL. This observation indicates that p38 depleting mAb can not be used to selectively eliminate the high avidity CTL pool. However, the p38 molecule might serve as a prognostic marker for acute cardiac rejection. Kinetic studies of donor specific, high avidity CTL demonstrated that these cells are absent within stable cardiac allografts, but start accumulating approximately 2 weeks prior to the diagnosis of acute rejection (chapter 2). Accordingly, increases in the number of high avidity CTL present within the cardiac allograft might predict the occurrence of acute rejection. As p38 distinguishes high avidity $CD4^+$CTL from additional CD4-bearing lymphocytes and as $CD4^+$CTL are found in a significant proportion of cardiac biopsies (chapter 3), immunohistochemical analysis of $CD4^+p38^+$lymphocytes would inform us about the number of high avidity, donor specific $CD4^+$CTL and hence about the rejection status of the cardiac allograft.

1.4. Aim of the study.

As discussed above, not all CTL but only those with a high avidity may have immunological potency. In this thesis, we analyzed the importance of high avidity CTL in clinical cardiac rejection. In **chapter 2**, the kinetics of graft infiltrating CTL with a high avidity for donor HLA class I antigens were studied and compared with the rejection status of the cardiac allograft. In **chapter 3**, the frequency and avidity of donor HLA class II specific CTL were examined and compared with the rejection status of the cardiac allograft. Both studies revealed that high avidity CTL migrate to the allograft during rejection. Accordingly, the kinetics of high avidity CTL within the peripheral blood might inform us about the immunological status of the transplanted heart and by that provide a method for non-invasive monitoring (i.e. without the requirement for heart biopsies) of rejection. The results of this study are shown in **chapter 4**.
To further characterize high avidity CTL and to understand their relevance in transplant rejection, the cytokine secretion profile and phenotype of these cells was studied. The results are shown in **chapter 5** and **chapter 6**, respectively.

Chapter 2

Progressive accumulation of CTL with high avidity for donor antigens during the development of acute cardiac rejection

To study the importance of cytotoxic T lymphocytes (CTL) with high avidity for donor antigens (Ag) in the development of acute cardiac allograft rejection, their appearance within the graft in relation to rejection was analyzed. For this study, donor directed CTL propagated from sequentially taken endomyocardial biopsies (EMB) were enumerated by limiting dilution analysis (LDA). Subsequentially, the fraction of these CTL having high avidity for donor Ag was determined by addition of a CD8 monoclonal antibody (mAb) to the cytotoxic phase of the LDA. Analysis of 37 EMB cultures obtained from 11 heart transplant (HTx) patients before, during, or after they experienced rejection, revealed the kinetics of donor specific CTL in relation to rejection for HTx patients in general. For 5 individual recipients, a more detailed analysis was performed.

The kinetics found for individual patients confirmed the pattern found for the total group of HTx recipients tested. Frequencies of donor specific precursor CTL (pCTL) as well as of *in vivo* primed donor reactive CTL (committed CTL or cCTL) increased towards rejection and decreased after successful rejection therapy. More than 2 weeks before rejection was diagnosed, only a small fraction of the graft infiltrating donor specific pCTL and cCTL had high avidity for donor Ag (median= 35% and 11%, respectively). Within 2 weeks preceding rejection, this fraction increased gradually (median= 52% and 55%, respectively) and became dominant during rejection (median= 87% and 78%, respectively). After successful rejection therapy, a decrease to basal levels (median= 18% and 24%, respectively) was observed.

Conclusively, intragraft accumulation of high avidity, donor specific pCTL and cCTL may cause transplant rejection.

2.1. Introduction

During the last several years, immunological events associated with lymphocytic infiltration of rejecting allografts have been studied. Data of murine (Orosz et al, 1989; Bishop et al, 1990,1992) and human cardiac allografts (Suitters et al, 1990) as well as of a rat kidney allograft model (Mason and Morris, 1984; Bradley et al, 1985), conclusively demonstrated that the presence of donor specific CTL within the transplant coincided with extensive tissue destruction. Intragraft granzyme B and perforin gene expression, indicative for the presence of activated CTL, was highly restricted to acutely rejecting renal allografts (Lipman et al, 1994). These results suggest a key role for CTL in rejection. An intriguing finding, however, was the observation that allo-antigen (alloAg) specific CTL were also present within transplants in which no histological evidence of rejection was found (Dallman et al, 1987; Weber et al, 1989; Ouwehand et al, 1993). Little is known about these latter CTL which apparently do not interfere with graft destruction.

Previously, graft infiltrating lymphocytes (GIL) of patients who experienced several acute cardiac rejection episodes were compared to those obtained from patients who never clinically rejected their cardiac allograft. Significant higher donor specific CTL frequencies were found within the rejector group compared to the non-rejector group. More importantly, donor specific CTL from both groups proved to have different characteristics. The majority of CTL of the rejector group had high avidity for donor Ag whereas CTL of the non-rejector group mainly had low avidity for donor Ag (Vaessen et al, 1994). In line with this observation, high avidity CTL were found in the peripheral blood of patients with ongoing rejection of their corneal allograft, but were hardly detectable in the blood of patients with good graft function (Roelen et al, 1995).

To elucidate the importance of CTL with high avidity for donor Ag in the development of acute cardiac allograft rejection, the kinetics of these CTL in relation to rejection was analyzed. For this study, graft infiltrating donor directed CTL propagated from EMB taken before, during, or after histologically proven acute rejection, were enumerated and their avidity for donor Ag was determined. Analysis of 37 EMB cultures obtained from 11 HTx patients, portrays the kinetics of donor specific CTL in general. From 5 patients, EMB taken before and during rejection as well as after rejection therapy were successfully cultured. These serial EMB cultures were used to analyze the kinetics of donor specific CTL in relation to rejection within individual patients.

To enumerate naive donor specific pCTL as well as CTL that have been activated in vivo by contact with donor Ag (cCTL), LDA as described by Orosz et al.(1989) was performed. Subsequently, the fraction of pCTL and cCTL with high avidity for donor Ag was determined by addition of CD8 mAb to the cytotoxic phase of the LDA. The CD8 molecule stabilizes the interaction between a T cell receptor (TCR) and a HLA-peptide complex. CTL which benefit most from such interactions and therefore are most dependent on CD8, express low affinity TCR for Ag. Likewise, CD8 independent CTL express high affinity TCR for Ag (MacDonald et al, 1982; Cai and Sprent, 1994; Auphan etal, 1994). Hence, the effect

of CD8 blocking on the cytotoxic capacity of CTL reveals their avidity for donor Ag. Here we report that donor specific pCTL and cCTL are present within the graft irrespective of acute cardiac rejection. During and immediately preceding acute rejection, graft infiltrating donor specific pCTL and cCTL mainly have *high* avidity for donor Ag. In contrast, during periods of "non-rejection", intragraft donor specific pCTL and cCTL mainly have *low* avidity for donor Ag. That high avidity CTL are able to induce myocyte destruction, whereas low avidity CTL are not, is discussed.

2.2. Materials and Methods

Patients. Endomyocardial biopsies obtained from HTx recipients prior to, during, and after histologically proven rejection, were used to study the kinetics of graft infiltrating donor specific pCTL and cCTL in the pathogenesis of human cardiac transplant rejection. Patients received a preoperative blood transfusion while cyclosporin A and low dose steroids were used as maintenance immunosuppression.

Endomyocardial biopsies were routinely taken at a weekly interval during the first 6 weeks post-transplantation and once in two weeks in the following 4 weeks. Later EMB were taken less frequently, declining to once every 4 months at 1 year. For the present study, EMB taken during the first 4 months following cardiac transplantation were cultured in IL2 conditioned medium to obtain GIL. This period was chosen because acute rejections occur most frequently during this timespan. Acute cardiac rejection was diagnosed by histopathology of EMB according to ISHLT criteria (Billingham et al, 1990). Anti-rejection treatment was instituted when mononuclear cell infiltrates coexisted with myocyte damage or myocyte necrosis and consisted of bolus steroids or a 2-weeks course of a polyclonal rabbit anti-thymocyte globulin preparation in case of ongoing rejection.

Lymphocyte cultures. Lymphocyte cultures were established from EMB as described previously (Ouwehand et al, 1991). Briefly, each biopsy was cultured in a 96-well round bottom tissue culture plate (Costar, Cambridge, MA) with 200 μl culture medium per well, in the presence of 10^5 irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMC) as feeder cells. Due to the absence of donor Ag in this system, no de novo stimulation of donor specific lymphocytes can take place in vitro. Culture medium consisted of RPMI-1640, Dutch Modification (Gibco, Paisley, Scotland), supplemented with 4mM L-glutamine, 100 IU/ml Penicillin, 100 μg/ml streptomycin and 10% pooled human serum. Additionally, 10% v/v lectin free lymphocult-T (Biotest GmbH, Dreieich, Germany) was added as exogenous source of IL2. It is postulated that under these circumstances only those lymphocytes which have acquired IL2 receptors due to a previous encounter with Ag can be cultured (Mayer et al, 1985; Zeevi et al, 1986). The plates were incubated in a humidified atmosphere at 37°C in 5% CO_2.

PBMC were isolated from heparinized venous blood of the transplant recipient by Ficoll-Hypaque density gradient centrifugation. Cells were washed twice in HBSS (Gibco) and once in RPMI-1640. PBMC were irradiated (30 Gy) and used as feeder cells.
GIL cultures were analyzed for WT31, CD8 (Becton Dickinson), and CD4 (Immunotech, Marseille, France) expression by three-color flow cytometry on a FACScan after staining with mAb directly conjugated to fluorescein, PERPC, and phycoerythrin, respectively. After phenotyping, a standard 4-hour ^{51}Cr release assay was performed to assess the ability of GIL cultures to specifically lyse target cells expressing HLA class I and II Ag for which the donor and recipient were mismatched (Tissue Antigens 1980; 16: 335). Tested GIL cultures were stored at -140°C in IL2-free culture medium supplemented with 10% v/v DMSO (Sigma).

Allogeneic target cells. Phytohaemagglutinin (PHA) stimulated T cell blasts of donor origin were obtained by culturing donor spleen cells for 4-7 days in culture medium supplemented with 10% v/v lymphocult-T and 1% PHA (Difco, Detroit, MI).

Limiting Dilution Analysis. GIL cultures considered for LDA had to exhibit donor HLA class I directed cytotoxicity in ^{51}Cr-release assays and were obtained from individual patients at various timepoints after transplantation. LDA was conducted simultaneously on all GIL samples of each patient to minimize experimental variation. Limiting dilution cultures were set up in 96-well round bottom microculture plates (Costar). Responder cells (GIL) were titrated in 8 double dilution steps starting from 5000 cells per well to 39 cells per well. All dilutions were performed in 24 replicates and 50,000 irradiated (30 Gy) stimulator cells were added to each well in a total volume of 0.2 ml culture medium supplemented with 20 U/ml rIL2 (Biotest). As stimulator cells we used either donor spleen cells (or, when not available, allogeneic mononuclear cells that shared HLA Ag with the donor) or autologous PBMC. When autologous PBMC are used, in vivo activated cCTL are cultured that maintain their cytolytic responsiveness to alloAg encountered in vivo (Orosz et al, 1989). Stimulation by donor spleen cells leads to expansion of the total pool of donor specific CTL (tCTL) consisting of both donor directed cCTL and their naive precursors (pCTL). Hence, the combination of these LDA allows a quantitative comparison between cCTL and pCTL (tCTL-cCTL) with similar Ag specificity.
After 7 days of culture, all 96-well plates were split into two and CD8 mAb was added to half of the split wells as described below. Subsequently, each well was individually tested for its capacity to lyse 2500 ^{51}Cr-labelled PHA blasts of donor origin. LDA cultures were incubated for 4 hrs at 37°C in 5% CO_2. Supernatants were harvested using a Skatron harvesting system (Skatron-AS, Lierse, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA). Spontaneous and maximum release were defined by incubation of target cells with culture medium in the absence or presence of Triton X-100 detergent (5% v/v solution in 0.01 TRIS buffer), respectively. Microcultures were considered cytolytic when the experimental lysis percentages

exceeded 10%. As a control for specificity, the reactivity of cultures with the highest responder cell concentration was tested against PHA blasts of third party cells and against the K562 cell line.

CD8 inhibition study. A 1:500 dilution of ascitic fluid was used for FK18, a mouse anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule (Koning et al, 1986; a kind gift of Dr F Koning, Dept of Immunohematology and Bloodbank, University Hospital Leiden, the Netherlands). This concentration totally inhibited the cytotoxic capacity of CD8-dependent CTL clones but did not affect target cell lysis by CD8-independent CTL clones. Before addition of ^{51}Cr-labelled target cells to the LDA, half of the split wells was preincubated with FK18 during 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2.

Cell mediated lympholysis (CML) assay. To control whether the difference in avidity reflected by FK18 sensitivity is indeed caused by blocking the interaction between a CD8 molecule and the α_3-domain of a HLA class I molecule, CD8-(in)dependent CTL clones were tested for donor directed cytotoxicity in a standard 4-h ^{51}Cr release assay in the absence and presence of FK18 or TP25.99, a mAb directed against the α_3-domain of HLA class I molecules (Pouletty et al, 1993; a kind gift of Dr S Ferrone, Dept of Microbiology, New York Medical College Valhalla, NY). Briefly, CTL clones were incubated with 2500 ^{51}Cr-labelled PHA blasts of donor origin at different effector:target (E:T) ratios in 0.2 ml culture medium. Alternatively, CML were performed either with CTL clones preincubated with FK18 (a 1:500 dilution of ascitic fluid) or with ^{51}Cr-labelled target cells preincubated with TP25.99 (50 μg/ml). After 4 hours of effector-target cell incubation, supernatants were harvested and the release of ^{51}Cr was measured as described in the LDA section above. To control for specificity, the cytotoxicity of the clones against ^{51}Cr-labelled third party PHA blasts and the K562 cell line was tested.

Frequency calculation. Minimal estimates of CTL frequencies were calculated by analysis of the Poisson distribution relationship between the percentage of cultures that failed to develop cytotoxicity and the number of responder cells per well. Both pCTL and cCTL frequencies, expressed as number of cytotoxic cells per 10^6 cells, were calculated with the maximum likelihood estimation, adapted with a jackknife method as described by Strijbosch et al (1987). Standard deviation and 95% confidence intervals (95% CI) were calculated as well. The calculated frequencies were accepted when the goodness-of-fit did not exceed 12. Subsequently, the fraction of donor specific CTL having high avidity for donor Ag could be calculated using the following formula:

$$\text{fraction (\%)} = 100\text{x} \frac{\text{freq of donor spec. CTL with CD8 mAb}}{\text{freq of donor spec. CTL without CD8 mAb}}$$

Statistics. To determine the importance of allograft specific CTL in the process of transplant rejection, the frequency and avidity of donor directed CTL present during rejection were compared to values found before and after this period. Differences between these categories were analyzed using the Mann-Whitney-U-test.

2.3. Results

Endomyocardial biopsies, routinely taken from HTx patients for the diagnosis of acute cardiac transplant rejection, were cultured to obtain GIL. Only EMB obtained within 4 months after transplantation were considered for the present study. Thirty-three percent of EMB without histological evidence of acute rejection grew sufficient GIL to allow for functional analysis. A significant higher percentage (50%; $p=0.01$) was found for rejecting EMB. Cytotoxicity towards donor HLA class I Ag was found in 83% and 61% of the GIL cultures established from EMB with and without histological signs of rejection, respectively. For the present study, GIL cultures established from 37 EMB of 11 patients were examined. No differences were found for the GIL cultures obtained from EMB with ($n=7$) or without ($n=30$) histological signs of rejection, regarding culture time (median= 25 days: range= 18-36 days, and 29 days: range= 14-47 days, respectively) nor percentage of $CD8^+$lymphocytes present (median= 58%: range= 8-84%, and 46%: range= 1-100%, respectively). Phenotypic analysis demonstrated that all GIL cultures examined consisted of T cells only. In addition to $CD8^+$lymphocytes, $CD4^+$T cells were present whereas TCR$\gamma\delta$ cells and NK cells were absent.

Control experiments. Both frequency and avidity of donor specific CTL present within GIL cultures were determined using LDA. To control for donor specific target cell lysis in the cytotoxic phase of the LDA, the reactivity of GIL cultures against PHA blasts of third party cells and against the K562 cell line was tested. No lysis of third party nor K562 cells could be detected.

Five $CD8^+$CTL clones obtained from GIL cultures of HTx patient FO were tested for donor directed cytotoxicity in a CML assay. Representative results are shown for 2 clones; FO810 and FO84. Addition of CD8 mAb (FK18) totally inhibited donor target cell lysis by FO810 cells but did not affect the cytotoxic capacity of FO84 cells (figure 1). Consequently, FO810 cells have low avidity for donor Ag whereas FO84 cells have high avidity for donor Ag. In addition, FO810 cells could not lyse donor target cells pretreated with TP25.99 whereas FO84 cells could. This observation demonstrates that differences in avidity reflected by FK18 sensitivity are indeed caused by blocking the interaction between a CD8 molecule on CTL and their counterpart (the α_3-domain of HLA class I molecules) on target cells.

GIL cultures regularly exhibited cytotoxicity towards donor HLA class II determinants. Analysis of donor HLA class II specific, $CD4^+$CTL clones ($n=8$) demonstrated that these cells efficiently lyse donor B-LCL but fail to lyse PHA blasts of donor origin. Since, donor

PHA blasts were used as target cells in the present study, all frequencies of donor specific CTL estimated by LDA concern donor HLA class I specific CTL and not donor HLA class II specific CTL.

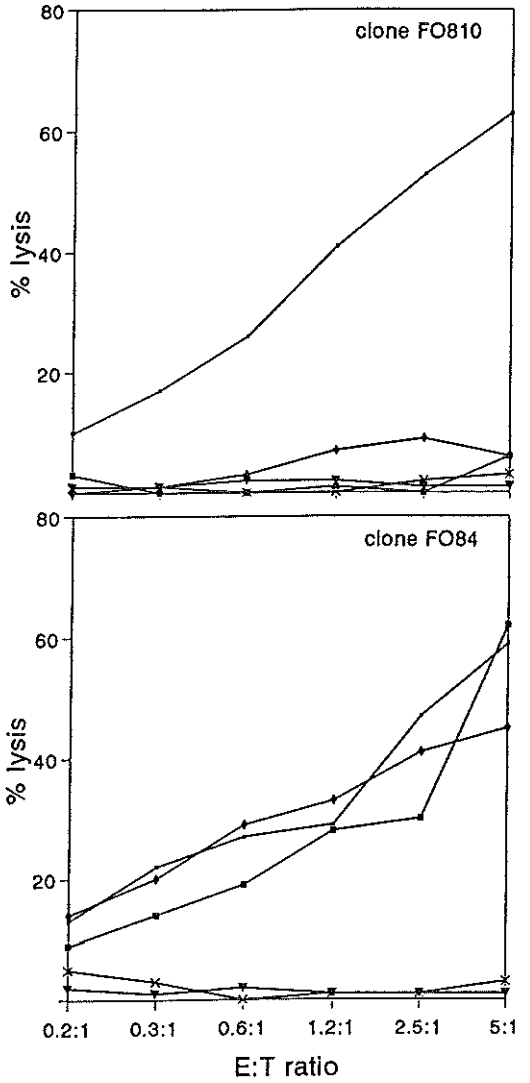

Figure 1: Lysis of ^{51}Cr-labelled PHA-blasts of donor origin (→) by CD8$^+$CTL clone FO810 (upper panel) and FO84 (lower panel) at different effector:target cell (E:T) ratios. Addition of either FK18 (■; a CD8 mAb) or TP25.99 (◆–; a mAb directed against the α_3-domain of HLA class I molecules) inhibited target cell lysis by FO810 cells but did not affect the cytotoxic capacity of FO84 cells. To control for donor specificity, the cytotoxicity of the clones towards an unrelated third party PHA-blast (–▼) and towards the K562 cell line (x) was tested.

The frequency of graft infiltrating donor specific CTL increases during the development of cardiac transplant rejection.

Total group of patients: 37 GIL cultures of 11 HTx patients were classified into 4 groups: those propagated from EMB obtained more than 2 weeks preceding rejection (>2 wks), within 2 weeks preceding rejection (2-1 wks), at time of rejection (R) and after rejection therapy (post R). Subsequently, donor specific pCTL and cCTL present within these GIL cultures were enumerated by LDA. Results are shown in figure 2. When several GIL cultures of a single patient were assigned to one group, the average pCTL and cCTL frequency was used.

Precursor CTL frequencies found during rejection (median= $3688/10^6$) did not significantly exceed values found more than 2 weeks preceding rejection (median= $697/10^6$, p=0.181), within 2 weeks preceding rejection (median= $497/10^6$, p=0.180), nor after rejection therapy (median= $1799/10^6$, p=0.456). A distinct pattern was found for donor directed cCTL (figure 2). More than 2 weeks before histological diagnosis of rejection, a median frequency of 26 cCTL/10^6 was found. Within 2 weeks preceding rejection this frequency increased 9-fold, reaching maximal levels during rejection (median= $1376/10^6$). This increase was significant (p=0.008) as was the decrease in cCTL frequency after therapy (median= $459/10^6$; p=0.018).

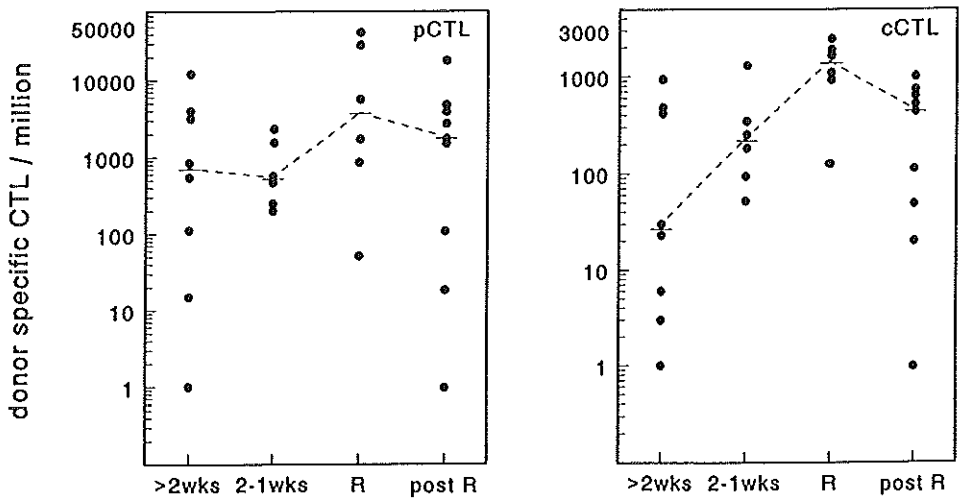

Figure 2: Appearance of donor specific CTL in relation to acute cardiac rejection: kinetics within the total group of HTx recipients. Frequencies of graft infiltrating donor specific pCTL (left) and cCTL (right) found more than 2 weeks preceding rejection (>2wks), within 2 weeks preceding rejection (2-1 wks), during rejection (R) and after rejection therapy (post R) are shown. No significant difference in pCTL frequency was found. In contrast, cCTL frequencies increased significantly (p=0.008) towards rejection and recovered to basal levels after treatment (p=0.018).

Individual patients: Of 5 HTx patients, GIL cultures were obtained from EMB taken before, during, as well as after cardiac transplant rejection. Using these serial GIL cultures, the kinetics of graft infiltrating donor specific CTL in the pathogenesis of rejection could be studied for individual patients. Table 1 shows donor specific tCTL, cCTL, and pCTL (tCTL-cCTL) frequencies found during rejection (R) as well as n days before (-n) or after (+n) rejection. Donor directed pCTL and cCTL frequencies found during clinical rejection were compared to values found before and after this period. In figure 3, mean pre- and post-rejection frequencies are depicted for each patient and combined with the CTL frequency found at the time of rejection.

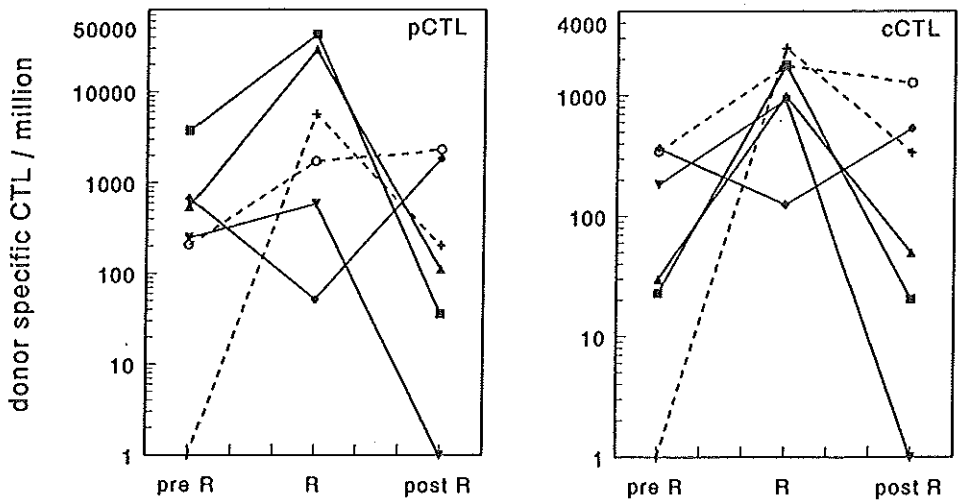

Figure 3: Appearance of donor specific CTL in relation to acute cardiac rejection: kinetics within patient ER (▲),KO (+; R1), KO (○; R2), MO (▼), VE (◆) and FO (■). Donor specific pCTL (left) and cCTL (right) frequencies found within the graft during rejection (R) exceeded values found before as well as after rejection.

With exception of patient VE, the frequency of donor directed pCTL and cCTL increased during rejection. The median donor directed pCTL frequency found before rejection, $265/10^6$, increased to $3688/10^6$ during rejection. The median donor specific cCTL frequency increased from $107/10^6$ to $1376/10^6$. After successful rejection therapy, the median pCTL ($74/10^6$) and cCTL ($35/10^6$) frequency dropped below values found before rejection. Patient KO, who experienced 3 rejection periods, is depicted by dotted lines. Three weeks before rejection was diagnosed, donor specific CTL were hardly detectable. During rejection, the frequency of donor directed pCTL and cCTL increased enormously to $5649/10^6$ and $2459/10^6$, respectively. After rejection therapy, the number of pCTL ($209/10^6$) and cCTL ($346/10^6$) decreased but still exceeded pre-rejection levels. A second rejection was diagnosed 1 week later. At the time of this second rejection, the frequency of donor directed pCTL and cCTL increased again to $1728/10^6$ and $1767/10^6$, respectively. After rejection therapy, the

frequency remained rather high (2327/10^6 pCTL; 1299/10^6 cCTL) and a third rejection was diagnosed 2 weeks later.

CTL with high avidity for donor Ag gradually accumulate in the pathogenesis of acute cardiac rejection.

Total group of patients: Significant more CTL with high avidity for donor Ag could be propagated from EMB obtained within 2 weeks preceding rejection than from EMB with comparable rejection grades taken before this period ($p=0.007$) or after rejection therapy ($p<0.0001$). Thus, despite histological resemblance, graft infiltrating donor directed CTL present at various timepoints around rejection have different characteristics. To investigate the involvement of high avidity CTL in the development of cardiac rejection, the fraction of graft infiltrating donor specific CTL having high avidity for donor Ag was determined throughout the rejection process. Figure 4 shows the kinetics for the total group of HTx patients tested. When donor specific CTL were absent, their avidity could not be determined.

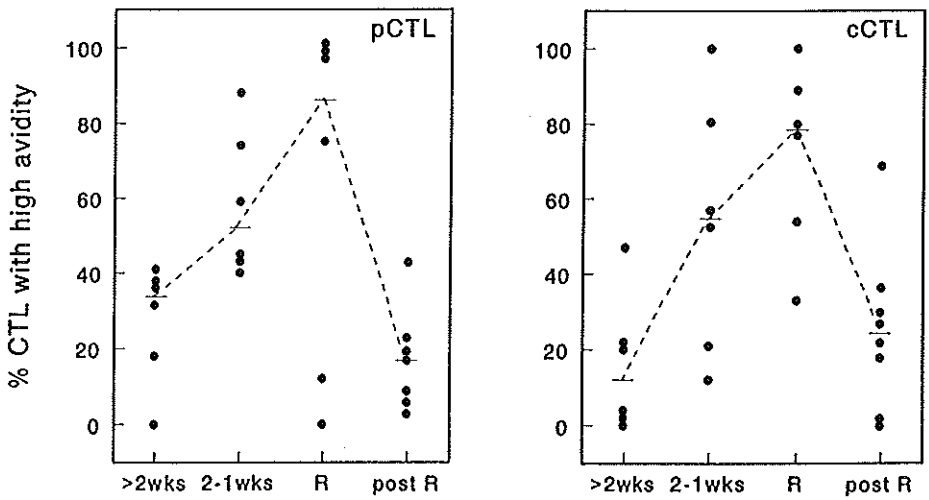

Figure 4: Appearance of high avidity CTL in relation to acute cardiac rejection: kinetics within the total group of HTx recipients. Fractions of graft infiltrating donor specific pCTL (left) and cCTL (right) with high avidity for donor Ag are depicted for the periods of more than 2 weeks before rejection (>2 wks), within 2 weeks preceding rejection (2-1 wks), during rejection (R) and after rejection therapy (post R). The fraction of donor directed cCTL with high avidity for donor Ag increases significantly during rejection ($p=0.004$) and decreases after treatment ($p=0.005$).

More than 2 weeks before rejection, a small fraction of graft infiltrating donor specific pCTL appeared to have high avidity for donor Ag (median= 35%). Within 2 weeks preceding rejection, this fraction increased significantly, (median= 52%; $p=0.004$) and became dominant during rejection (median= 87%). After successful rejection therapy, the fraction of high avidity pCTL dropped to levels found for patients who never experienced rejection (median= 18%). Similar results were obtained for graft infiltrating donor specific cCTL.

More than two weeks preceding rejection, only 11% (median) of donor specific cCTL had high avidity for donor Ag. Within 2 weeks preceding rejection, this fraction increased significantly (median= 55%; p=0.04) and became dominant during rejection (median= 78.5%; p=0.004). After successful rejection therapy, the fraction of donor directed cCTL with high avidity for donor Ag decreased significantly (median= 24% p=0.005).

Individual patients: The kinetics found for 5 single patients confirmed the pattern found for HTx patients in general (figure 5). During rejection, the fraction of graft infiltrating pCTL with high avidity for donor Ag (median 87.5%) exceeded the fractions found before rejection (median= 43%) as well as after rejection therapy (median= 6%) in 4 out of 5 patients. A comparable course was found for donor directed cCTL. For all patients, the fraction of cCTL with high avidity for donor Ag increased during rejection (median= 78.5%) and decreased after rejection therapy (median= 22%) to pre-rejection levels (median= 21%).

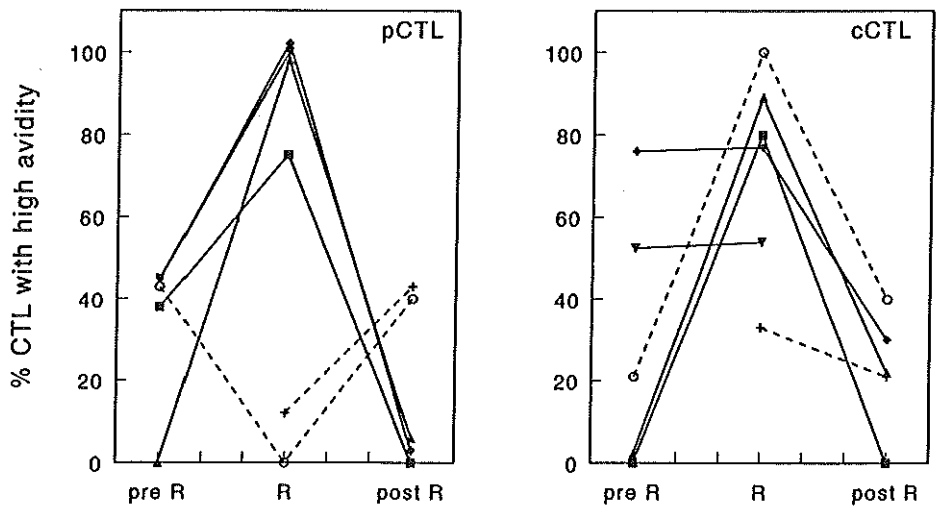

Figure 5: Appearance of high avidity CTL in relation to acute cardiac rejection: kinetics within patient ER (▲), KO (+; R1), KO (○; R2), MO (▼), VE (◆) and FO (■). During rejection (R), an increased fraction of donor specific pCTL (left) and cCTL (right) present had high avidity for donor Ag.

2.4. Discussion

To investigate the importance of donor specific CTL in the pathogenesis of acute cardiac rejection, the frequency of graft infiltrating donor directed pCTL and cCTL present during clinical rejection was compared to values found before and after this period within the same patient. From this study, it appeared that donor specific pCTL and cCTL are present in the graft during rejection as well as during periods in which no histological signs of myocyte damage are observed. Studies using rat allograft models, showed the presence of donor

specific CTL within the graft to correlate with tissue destruction and that these cells were absent in grafts that survived indefinitely (Mason and Morris, 1984; Bradley et al, 1985). Other studies described the presence of graft specific CTL within non-rejected rat kidneys (Dallman et al, 1987) and within cardiac allografts of patients who never clinically rejected their graft (Ouwehand et al, 1993). On first sight, these contradicting results question the importance of donor specific CTL in transplant rejection. The present report, however, demonstrates that the number of donor specific pCTL and cCTL present within the graft increases during rejection. Moreover, accumulation of donor reactive cCTL is initiated before rejection is diagnosed suggesting that the number of donor directed cCTL present within the graft is important for the induction of transplant rejection.

The presence of donor specific pCTL within GIL cultures was not expected since only activated IL2-receptor expressing T-lymphocytes were postulated to expand from EMB under the culture conditions used (Mayer et al, 1985; Zeevi et al, 1986). Possibly, the concentration of IL2 used resulted in expansion of all lymphocytes present within the biopsy. Alternatively, the donor directed pCTL measured were not totally naive pCTL nor fully mature cCTL but possibly resembled CTL between these two differentiation stages; the poised CTL as proposed by Gromo et al. (1987). These CTL express IL2 receptors but require donor Ag to differentiate into cCTL. The heterogeneity of the pCTL pool would also explain why the rejection process is more unambiguous reflected by changes in the cCTL frequency than by changes in the pCTL frequency.

According to the frequencies shown, GIL cultures do not totally consist of donor HLA class I specific CTL. Previous studies by LDA revealed that donor specific T-helper lymphocytes (unpublished results) and CTL specific for third party HLA determinants are present within EMB derived GIL cultures as well (Suitters et al, 1990; Ouwehand et al, 1993). Additionally, GIL cultures may contain donor HLA class II specific CTL (Ouwehand et al, 1991), T-helper lymphocytes specific for third party HLA Ag, or T-lymphocytes that recognize heat shock proteins (Moliterno et al, 1995).

The fraction of graft infiltrating donor specific CTL with high avidity for donor Ag could be estimated after addition of CD8 mAb to the cytotoxic phase of the LDA. The fraction of donor specific pCTL and cCTL with high avidity for donor Ag increased towards rejection and became dominant during rejection. After rejection therapy, this fraction recovered to low levels. The avidity of graft infiltrating CTL for donor Ag, therefore, seems to be a discriminating factor between rejection and "non-rejection".

Only 2 other groups have studied the presence of high avidity CTL in clinical allograft models. Both groups examined PBL instead of GIL. Prior to clinical signs of transplant rejection, donor specific, CD8 mAb resistant, tCTL were found in the peripheral blood of kidney transplant recipients. In contrast, circulating donor specific tCTL, whose lytic capacity was almost completely blocked by CD8 mAb, were found in patients with a well functioning graft two years post-transplantation (De Hoop et al, 1994). Similarly, circulating donor specific tCTL of patients with an ongoing rejection of their corneal allograft were

significantly less inhibited by CD8 mAb than circulating tCTL of patients with good graft functioning (Roelen et al, 1995). Comparable results were obtained by our group using PBL of HTx patients (Vaessen et al, 1995).
Since donor specific cCTL are seldomly, if ever, detected in the circulation (Orosz and Bishop, 1990; Vaessen et al, 1992) we postulate that, prior to clinical rejection, donor specific pCTL with high avidity migrate to the transplant. At the graft side, these pCTL accumulate or are selectively expanded and differentiate into donor reactive cCTL with high avidity. Sufficient numbers of high avidity cCTL will then cause graft damage as observed histologically. After successful rejection treatment, the frequency of donor specific CTL as well as the fraction with high avidity decreases to basal levels. These alloAg specific CTL possibly died due to the rejection therapy given.
Several observations support our assumption that donor specific CTL with high avidity for donor Ag are responsible for cardiac transplant rejection. First of all, this study shows that high avidity CTL are hardly detectable in periods of "non-rejection". Likewise, graft infiltrating CTL found after successful rejection therapy (Baan et al, 1995), in non-rejectors (Ouwehand et al, 1993; Vaessen et al, 1994) or in neonatally made tolerant mice (Wood and Steilein, 1987) mainly have low avidity for donor Ag. Secondly, donor myocyte damage coincides with an enormous intragraft accumulation of donor directed CTL with high avidity. It has been described that high avidity CTL are reactive to cells expressing even low levels of HLA molecules whereas an increase in HLA surface density on target cells is required to induce lysis by low avidity CTL (Shimonkevitz et al, 1985; Mentzer et al, 1990). This illuminates our observation that the presence of donor specific CTL within the transplant not necessarily leads to graft damage. During periods of non-rejection, the HLA class I and II expression on myocytes is low (Milton and Fabre, 1985). As a result, binding and subsequent lysis of donor target cells by the low avidity CTL present is unlikely to occur. However, despite their low surface expression of HLA alloAg, myocytes serve as targets when high avidity donor specific CTL enter the graft and a rejection period is encountered. Direct evidence for the innocent bystander activity of low avidity CTL in vivo was only recently provided. Anti-K^b Des-TCR transgenic mice able to reject K^b skin grafts failed to do so after exclusive deletion of high avidity K^b specific T cells (Hoffmann et al, 1995).
Some reports provided evidence that the avidity of CTL can be influenced by the HLA-alloAg expression on target cells. An increase in the number of interactions possible between CTL and target cells was found to reduce the CTLs susceptibility to CD8 blocking (Mentzer et al, 1990; Hill et al, 1992). Likewise, a decrease in alloAg density on target cells reduced the avidity of the CTL (Maryanski et al, 1988). In our test system, however, LDA was conducted simultaneously on all GIL samples of each individual patient. In this way, the enhanced CTL avidity found shortly before and during rejection can not be ascribed to the target cells used.
Conclusively, data presented in this article imply that sufficient numbers of donor specific CTL with high avidity induce transplant rejection.

Table 1. Donor specific tCTL, cCTL and pCTL frequencies in absence or presence of CD8 mAb.

RE[1]	R[2] -/+ n days	FREQ tCTL	(95% CI)	FREQ tCTLFK[3]	(95% CI)	FREQ cCTL	(95% CI)	FREQ cCTLFK	(95% CI)	FREQ pCTL	FREQ pCTLFK
ER	-49	566	(434-697)	11	(0-27)	30	(6-55)	20	(0-39)	546	0
ER	R	29889	(19304-40475)	32542	(20968-44117)	986	(752-1220)	877	(673-1080)	28903	31665
ER	+21	162	(85-240)	29	(0-63)	50	(6-94)	11	(0-33)	112	18
KO	-22	0	(0-0)	0	(0-0)	0	(0-0)	0	(0-0)	0	0
KO	R1[4]	8101	(6066-10137)	1458	(1115-1801)	2459	(1907-3012)	800	(618-982)	5649	658
KO	+6/-7	555	(420-689)	161	(103-219)	346	(254-437)	72	(33-110)	209	89
KO	R2	3495	(2469-4520)	1549	(1179-1918)	1767	(1305-2228)	1937	(1488-2387)	1728	0
KO	+21/-14	3626	(2769-4484)	1671	(1296-2046)	1299	(955-1643)	742	(581-902)	2327	929
KO	R3	ND		ND		ND		ND		ND	ND
MO	-14	279	(193-365)	132	(79-186)	222	(150-295)	132	(79-186)	57	0
MO	-7	587	(440-734)	464	(331-597)	145	(71-219)	65	(20-110)	442	399
MO	R	1790	(1429-2151)	1538	(1186-1890)	925	(702-1148)	502	(361-643)	865	1036
MO	+21	0	(0-0)	0	(0-0)	0	(0-0)	0	(0-0)	0	0
MO	+24	graft failure									
VE	-21	1328	(1035-1621)	376	(276-475)	480	(363-598)	226	(157-295)	848	150
VE	-14	983	(757-1209)	723	(526-921)	448	(346-550)	505	(380-630)	535	218
VE	-7	510	(350-669)	381	(246-516)	59	(25-94)	36	(7-65)	451	345
VE	R	178	(119-238)	218	(142-294)	126	(75-178)	97	(48-147)	52	121
VE	+35	2388	(1869-2907)	214	(141-288)	537	(384-691)	160	(103-217)	1851	54
FO	-27	3799	(2594-5004)	1437	(1017-1858)	23	(15-31)	11	(0-33)	3776	1426
FO	R	44305	(25423-63187)	33165	(21339-44991)	1784	(1410-2157)	1433	(1141-1724)	42521	31732
FO	+7	36	(7-65)	6	(0-17)	0	(0-0)	0	(0-0)	36	6
FO	+76	43	(11-74)	11	(0-27)	41	(12-70)	17	(0-37)	2	0

Donor specific tCTL, cCTL and pCTL (tCTL-cCTL) present within cardiac allografts of 5 heart transplant recipients[1] at the time of rejection (R) as well as n days before (-n) or after rejection (+n)[2] were enumerated by LDA. Subsequently, the frequency of donor specific CTL with high avidity for donor Ag was determined by addition of CD8 mAb (FK18)[3] to the LDA. The frequency of donor specific CTL as well as the fraction of these CTL having high avidity for donor Ag increases during rejection and decreases after rejection therapy. Primary rejections are stated as R1[4]. Likewise, secondary and third rejections are stated as R2 and R3, respectively. One patient (MO) suffered from total graft disfunctioning, as a result graft infiltrating CTL frequencies were below detection limits at that time.

Chapter 3

The avidity, not the mere presence, of primed CTL for donor HLA class II antigens determines their clinical relevance after heart transplantation

To analyze the relevance of CD4-positive cytotoxic T lymphocytes (CTL) in clinical cardiac rejection, we studied the frequency and avidity of donor HLA class II specific CTL present within the graft during a rejection episode and during a period without rejection. For this analysis, endomyocardial biopsies (EMB) of heart transplant recipients were cultured to obtain graft infiltrating lymphocytes (GIL). GIL cultures exhibiting donor class II directed cytotoxicity were considered for the present study. Using limiting dilution analysis (LDA), the frequency of donor class II specific CTL that had been primed by donor antigens (Ag) in vivo (designated cCTL) was determined in GIL cultures established from EMB taken during a rejection episode ($n=10$) or during a period without rejection ($n=11$). Addition of anti-CD4 to the LDA, revealed the fraction of donor class II specific cCTL having a high avidity for donor Ag. During a rejection episode, 196 (median) donor class II specific cCTL/10^6 GIL were present. In a period without rejection, the frequency of donor class II specific cCTL was not significantly different (median=330/10^6;$p=0.1$). Addition of anti-CD4, however, revealed that donor class II specific cCTL with a high avidity for donor Ag are predominant during a rejection episode (median=100%) but are in minority during a period without rejection (median=35%; $p<0.0001$). These results suggest that graft infiltrating $CD4^+$CTL can mediate cardiac rejection provided they have a high avidity for donor Ag.

3.1. Introduction

Acute rejection is most commonly seen within the first 3 months after cardiac transplantation (Balk et al, 1991) and is characterized by the presence of mononuclear cell infiltrates and myocyte damage or myocyte necrosis in the graft. In general, CTL are postulated to function as terminal effector cells, directly mediating tissue damage. Indeed, enumeration of donor HLA class I specific CTL propagated from sequential EMB of heart transplant (HTx) patients revealed that increased numbers of donor class I reactive CTL were present within the graft at the time of acute rejection. More importantly, during and immediately preceding acute rejection, these cells mainly had a high avidity for donor Ag; i.e. these CTL did not require CD8 molecules to stabilize their binding with donor target cells. In contrast, in the absence of rejection, graft infiltrating donor class I specific CTL mainly had a low avidity for donor Ag (Van Emmerik et al, 1996). This relationship between the CTLs avidity for donor class I Ag and the occurrence of graft rejection is supported by data of other groups (roelen et al, 1995; Hoffmann et al, 1995).

Unlike CTL specific for allogeneic HLA class I determinants, HLA class II allospecific CTL are rarely reported in correlation with clinical transplant rejection. In only 2 studies data have been obtained by testing GIL cultures for cytotoxicity towards donor class II determinants (Micelli et al, 1988; Ouwehand et al, 1991). Using GIL cultures established from biopsies performed at different time points after cardiac transplantation, we reported a significant decrease in the incidence of donor class II directed cytotoxicity more than 3 months after transplantation (Ouwehand et al, 1991). This decrease run parallel with a decline in the occurrence of acute rejection. Although this observation suggested an involvement of class II specific CTL during the most reactive phase after transplantation it did not prove a direct causal relation between class II alloreactivity and transplant rejection.

In the present report, we analyzed whether donor HLA class II specific CTL propagated from EMB taken during rejection episodes or during periods without rejection were distinct with respect to their frequency and avidity for donor Ag. LDA was employed to selectively quantitate CTL that had been primed by donor class II Ag in vivo (designated cCTL; Orosz et al, 1989). The fraction of these cCTL having a high avidity for donor class II Ag was determined by addition of CD4 mAb to the cytotoxic phase of the LDA. The CD4 molecule stabilizes the interaction between a T cell receptor (TCR) and a HLA class II-peptide complex. CTL which benefit most from such interactions and therefore are most dependent on CD4, express TCR with low affinity for their Ag. Likewise, CD4 independent CTL express TCR with high affinity for their Ag (Biddison et al, 1982; Marrack et al, 1983; Greenstein et al, 1985; Gougeon et al, 1985; Sleckman et al, 1987). Hence the effect of CD4 blocking on the cytotoxic capacity of CTL reveals their overall avidity for HLA class II determinants. Alternatively, it has been suggested that CD4 blocking directly delivers negative signals to the $CD4^+$CTL (Fleischer et al, 1986). Thus, inhibition of $CD4^+$CTL reactivity by CD4 mAb would reflect a negative signal transmitted by this antibody rather than their low avidity. However, as reviewed by De Vries et al. (1989) the "negative signal"

hypothesis is only valid in case of non-antigen specific T cell activation by CD3 mAb and therefore does not apply to the present report.
The results obtained demonstrate that cCTL with a high avidity for donor class II Ag are predominant within the graft during an acute rejection episode whereas cCTL with a low avidity for donor class II Ag are predominant within the graft during periods without rejection.

3.2. Materials and Methods

Patients. We studied 1409 EMB (135 with and 1274 without histological signs of acute rejection) obtained from 94 heart transplant patients during the first year after transplantation. These patients had mismatches for both HLA class I (HLA-A and B) and class II (HLA-DR) with their donor. All patients received a preoperative blood transfusion while cyclosporin A and low dose steroids only were used as maintenance immunosuppression. EMB were routinely taken at a weekly interval during the first 6 weeks after transplantation and once in two weeks in the following 4 weeks. Later EMB were taken less frequently, declining to once every 4 months at 1 year. Acute cardiac rejection was diagnosed by histopathology of EMB according to ISHLT criteria (Billingham et al, 1990). Anti-rejection treatment was instituted when mononuclear cell infiltrates coexisted with myocyte damage or myocyte necrosis (grade 3A or more) and consisted of bolus steroids or a 2-weeks course of a polyclonal rabbit anti-thymocyte globulin preparation in case of ongoing rejection.

Lymphocyte cultures. Graft infiltrating lymphocyte (GIL) cultures were established as described previously (Ouwehand et al, 1991). Briefly, each biopsy was cultured in a 96-well round bottom culture plate (Costar, Cambridge, MA) with 200 μl culture medium per well, in the presence of 10^5 irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMC) as feeder cells. Due to the absence of donor Ag in this system, no de novo stimulation can take place in vitro. As a result only those CTL that have already been primed by donor Ag in vivo (cCTL) will be functional against donor Ag. Naive precursor CTL (pCTL) will not differentiate into cCTL. Culture medium consisted of RPMI-1640 Dutch Modification (Gibco, Paisley, Scotland), supplemented with 4mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% pooled human serum. Additionally, 10% v/v lectin free lymphocult-T-LF (Biotest GmbH, Dreieich, Germany) was added as source of IL2. The plates were incubated in a humidified atmosphere at 37°C in 5% CO_2. Established GIL cultures were phenotyped and tested for cytotoxicity against HLA-A, B and DR determinants for which the cardiac donor and the recipient were mismatched.
Donor specific T cell clones were generated from GIL cultures by limiting dilution at 0.3 cells/well in culture medium supplemented with lymphocult-T-LF and 0.5-1% phytohaemagglutinin (PHA; Difco, Detroit, MI). Irradiated (30 Gy) donor B-LCL ($5x10^4$ c/ml) and PBMC of healthy blood donors ($5x10^5$ c/ml) were added as feeder cells. After 2

weeks, wells with visible cell growth were restimulated with irradiated donor B-LCL and PBMC of healthy blood donors. 7-14 days later, clones were phenotyped as described below and assayed for donor specific cytotoxicity. Clones used in the present study totally consisted of $CD4^+WT31^+$cells and exhibited cytotoxicity towards 1 HLA-DR determinant for which the recipient and donor were mismatched.

PBMC were isolated from heparinized venous blood of the transplant recipient by Ficoll-Hypaque density gradient centrifugation. Cells were washed twice in HBSS (Gibco) and once in RPMI-1640.

T-cell blasts were obtained by culturing PBMC or spleen cells for 4-7 days in culture medium supplemented with 10% v/v lymphocult-T (Biotest) and 1% PHA.

B-lymphoblastoid cell lines (B-LCL) originated from infection of fresh PBMC or spleen cells with Epstein Barr Virus obtained from the marmoset cell line B95-8 as described by Moreau et al. (1986). These cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, Utah).

Phenotypic analysis. GIL cultures and T cell clones established from GIL cultures were analyzed for WT31, CD8 (Becton Dickinson), and CD4 (immunotech, Marseille, France) expression by three-color flow cytometry on a FACScan after staining with mAb directly conjugated for fluorescein, PERPC, and phycoerythrin, respectively.

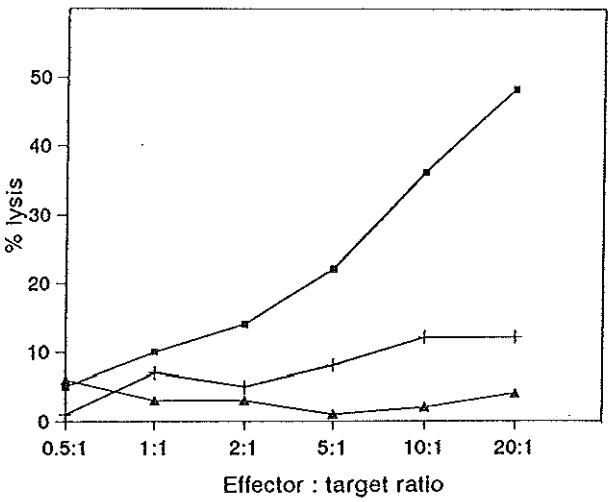

Figure 1: Lysis by $CD4^+$CTL clones of T cell blasts (+) and B-LCL (■) expressing similar HLA class II determinants. Lysis of unrelated B-LCL (▲) was used as a control for specificity.

Cell mediated lympholysis (CML). The cytotoxic capacity of GIL cultures (or T cell clones established from GIL cultures) was tested against donor cells and a panel of target cells sharing either HLA class I or class II determinants with the donor. Briefly, effector cells were incubated with 2500 ^{51}Cr-labelled target cells at different effector:target (E:T) ratios in

0.2 ml culture medium. After 4 hours of incubation, supernatants were harvested and the release of ^{51}Cr was measured as described in the LDA section.
B-LCL served as target cells to measure donor class I (HLA-A and B) directed cytotoxicity as well as donor class II (HLA-DR) directed cytotoxicity. T cell blasts served as target cells to measure donor class I directed cytotoxicity exclusively. The incapability of T cell blasts to serve as target cells for class II alloreactive CTL is based on CML assays using donor class II specific $CD4^+$CTL clones established from GIL cultures. These CTL clones (n=8) efficiently lysed donor B-LCL but failed to lyse donor T cell blasts even when used at high E:T (20:1) ratios (figure 1).
As a control for specificity, GIL cultures were tested against unrelated target cells and the K562 cell line. A CML assay was considered positive when the percentage of specific lysis of target cells exceeded 10% and the slope of the graph was positive.

Limiting Dilution Analysis. Limiting dilution cultures were set up in 96-well round bottom microculture plates (Costar). Responder cells (GIL) were titrated in 8 double dilution steps starting from 5000 cells per well to 39 cells per well. All dilutions were performed in 24 replicates and 50,000 irradiated (30 Gy) autologous PBMC were added to each well in a total volume of 0.2 ml culture medium supplemented with 20 U/ml rIL2 (Biotest). When autologous PBMC are used as stimulators, only in vivo activated CTL (cCTL) and not their naive precursors (pCTL) proliferate and develop LDA-detectable cytolytic activity (Orosz et al, 1989). After 7 days of culture, all 96-well plates were split into two and CD4 mAb was added to half of the split wells as described below. Subsequently, each well was individually tested for its capacity to lyse 2500 ^{51}Cr-labelled target cells.
As described in the CML section, B-LCL can serve as target cells for HLA class II specific CTL. However, by using donor B-LCL as target cells in LDA, the total sum of donor class I and II specific cCTL would be quantitated. To selectively quantitate donor class II specific CTL, B-LCL that lack HLA class I similarities but share HLA class II similarities with the donor are required. In the present report, we used B-LCL sharing HLA-DR Ag with the donor. LDA cultures were incubated for 4 hrs at 37°C in 5% CO_2. Supernatants were harvested using a Skatron harvesting system (Skatron-AS, Lierse, Norway) and release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Downers Grove, USA). Spontaneous and maximum release were defined by incubation of target cells with culture medium in the absence or presence of triton X-100 detergent (5% v/v solution in 0.01 TRIS buffer), respectively. Microcultures were considered cytolytic when the experimental lysis percentages exceeded 10%. As a control for specificity, the reactivity of cultures with the highest responder concentration was tested against third party B-LCL and against the K562 cell line.

CD4 inhibition study. Before addition of ^{51}Cr-labelled target cells to the LDA, half of the split wells were preincubated with 1 μg/ml RIV6, a mouse anti-human antibody of the IgG2A subclass directed against the CD4 molecule (a kind gift of Dr. M.F. Leerling, RIVM,

Bilthoven, The Netherlands) during 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2. This concentration of CD4 mAb totally inhibited the cytotoxic capacity of CD4-dependent CTL clones but did not affect target cell lysis by CD4-independent CTL clones (figure 4). As a control antibody, FK18 (a mouse anti-human antibody of the IgG3 subclass directed against the gp32 chain of the CD8 molecule) was used at a 1:500 dilution of ascitic fluid (2). Irrespective of their susceptibility to inhibition by RIV6, $CD4^+$CTL clones were not affected by FK18. Likewise, addition of FK18 to LDA cultures with the highest responder cell concentration did not influence their cytolytic response towards donor class II Ag.

Frequency calculation. Minimal estimates of graft infiltrating cCTL 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 (Strijbosch et al, 1987). In our test system, microcultures were only considered cytolytic when the experimental lysis percentages exceeded 10%. The frequency of donor reactive cCTL, expressed as the number of cCTL per 10^6 GIL, was calculated with the maximum likelihood estimation adapted with a jackknife method. The calculated frequencies were accepted when the goodness-of-fit did not exceed 12.
Subsequently, the fraction of class II allospecific cCTL having high avidity for donor HLA class II Ag could be calculated using the following formula:

$$\text{fraction (\%)} = 100x \frac{\text{freq of donor spec. CTL with CD4 mAb}}{\text{freq of donor spec. CTL without CD4 mAb}}$$

Statistics. The frequency and avidity of donor directed cCTL present during a rejection episode or during a period without rejection were compared using the Mann-Whitney-U-Test.

3.3. Results

CTL specific for donor class II Ag can be propagated from EMB of cardiac transplant recipients. To analyze the participation of both $CD8^+$CTL and $CD4^+$CTL in acute cardiac rejection, we tested the cytotoxic function of 525 lymphocyte cultures established from 1409 EMB obtained from 94 heart transplant recipients during the first year following transplantation. To distinguish between the activity of donor specific $CD8^+$CTL and $CD4^+$CTL, target cells were used sharing either HLA class I or class II determinants with the donor. Results of the CML assays are shown in figure 2. The presence of donor specific CTL appeared not to be restricted to grafts showing histological evidence of acute rejection (i.e. myocyte damage). Donor directed cytotoxicity was observed in 87% and 76% of the lymphocyte cultures established from EMB with and without myocyte damage, respectively.

Of these donor reactive cultures, no less than 60% exhibited cytotoxicity towards donor HLA class II Ag (either alone or in combination with reactivity towards donor class I Ag).

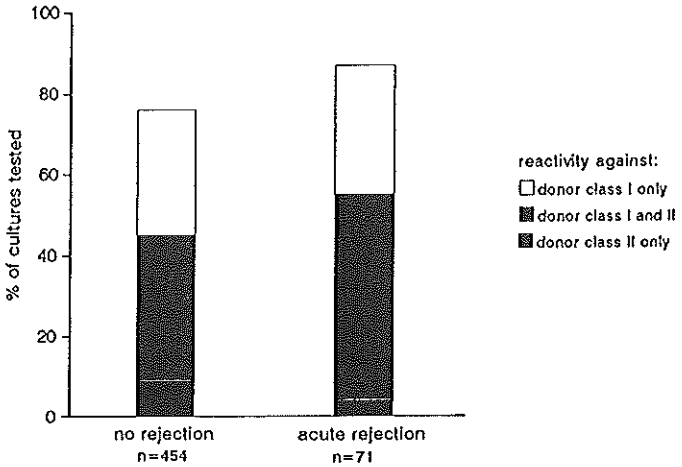

Figure 2: The cytotoxic capacity of lymphocyte cultures established from EMB of cardiac transplant recipients was tested against a panel of target cells sharing either HLA class I or class II Ag with the donor. Reactivity towards donor HLA class I and/or class II determinants was observed both in cultures obtained from EMB with and without histological signs of rejection.

Acute cardiac rejection does not coincide with an increased frequency of donor class II specific cCTL. Donor class II specific cCTL were enumerated within 21 GIL cultures showing donor class II specific cytotoxicity in CML assays. Ten GIL cultures were established from EMB taken during a rejection episode (8 EMB were obtained at the time of acute rejection, 2 EMB were obtained 1 week preceding acute rejection). Eleven GIL cultures were obtained from EMB taken during a period without rejection. EMB depicting a rejection episode were taken early after transplantation [median=40 days post-transplant (range=21-125 days)] whereas EMB representing a period without rejection were obtained at later timepoints [median=213 days post-transplant (range=122-280 days)]. No differences were found in the percentage of $CD4^+$lymphocytes present [median=37% (range=2-99%) and 45% (range=1-98%), respectively], the culture time [median=29.5 days (range=20-45 days) and 34 days (range=25-79 days) respectively] nor the number of HLA-DR disparities between donor and acceptor [mean=1.50 and 1.64, respectively].

In figure 3A, frequencies of cCTL reactive to target cells expressing donor HLA class II Ag are depicted. During a rejection episode, 196 (median; range=48-404) donor class II reactive cCTL/10^6 GIL were found. In a period without rejection, the frequency of donor class II reactive cCTL was not significantly different (median=330/10^6 GIL; range=95-2799; p=0.10). No reactivity towards the K562 cell-line nor towards third party B-LCL was observed.

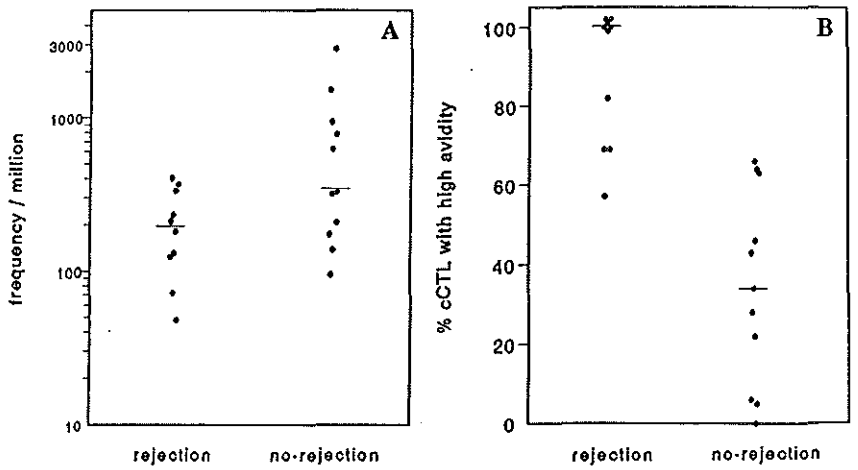

Figure 3: Donor HLA class II specific cCTL propagated from EMB taken during a rejection episode or during a period without rejection were enumerated by LDA. The frequency (figure 3A; median) of donor class II specific cCTL found within both periods is comparable ($p=0.1$; Mann-Whitney-U-test). The fraction of these cCTL having a high avidity for donor Ag was determined by addition of RIV6 to the cytotoxic phase of the LDA (figure 3B). A significant higher fraction (median) of the donor class II specific cCTL had a high avidity for donor Ag during a rejection episode than during a period without rejection ($p<0.0001$; Mann-Whitney-U-test).

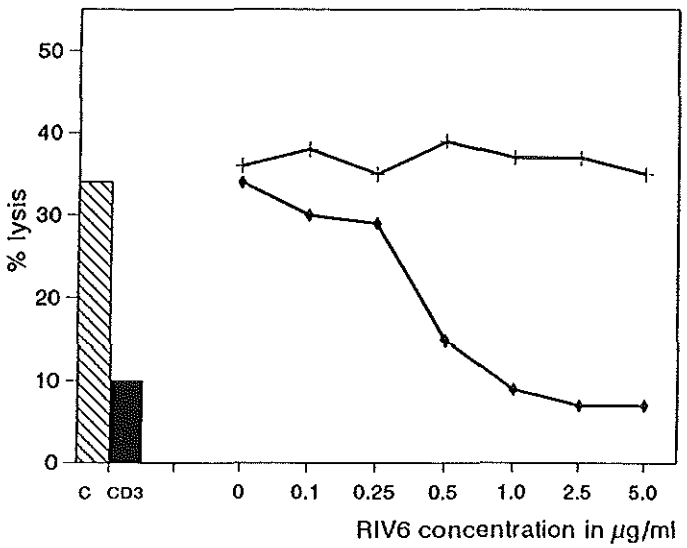

Figure 4: The susceptibility of class II directed CD4⁺CTL clones for RIV6 is depicted. RIV6 did not affect the cytolytic capacity of clone 39 (+; E:T= 2.5:1) but effectively inhibited target cell lysis by clone 18 (■; E:T= 20:1). Based on the dose-response curves, 1 µg/ml RIV6 was added to the cytotoxic phase of LDA to discriminate between CTL with a high (RIV6 resistant) and those with a low avidity for class II Ag (RIV6 sensitive). Addition of the control antibody, FK18 (a murine anti-human CD8 mAb) did not affect the cytotoxic activity of the clones.

Acute cardiac rejection is associated with the quality of the donor class II specific cCTL population present. The ability of $CD4^+$CTL clones to exhibit class II directed cytotoxicity in the presence of different doses of RIV6 (a CD4 mAb) was tested in a CML assay. Representative results are shown in figure 4. Concentrations of RIV6 as high as 5 μg/ml did not affect the cytolytic capacity of clone 39. In contrast, target cell lysis by clone 18, even when used at an E:T ratio of 20:1, was strongly inhibited by RIV6. Hence, clone 39 cells have a high avidity for Ag whereas clone 18 cells have a low avidity for Ag. As a control antibody, FK18 (a CD8 mAb) was used (2). This antibody did not affect target cell lysis by $CD4^+$CTL clone 18 nor by $CD4^+$CTL clone 39.

We added RIV6 at a concentration of 1 μg/ml to the cytotoxic phase of the LDA. As depicted by figure 4, this concentration of RIV6 is very effective to discriminate between class II specific CTL with either a high or a low avidity for Ag. The fraction of donor class II specific cCTL having a high avidity for donor Ag is shown in figure 3B. A prominent fraction of the donor class II specific cCTL propagated from EMB taken during a rejection episode had a high avidity for donor Ag (median=100%; range= 57-100%). In contrast, a significant smaller fraction of the donor class II specific cCTL propagated from EMB obtained during a period without rejection had a high avidity for donor Ag (median=35%; range= 0-66%; $p<0.0001$). These data show that cCTL with a high avidity for donor class II Ag are selectively present within the cardiac allograft immediately preceding and during acute rejection.

3.4. Discussion

Although it is well accepted that T cells mediate acute allograft rejection, there is still controversy over which T-cell subset is responsible for the tissue destruction. Since accumulation of donor specific CTL in allografts coincides with graft destruction (Suitters et al, 1990; Bishop et al, 1992; Van Emmerik et al, 1996), it is tempting to believe that CTL act as terminal effector cells. Support for the involvement of CTL is provided by experiments showing the exquisite antigen specificity of the alloresponse (Mintz et al, 1967; Sutton et al, 1989) and the ability of CTL clones grown from rejecting allografts to destroy allogeneic tissue when injected into appropriate hosts (Snider et al, 1986). In contrast, experiments showing the occurrence of graft rejection in the absence of $CD8^+$T cells have been interpreted as evidence against a critical role for CTL in transplant rejection (Simpson, 1993). This interpretation, however, is dependent on the assumption that all cytotoxic T cells are contained in the $CD8^+$subset thereby neglecting the existence of $CD4^+$CTL.

In the present report, the contribution of $CD4^+$CTL to clinical cardiac rejection is analyzed and compared to data on $CD8^+$CTL obtained by previous studies. First, we studied whether the presence of donor specific $CD8^+$CTL and $CD4^+$CTL within the graft is associated with acute cardiac rejection. For this analysis, graft infiltrating lymphocytes propagated from routinely taken EMB were tested for cytotoxicity towards HLA class I and II determinants

for which the acceptor and donor were mismatched. In general, class I directed cytotoxicity is more frequently observed than class II directed cytotoxicity (figure 2). This might be due to the distribution of potential target cells in the transplanted human heart. Whereas HLA class I expression is observed on both myocytes and interstitial structures, HLA class II is only expressed on interstitial structures.

Lymphocyte cultures established from EMB with histological signs of acute rejection frequently displayed cytotoxicity towards donor HLA class I and/or class II determinants. Yet, EMB without histological signs of rejection also regularly grew lymphocytes exhibiting donor class I and/or class II directed cytotoxicity in vitro. If CTL are implicated in allograft rejection, the question arises why these latter CTL do not mediate destruction of the graft from which they were recovered.

Enumeration of donor HLA class I specific cCTL propagated from sequential EMB of heart transplant recipients recently revealed that increased numbers of donor class I reactive cCTL were present within the graft at the time these patients experienced acute rejection. More importantly, it appeared that class I specific cCTL present within the cardiac allograft during a rejection episode or during a period without rejection had different characteristics. During and immediately preceding acute rejection, graft infiltrating donor class I specific cCTL mainly had a high avidity for donor Ag. During a period without rejection, intragraft donor class I specific cCTL mainly had a low avidity for donor Ag (Van Emmerik et al, 1996). Accordingly, the frequency and avidity of donor specific cCTL seem to be discriminating factors between rejection and no-rejection.

Subsequently, we analyzed whether donor class II specific cCTL propagated from EMB taken during a rejection episode or during a period without rejection were distinct with respect to their frequency and avidity for donor Ag. No differences were found for the number of donor class II specific cCTL present. During a rejection episode, however, the majority of the graft infiltrating donor class II specific cCTL had a high avidity for donor Ag. In contrast, during a period without rejection, intragraft donor class II specific cCTL were generally found to have a low avidity for donor Ag. These data imply that a predominance of high avidity cCTL among the graft infiltrating donor specific cCTL population is relevant for acute cardiac rejection.

Only a few other studies have been reported on the avidity of donor specific CTL in relation to transplant rejection. All of these studies considered $CD8^+$CTL directed towards donor class I Ag. Donor specific CTL present within the peripheral blood of corneal transplant patients experiencing rejection mainly had a high avidity for donor Ag whereas circulating donor specific CTL of corneal transplant patients with a good graft function mainly had a low avidity for donor Ag (Roelen et al, 1995). Kinetic analysis within individual heart transplant recipients demonstrated that a progressive accumulation of high avidity, donor specific cCTL within the allograft coincided with acute rejection (Van Emmerik et al 1996). In addition, early after cardiac transplantation when acute rejection is most frequently experienced, graft infiltrating CTL of patients that never rejected their allograft generally had a low avidity for

donor Ag (Vaessen et al, 1994). Likewise, CTL in heart transplants of neonatally made tolerant mice (Wood and Streilein, 1987) had a low avidity. According to these observations and the data presented in this report, high avidity donor specific CTL might be crucial for the occurrence of transplant rejection whereas low avidity donor specific CTL might be insignificant in vivo.

To explain this concept we suggest the following model. High avidity CTL have been described to lyse cells expressing even low levels of HLA molecules whereas a strong HLA surface expression on target cells is required for target cell lysis by low avidity CTL (Marrack et al, 1983; Gougeon et al, 1985; Shimonkevitz et al, 1995; Mentzer et al, 1990). In absence of acute rejection, the HLA class I and II expression on donor heart tissue is weak (Milton et al, 1985; Suitters et al, 1987). As a consequence, binding and subsequently lysis of donor target cells by the low avidity donor specific cCTL present is unlikely to occur. Nevertheless, donor cells with a low surface expression of HLA class I and II determinants might serve as targets when high avidity donor specific cCTL enter the cardiac allograft. Direct evidence for the inability of low avidity CTL to mediate tissue destruction in vivo was only recently provided. Anti-K^b TCR transgenic mice able to reject K^b skin grafts failed to do so after exclusive deletion of high avidity K^b specific T cells (Hoffmann et al, 1995).

Our opinion that cCTL exhibiting donor class II directed cytotoxicity might contribute to cardiac tissue destruction, is challenged by others. Rejection of cardiac allografts in mice depleted for CD8-bearing cells was reported to occur in the absence of detectable numbers of graft infiltrating $CD4^+$cCTL (Bishop et al, 1993). However, donor T cell blasts were used as target cells to enumerate these $CD4^+$cCTL. Since we have repeatedly failed to detect class II directed cytotoxicity in CML assays using T cell blasts (figure 1) we question whether $CD4^+$cCTL would still be undetectable in this experimental system when B cell blasts had been used as target cells instead.

To investigate the ability of CTL to mediate cardiac tissue destruction and if so, whether this ability depends on their avidity for donor Ag, one should actually use donor myocytes as target cells. Unfortunately, up to now one has failed to grow adult type myocytes to solve this issue.

In conclusion, cited literature and the results of the present study suggest that graft infiltrating cCTL with a high avidity for donor HLA class I or II Ag may be involved in cardiac allograft rejection whereas intragraft cCTL with a low avidity for either donor class I or class II Ag may not.

Chapter 4

Kinetics of circulating CTL precursors that have a high avidity for donor antigens: Correlation with the rejection status of the human cardiac allograft

Studies on graft infiltrating cells demonstrated that accumulation of cytotoxic T lymphocytes (CTL) with a high avidity for donor antigens (Ag) coincided with acute cardiac rejection. In the present study, we analyze whether such high avidity CTL are present within the peripheral blood of cardiac transplant recipients and whether their kinetics correspond with the rejection status of the allograft. Using limiting dilution analysis (LDA), donor specific CTL were enumerated in serial blood samples of 7 patients. From each patient, 7-11 samples were obtained during the first year after transplantation and 1-3 samples were obtained later. Enumerated donor specific CTL were divided into CTL with a high or a low avidity for donor Ag depending on their sensitivity to CD8-blocking. In contrast to the situation in the graft, donor specific CTL present within the peripheral blood were CTL precursors (pCTL) and not fully mature CTL (cCTL). The number of donor specific pCTL among peripheral blood cells fluctuated irrespective of the rejection grade of the allograft indicating that the frequency of circulating donor specific pCTL does not reflect the immunological status of the allograft. During acute cardiac rejection, 66% (median) of the circulating donor specific pCTL had a high avidity for donor Ag. This percentage significantly exceeded pre- and post-rejection values obtained during the first year post-transplantation (median=39% and 37%, respectively). The disparity in avidity increased even further more than 1 year after transplantation when stable engraftment was achieved. Among peripheral donor specific pCTL those with a high avidity were absent (median=0%). Hence, the avidity of circulating donor specific pCTL might inform us about the immune status of the cardiac allograft.

4.1. Introduction

Several attempts have been made to associate the number of donor specific CTL among peripheral blood mononuclear cells (PBMC) with the immunological status of the human allograft. Stable engraftment achieved long after transplantation was found to correspond with a reduction of circulating donor specific CTL in some (Herzog et al, 1987; Mathew et al, 1993; Hu et al, 1994) but not all cases (Zanker et al, 1993; Eberspächer et al, 1994). During a period of immunological activity, the relationship between the frequency of circulating donor specific CTL and the rejection status of the allograft appeared to be controversial as well. Reader et al. (1990), demonstrated that donor specific CTL frequencies found in blood samples of heart transplant recipients at the time of acute rejection exceeded those found in blood samples obtained in the absence of acute rejection. In contrast, no such distinction could be made according to data reported by others (Steinmann et al, 1990; Roelen et al, 1995). Apparently, the frequency of circulating CTL does not adequately reflect the rejection grade of the allograft.

In a previous study on heart transplant recipients, we demonstrated that donor specific CTL propagated from endomyocardial biopsies (EMB) obtained during a rejection episode were distinct from those obtained during a period without rejection. Whereas the former CTL mainly had a high avidity for donor Ag, the latter CTL generally had a low avidity for donor Ag (Ouwehand et al, 1993; Vaessen et al, 1994; Van Emmerik et al, 1997). These data suggest that the avidity of donor specific CTL might serve as a parameter to distinguish between "acute rejection" and "non-rejection". Subsequent kinetic studies within individual patients demonstrated that CTL with a high avidity for donor Ag accumulated within the graft shortly before and during acute rejection (Van Emmerik et al, 1996), which made us reasoning that these CTL might be present within the peripheral blood before they enter the graft.

In order to assess the relationship between the avidity of circulating donor specific CTL and the immunological status of the cardiac allograft, donor specific CTL present within serial PBMC samples of heart transplant recipients were enumerated by limiting dilution analysis (LDA). Subsequently, these CTL were divided into CTL having a high or a low avidity for donor Ag based on their resistance or sensitivity to CD8 mAb, respectively (MacDonald et al, 1982; Cai and Sprent, 1994; De Vries et al, 1989; Auphan et al, 1994). The fraction of donor specific CTL that had a high avidity for donor Ag was then compared with the rejection status of the graft in order to determine whether the avidity of circulating donor specific CTL is indicative of cardiac rejection.

4.2. Materials and Methods

Patients. Seven recipients of cardiac allografts form the basis of this report. These patients received a single preoperative blood transfusion. After transplantation, a 7 days course of OKT3 was given as induction therapy while only cyclosporin A and low dose steroids were

used as maintenance immunosuppression. The number of HLA mismatches between donor and recipient for the A, B, and DR antigens are shown in table 1. In the early posttransplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to ones every 4 months at 1 year. The rejection status of the graft was determined by applying standard histological criteria (Billingham et al, 1990). Anti-rejection treatment was instituted when mononuclear cell infiltrates coexisted with myocyte damage or myocyte necrosis (ISHLT criteria: 3A or 3B) and consisted of solumedrol or a 2-weeks course of a polyclonal rabbit anti-thymocyte globulin preparation in case of an ongoing rejection. After an acute rejection episode the next biopsy was taken 1 week following anti-rejection therapy. Along with EMB, peripheral blood samples were collected. PBMC were isolated from these blood samples by Ficoll-Hypaque density gradient centrifugation and stored at -140°C. Serial PBMC samples of individual patients were then used to analyze the kinetics of circulating donor specific CTL.

Table 1. Number of HLA mismatches between donor and recipient

patients	mismatches with the donor HLA-A	HLA-B	HLA-DR
BO	1	1	1
SE	2	2	2
HE	1	2	2
VE	2	1	1
SC	1	1	2
DO	2	1	2
HU	2	1	0

Allogeneic target cells. Phytohaemagglutinin (PHA) stimulated T cell blasts of donor origin were obtained by culturing donor spleen cells for 4-7 days in culture medium supplemented with 10% v/v lymphocult-T (Biotest) and 1% PHA (Difco, Detroit, MI). Culture medium consisted of RPMI-1640, Dutch Modification (Gibco), supplemented with 4mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% pooled human serum (sera were tested in a mixed lymphocyte culture assay and excluded from the pool if inhibitory or stimulatory effects on proliferation were observed).

Limiting Dilution Analysis. LDA was conducted simultaneously on all early PBMC samples of each individual patient to minimize experimental variation. Limiting dilution cultures were set up in 96-well round bottom microculture plates (Costar). Responder cells were titrated

in 8 double dilution steps starting from 50000 cells per well to 390 cells per well. All dilutions were performed in 24 replicates and 50000 irradiated (30 Gy) stimulator cells were added to each well in a total volume of 0.2 ml culture medium supplemented with 20 U/ml rIL2 (Proleukin, Eurocetus, Amsterdam, the Netherlands). As stimulator cells we used donor spleen cells. Additionally, wells with the highest responder cell concentration were stimulated with autologous PBMC. When autologous PBMC are used as stimulator cells, only in vivo activated CTL (cCTL) and not their naive precursors (pCTL) proliferate and develop LDA-detectable cytolytic activity (Orosz et al, 1989). When donor spleen cells are used, the total pool of donor specific CTL consisting of both donor directed cCTL and pCTL will expand. At day 7, half of the medium of the limiting dilution cultures was replaced by fresh culture medium containing 20 U/ml rIL2. After 10 days of culture, all 96-well plates were split into two and CD8 mAb was added to half of the split wells as described below. Subsequently, each well was individually tested for its capacity to lyse 2500 ^{51}Cr-labelled PHA blasts of donor origin. LDA cultures were incubated for 4 hrs at 37°C in 5% CO_2. Supernatants were harvested using a Skatron harvesting system (Skatron-AS, Lierse, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA). Spontaneous and maximum release were defined by incubation of target cells with culture medium in the absence or presence of Triton X-100 detergent (5% v/v solution in 0.01 M TRIS buffer), respectively. Microcultures were considered cytolytic when the experimental lysis percentages exceeded 10%.

CD8 inhibition study. A 1:500 dilution of ascitic fluid ($\pm$1 μg) was used for FK18, a mouse anti-human antibody of the IgG3 subclass which recognizes the gp32 chain of the CD8 molecule (Koning et al, 1986; The FK18 hybridoma cell line was kindly provided by Dr F Koning, Dept of Immunohaematology and Bloodbank, University Hospital Leiden, the Netherlands). This concentration totally inhibited the cytotoxic capacity of CD8-dependent CTL clones but did not affect target cell lysis by CD8-independent CTL clones (Van Emmerik et al, 1996). Before addition of ^{51}Cr-labelled target cells to the LDA, half of the split wells was preincubated with FK18 during 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2.

LDA controls. As a control for specificity, the reactivity of LDA cultures with the highest responder cell concentration was tested against PHA blasts of autologous PBMC. LDA cultures mediating cytolysis of autologous target cells were excluded from the study.
It should be noticed that by using donor PHA-blasts as target cells in the cytotoxic phase of the LDA, only CTL directed against donor HLA class I Ag (and not those directed towards donor HLA class II Ag) are enumerated (Van Emmerik et al, 1997). As a result, addition of CD8 mAb in the absence of CD4 mAb, is sufficient to analyze the avidity of the CTL enumerated in the present report. Controls showing the specificity of the CD8 mAb used (FK18), have been reported previously (Van Emmerik et al, 1996).

Table 2. Frequency of peripheral donor specific pCTL in the presence and absence of CD8 mAb.

patient	day	PA[1]	freq[2] -mAb	95% CI[4]	freq[3] +mAb	95% CI	patient	day	PA	freq -mAb	95% CI	freq +mAb	95% CI
BO	9	0	2	0-4	0		VE	0	-	14	8-20	1	0-2
	23	0	6	2-10	0			8	0	7	3-11	1	0-2
	37	1A	28	20-36	19	13-26		22	1A	5	2-9	1	0-2
	45	3A	25	17-32	17	11-23		36	1A	20	13-27	15	9-21
	54	0	29	21-37	20	13-26		43	1A	8	4-12	6	2-10
		3A						57	3A	15	9-21	14	9-20
	89	1A	1	0-2	0			65	1A	8	4-12	2	0-4
	103	1A	29	20-38	11	6-15		113	1A	23	15-31	10	5-14
	129	1A	39	27-50	36	25-46		142	0	24	16-31	13	8-18
	156	3A	35	25-46	35	24-46		365	0	14	7-22	4	1-7
	164	2	15	9-21	10	6-15		715	1A	9	5-14	2	0-4
	732	1A	15	9-21	1	0-2	SE	0		26	19-34	1	0-2
HE	0	-	16	10-22	3	1-5		6	0	29	20-38	4	1-6
	8	0	7	3-10	1	0-2		13	1A	31	22-40	4	2-7
	14	2	10	5-14	3	1-6		20	0	115	82-147	18	7-29
	17	3A	12	7-17	4	1-6		27	2	119	84-154	36	20-51
	22	1A	18	11-24	2	0-5		34	3A	45	28-63	6	2-11
	28	0	18	12-25	0			41	2	109	79-139	10	6-15
	36	3A	13	8-18	3	1-6		48	3B	77	53-100	4	2-7
	45	3A	15	9-21	2	0-4		66	1A	29	21-36	5	2-8
	80	1A	15	9-21	0			109	1A	67	47-87	14	8-19
	115	2	4	1-6	0			312	0	120	94-146	17	11-23
	493	2	72	57-87	0			591	1A	18	11-24	4	1-6
	717	0	72	53-92	0			906	0	2	0-4	0	
	931	2	69	45-94	0		HU	7	0	40	30-50	15	10-21
SC	9	2	0		0			14	0	25	17-32	10	5-14
	23	1A	20	13-26	7	3-10		21	1A	38	27-48	13	8-18
	30	1A	7	3-10	3	1-5		35	0	18	12-25	7	3-11
	44	3A	13	8-18	9	5-14		41	0	39	29-49	20	14-26
	63	1A	18	12-24	11	6-15		55	1B	24	17-32	11	6-16
	84	1A	9	5-11	5	2-8		62	0	11	6-16	4	1-6
	91	3	9	5-13	6	2-9		175	1A	41	28-53	0	
	317	1A	9	5-14	4	1-6		910	0	10	5-15	0	
	545	1A	2	0-6	0								
DO	0	-	42	31-52	28	20-36							
	6	1A	41	31-51	29	21-37							
	13	1A	37	28-46	12	7-17							
	20	3A	54	42-66	32	23-41							
	27	1A	71	55-87	27	19-35							
	55	1A	51	39-63	26	18-33							
	433	0	7	3-10	0								

Donor specific pCTL present within serial blood samples of individual heart transplant patients were enumerated and their avidity was determined. The table depicts the days after transplantation at which the blood samples were collected, the rejection grade (PA) of the transplant as defined by histological evaluation of EMB taken simultaneously with the blood samples[1], the frequency of donor specific pCTL[2], the frequency of donor specific pCTL with a high avidity[3] and the corresponding 95% confidence intervals[4]. The rejection status was defined according to ISHLT criteria[16]: PA 0, no infiltrate; PA 1A/B, focal/diffuse but sparse infiltrate without necrosis; PA 2, one focus with agressive infiltration and/or myocyte damage; PA 3A/B, multifocal aggressive infiltrates and/or myocyte damage.

Frequency estimation. Minimal estimates of pCTL frequencies were calculated by analysis of the Poisson distribution relationship between the percentage of cultures that failed to develop cytotoxicity and the number of responder cells per well. The pCTL frequencies, expressed as number of cytotoxic cells per 10^6 cells, were calculated with the maximum likelihood estimation, adapted with a jackknife method as described by Strijbosch et al. (1987). Standard deviation and 95% confidence intervals (95% CI) were calculated as well. The calculated frequencies were accepted when the goodness-of-fit did not exceed 12. Subsequently, the fraction of donor specific CTL having high avidity for donor Ag could be calculated using the following formula:

$$\text{fraction (\%)} = 100\text{x} \frac{\text{freq of donor spec. CTL with CD8 mAb}}{\text{freq of donor spec. CTL without CD8 mAb}}$$

Statistical analysis of the kinetics within individual patients (Figure 1) was performed by using the non-parametric Wilcoxon signed rank test. For the statistical analysis of differences between groups (Figure 3), the Mann-Whitney U test was performed. Both tests are part of the statistical program INSTAT.

4.3. Results

Kinetics of circulating donor specific pCTL in relation to acute rejection. The frequency of donor specific CTL was measured in 60 PBMC samples of 7 heart transplant recipients obtained immediately before and at various time points after transplantation. In all but three PBMC samples, donor specific CTL were present. In only 5 cultures, in vivo activated donor specific CTL (cCTL) could be identified demonstrating that circulating CTL are primarily CTL precursors (pCTL). Figure 1 and 2 show the longitudinal changes in donor specific pCTL frequencies for each patient (bars; left Y-axes). Data of patients who experienced acute cardiac rejection are shown in figure 1. Data of a patient that never experienced acute cardiac rejection are shown in figure 2. The occurrence of acute cardiac rejection as defined by histological examination of associated EMB (ISHLT criteria: 3A/3B) is depicted by arrows. For 95% CI intervals and the rejection score of individual EMB, we refer to Table 2.
The number of donor specific pCTL among PBMC fluctuates irrespective of the rejection grade of the allograft. Hence, the frequency of circulating donor specific pCTL not simply reflects the immunological status of the allograft. Statistical analysis of the individual profiles revealed that pCTL frequencies found during acute rejection (median=15/10^6; range=9-53/10^6) did not significantly exceed values found before acute rejection (median=16/10^6; range=7-73/10^6, P=0.38) nor those found after successful rejection therapy (median=16.5/10^6; range=1-61/10^6, P=0.81).

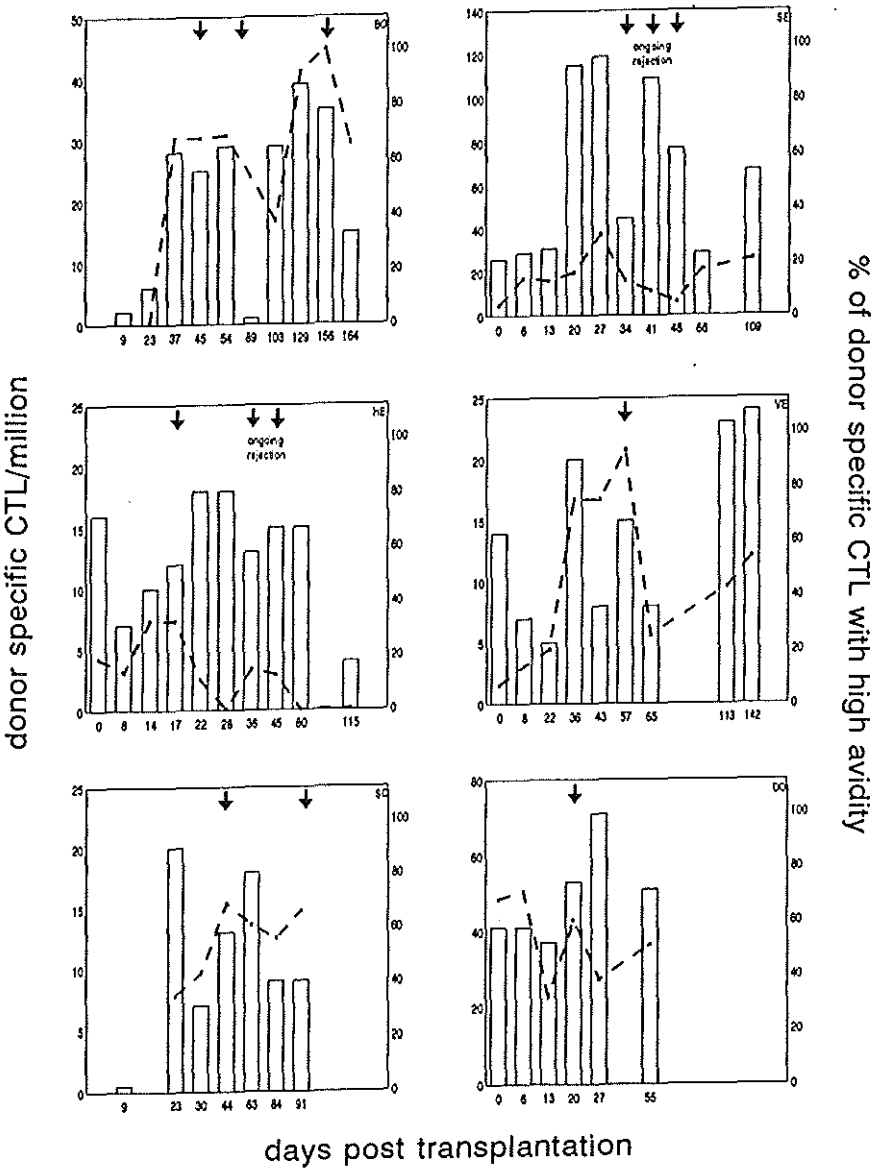

Figure 1: The frequency (bar; left Y-axes) and avidity (broken line; right Y-axes) of donor specific pCTL present within peripheral blood samples of heart transplant recipients obtained before and at various time-points after transplantation were determined by LDA. Values were compared with the rejection grade of the cardiac allograft. Within the individual patients, the CTL frequency found during acute cardiac rejection (arrow) did not significantly exceed frequencies found before the diagnosis of acute rejection (P=0.38) nor those found after rejection therapy (P=0.81). During acute rejection, the percentage of donor specific pCTL with a high avidity for donor Ag had increased significantly when compared to values found before (P=0.008) or after this period (P=0.03).

The avidity for donor Ag of above quantitated donor specific pCTL was determined in split well analysis by addition of a CD8 mAb (FK18) to the cytotoxic phase of the LDA as low avidity CTL are susceptible to CD8 blocking whereas high avidity CTL are resistant to CD8 blocking (MacDonald et al, 1982; De Vries et al, 1989; Cai and Sprent, 1994; Auphan et al, 1994). Donor specific pCTL frequencies found in the presence of CD8 mAb along with their 95% CI intervals are depicted in Table 2. The fraction of donor specific pCTL with a high avidity for donor Ag is shown in figure 1 and 2 (broken line; right Y-axes). In case donor specific pCTL were hardly detectable, their avidity could not be determined.

The individual profiles of the rejectors show that one pattern of changes is predominant. The fraction of the donor specific pCTL with a high avidity for donor Ag increases towards rejection (arrow) and decreases after successful rejection therapy. During acute rejection, 66% (median; range=13-100%) of the donor specific pCTL enumerated had a high avidity for donor Ag. This fraction significantly exceeded both pre-rejection values (median=39%; range=0-65%, $P<0.008$) and post-rejection values (median=37%; range=0-66%, $P<0.03$) of the same patient. Nevertheless, as demonstrated for the non-rejector HU (figure 2), a considerable part of the circulating donor specific CTL might also have a high avidity for donor Ag in the absence of histological signs of graft damage. However, the profile of this patient only showed minor fluctuations during the early posttransplant period. According to these results, the kinetics of circulating high avidity pCTL rather than an absolute proportion of these cells corresponds with the immunological status of the transplanted heart.

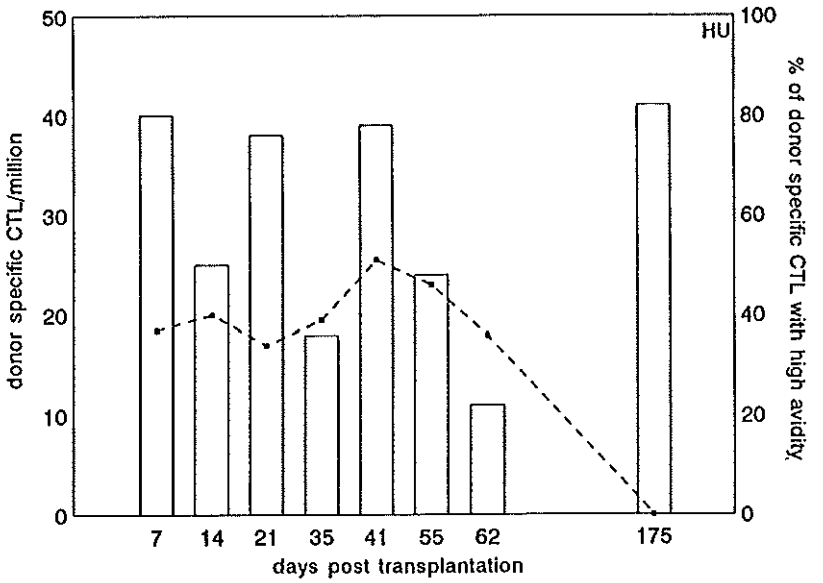

Figure 2: Kinetics of donor specific pCTL within the peripheral blood of a cardiac transplant recipient that never experienced clinical rejection. The numbers of donor specific pCTL per million PBMC (left Y-axes) are shown as bars. The fraction of these pCTL that has a high avidity for donor Ag (right Y-axes) is depicted by the broken line.

Presence of circulating donor specific pCTL during stable engraftment. The previous experiments evaluated the kinetics of high avidity pCTL during the peak of the alloresponse. We questioned whether these cells do persist long after the immune response has subsided. To evaluate this possibility, 11 PBMC samples obtained from the 7 patients described above more than 1 year after transplantation (range: 1-2.5 year) were analyzed for their frequency and avidity of donor specific pCTL. At this time, all patients had achieved a state of stable engraftment as they did not experience allograft rejection hereafter. The results (Table 2) were compared with values of these patients acquired during the first year after transplantation.

As depicted by figure 3A, donor specific pCTL frequencies found more that 1 year after transplantation (> 12 months; median = $12/10^6$) were comparable to values found during the first 3 months (90 days) after transplantation (< 3 months; n = 47; median = $19/10^6$; P = 0.39) and those found during the intermediate period (3-12 months; n = 11; median = $29/10^6$; P = 0.24). It should be noted, however, that donor specific pCTL were absent in 2 samples obtained more than 1 year after transplantation and only in 3 samples obtained during the early period. As a consequence these samples could not be evaluated for their avidity and therefore are not depicted in figure 3B.

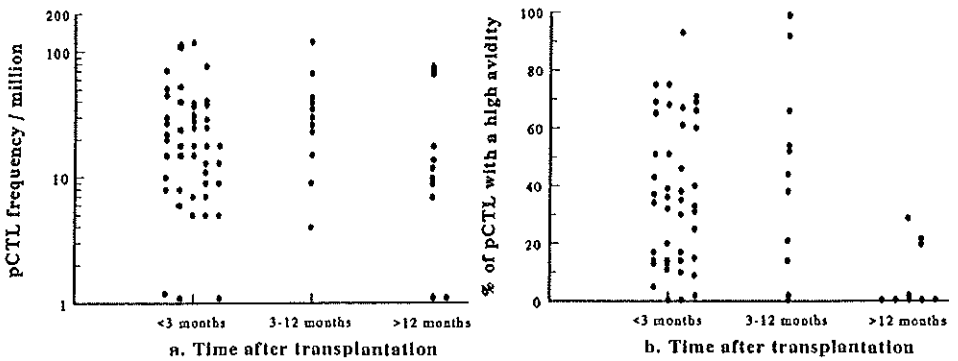

Figure 3: Peripheral blood samples of cardiac transplant recipients obtained during the first 3 months after transplantation (<3 months), between the 3rd and 12th month after transplantation (3-12 months), and more than one year after transplantation (>12 months) were compared for their frequency of donor specific pCTL (panel a). No significant difference was observed between these 3 groups. Subsequently, the avidity of these pCTL for donor Ag was determined by analyzing their resistance to CD8 mAb. The percentages of the donor specific pCTL enumerated that had a high avidity for donor Ag are depicted in panel b. A significant smaller fraction of the donor specific pCTL had a high avidity for donor Ag more than 1 year after transplantation when compared to values found during the early (<3 months; P<0.001) and intermediate period of time (3-12 months; P<0.02).

Addition of CD8 mAb revealed what part of the circulating donor specific pCTL pool could be scored as high avidity pCTL (figure 3B). During the first year after transplantation, the proportion of donor specific pCTL with a high avidity showed a broad range. In several

blood samples, the donor specific pCTL pool mainly consisted of pCTL with a high avidity for donor Ag. In other samples, high avidity pCTL were outnumbered by low avidity pCTL. Within 3 months after transplantation, 35% (median) of the donor specific pCTL enumerated had a high avidity for donor Ag. During the intermediate period a comparable fraction was found (median=44%). More than one year after transplantation, however, a significant lower percentage of the donor specific pCTL had a high avidity for donor Ag (median=0%) when compared to the early ($P<0.001$) and intermediate period ($P<0.02$).
As shown in figure 3B, 3 samples obtained more than 1 year after transplantation still contain some high avidity pCTL. These samples were obtained from patient VE at day 365 and 715 post-transplantation and from patient SE at day 591 post-transplantation. For patient SE, donor specific pCTL became totally absent at a later timepoint (day 906) and with that high avidity pCTL are suspected to be absent as well. For patient VE, the proportion of donor specific pCTL with a high avidity also successively decreased from 50% (mean at <3 months) and 49% (mean at 3-12 months) to 29% (at day 365) and 20% (at day 715). In 4 patients, donor specific pCTL with a high avidity became absent more than 1 year after transplantation whereas in another patient both high and low avidity donor specific pCTL became absent. Based on these individual profiles, it appears that high avidity pCTL are absent (6 patients) or are reduced (1 patient) more than 1 year after transplantation when stable engraftment is achieved.

4.4. Discussion

During the last several years we have focused our studies on the relevance of donor specific CTL in human cardiac allograft rejection. Initially, EMB, routinely taken from heart transplant recipients for the diagnosis of acute rejection, were cultured to obtain the graft infiltrating lymphocytes. The number of donor specific CTL present among these graft infiltrating lymphocytes was then estimated using LDA. Obtained data demonstrated that donor specific cCTL and their precursors (pCTL) were both present within the cardiac allograft irrespective of its rejection grade (Vaessen et al, 1994). On first sight, this observation questions the importance of donor specific CTL in acute cardiac rejection. However, donor specific CTL grown from EMB with and without histological evidence of acute rejection appeared to have different characteristics. Whereas the former CTL generally had a high avidity for donor Ag, the latter CTL mainly had a low avidity for donor Ag (Ouwehand et al, 1993; Vaessen et al, 1994; Van Emmerik et al, 1997). According to these data, we reasoned that high avidity CTL might be important in allograft rejection. The clinical relevance of high avidity CTL in cardiac transplant rejection was strengthened by longitudinal studies within individual heart transplant recipients. Analysis of donor specific CTL propagated from sequential EMB revealed that intragraft accumulation of pCTL and cCTL with a high avidity for donor Ag coincided with myocyte destruction (Van Emmerik et al, 1996).

The intragraft accumulation of high avidity CTL during a rejection episode implies the migration of these cells towards the allograft before acute rejection becomes evident. In the present study we analyzed the kinetics of circulating donor specific CTL within 7 patients and compared the results with the rejection status of their cardiac allograft as defined by histological evaluation of associated EMB.

The number of donor specific CTL within PBMC samples obtained immediately before and at various time points after cardiac transplantation was estimated by LDA. Donor specific cCTL were rarely detected among PBMC indicating that donor specific CTL circulate as CTL precursors. This is in line with earlier data of our group using a different approach (Vaessen et al, 1992). Since the induction of the (allo) immune response is thought to occur within the secondary lymphoid organs (Austyn and Larsen, 1990; Larsen et al, 1990), donor specific pCTL present within the peripheral blood not necessarily are totally naive CTL precursors. Instead they might be activated CTL that have not yet differentiated into functional cCTL; the so-called poised CTL (Gromo et al, 1987). During a rejection episode, numerous cCTL are present within the allograft (Vaessen et al, 1994; Van Emmerik et al, 1996). This observation implies that the final differentiation of pCTL into cCTL occurs at the graft side. Similar results have been obtained by others in a murine transplant model (Orosz and Bishop, 1990).

According to the present study, donor specific pCTL (either naive or poised) are detectable within the peripheral blood of heart transplant patients throughout the whole follow-up period. Their number, however, fluctuated irrespective of the rejection status of the cardiac allograft. Hence, alterations in the frequency of circulating donor specific pCTL do not correlate with the clinical outcome after heart transplantation. In contrast, Reader et al. (1990) reported that the frequency of donor specific CTL within the PBMC population of cardiac transplant patients correlated well with the rejection grade of associated EMB. The kinetics of 2 representative patients depicted in their report, however, did not confirm this hypothesis.

Addition of CD8 mAb revealed which proportion of the circulating donor specific pCTL pool had a high avidity for donor Ag. Acute rejection episodes were found to correspond with increased proportions of donor specific pCTL with a high avidity. Although this observation implies that the overall avidity of circulating pCTL might reflect immunological processes occurring within the allograft, the current method can not be used to monitor for acute cardiac rejection. As shown by the present kinetic studies, acute cardiac rejection is associated with an increase in the number of high avidity pCTL among the circulating donor specific pCTL present. The percentage of donor specific pCTL scored as high avidity pCTL during acute cardiac rejection, however, differs from patient to patient. High avidity pCTL may become the predominant subset or still be outnumbered by low avidity pCTL. Accordingly, changes in the percentage of donor specific pCTL with a high avidity rather than the percentage itself inform us about the rejection status of the allograft. Therefore, to evaluate a blood sample of a particular patient, the results of this blood sample should be

analyzed in the light of the results of preceding samples. The LDA technique described here, however, requires a 10 day culture period which can not be reduced without losing its sensitivity (data not shown). As a result, analysis of the avidity of donor specific pCTL is too time consuming to actually monitor for acute cardiac rejection. Another argument against the application of this technic to monitor for acute cardiac rejection is that the increase in the percentage of donor specific pCTL with a high avidity can be very small and therefore might not be regarded as being one.

Next, we questioned whether these high avidity donor specific pCTL persist long after transplantation when the immune response has subsided. For this analysis, PBL samples obtained more than 1 year after heart transplantation were analyzed for their frequency and avidity of donor specific pCTL. The results were compared with values acquired during the first 3 months after transplantation and with values obtained between the 3rd and 12th month post-transplantation. No significant reduction in the number of donor specific pCTL was observed with time after transplantation. More than 1 year after transplantation, however, a significant smaller fraction of the donor specific pCTL present had a high avidity for donor Ag. This observation implies that high avidity pCTL disappear from the circulation with time after transplantation.

Only one other group has analysed the avidity of donor specific CTL in PBMC samples obtained long after transplantation. This study was performed on corneal transplant patients that either had a well functioning graft or experienced ongoing rejection more than 1 year posttransplantation (Roelen et al, 1995). Whereas high avidity CTL were hardly found among peripheral donor specific CTL of patients with a well functioning graft, they were extensively present within patients with a rejecting graft. Along with our data, this observation implies that stable engraftment corresponds with an absence of high avidity CTL within the peripheral blood. As such, quantification of high avidity CTL within peripheral blood samples might inform us whether stable engraftment has been achieved.

According to the present data, a rejection episode is depicted by an increase in the fraction of donor specific pCTL with a high avidity whereas stable engraftment is depicted by a decrease in this fraction. Kinetic studies on IL-2 producing T cells showed that a similar correlation exists between the number of donor specific IL-2 producing T cells and the immune status of the cardiac allograft (DeBruyne et al, 1993). Accordingly, IL-2 producing cells and high avidity CTL might represent distinct functional activities of the same population of donor reactive T cells. In line with this hypothesis, Cai and Sprent (1994) and Heath et al. (1993) reported that CD8$^+$T cells that interacted with a high avidity with Ag-bearing cells produced IL-2 after stimulation whereas CD8$^+$T cells that interacted with a low avidity with Ag-bearing cells did not. Recently, we analyzed high (n=8) and low (n=7) avidity CTL clones obtained from the peripheral blood and graft of heart transplant patients for their IL-2 production profile (chapter 5). Only 2 high avidity CTL clones and 1 low avidity CTL clone produced IL-2 when stimulated by donor cells. This observation indicates that the IL-2 producing T cells found in correlation with acute cardiac rejection do not belong

to the same T cell population as high avidity CTL.

As reported previously, donor specific pCTL and cCTL with a high avidity for donor Ag accumulate within the cardiac allograft during and immediately preceding acute rejection. In absence of acute rejection, however, these CTL are hardly present within the graft (Vaessen et al, 1994; Van Emmerik et al, 1996,1997; Baan et al, 1995). In combination with the present data it is tempting to speculate that, prior to acute rejection, circulating donor specific pCTL with a high avidity for donor Ag selectively home to the allograft. However, aside from their expansion during acute rejection, high avidity pCTL can be found within the peripheral blood during the first year after transplantation. To explain their selective homing to the allograft in case of a rejection episode, only speculations can be made. An explanation might be that, prior to visible sights of graft damage, pCTL with a high avidity for donor Ag become activated in the spleen or draining lymphnodes of the cardiac transplant recipient. In contrast to naive donor specific pCTL, the resulting poised CTL might have characteristics that allow their passage from the peripheral blood into the allograft. At the graft side, these poised CTL are triggered to differentiate into fully mature donor specific cCTL with a high avidity. Sufficient numbers of these cCTL then cause graft destruction as observed by histology. The relevance of high avidity pCTL for the initiation of the acute rejection cascade is confirmed by our observation that the disappearance of these cells from the peripheral blood correlates with stable engraftment.

Several mechanisms for "transplant tolerance" have already been suggested. With respect to the downregulation of the activity of alloreactive CTL, clonal deletion, anergy, and active suppression have been put forward as possible mechanisms (Charpentier et al, 1982; Herzog et al, 1987; Wramner et al, 1987; Müller-Ruchholtz et al, 1992; Mathew et al, 1993; Hu et al, 1994; Burlingham et al, 1995). In the present report, we demonstrated that peripheral pCTL with a high avidity for donor Ag were largely reduced in number more than 1 year after transplantation when the immune response had subsided. Similarly, Heeg and Wagner (1990) reported that peripheral donor specific pCTL with a high avidity became undetectable in mice made tolerant to allogeneic skin grafts. Hence, processes that selectively affect high avidity CTL might influence the induction of transplant unresponsiveness. According to the murine model, a functional deletion of peripheral pCTL with a high avidity for donor Ag accounts for the acquired unresponsiveness. Which mechanism causes the reduction of high avidity CTL in our study is yet unknown but should be studied as the results might lead to the establishment of conditions that would induce a permanent state of transplant unresponsiveness.

The question remains why only high avidity CTL are detrimental for cardiac allograft survival since both CTL with a high and low avidity display donor directed cytotoxicity in vitro. As suggested previously (Van Emmerik et al, 1996), low avidity CTL only lyse target cells expressing high levels of HLA molecules whereas high avidity CTL are even reactive to cells with a low HLA surface density (Shimonkevitz et al, 1985; Mentzer et al, 1990). The HLA expression on myocytes of the transplanted heart is low (Milton and Fabre, 1985). As

a result, binding and subsequent lysis by low avidity CTL does not occur. Nevertheless, donor myocytes serve as targets when high avidity CTL accumulate within the graft and a rejection episode is encountered. Target cells used in vitro seem to express sufficient HLA molecules for recognition and cytotoxicity by both high and low avidity CTL.
In conclusion, acute cardiac rejection correlates with an increase in the number of high avidity pCTL among donor specific pCTL whereas stable engraftment correlates with a (total) reduction in the number of high avidity pCTL among donor specific pCTL. Although this observation indicates that the kinetics of high avidity pCTL might inform us about the rejection status of the cardiac allograft, the technic in its present form can not be used to monitor for acute cardiac rejection. However, the current technic might distinguish patients who achieved stable engraftment and therefore might tolerate steroid withdrawal.

Chapter 5

The avidity of allospecific CTL determines their cytokine production profile

Donor specific cytotoxic T lymphocytes (CTL) present within the cardiac allograft during a rejection episode are distinct from those that populate the cardiac allograft in the absence of rejection. Whereas the former generally have a high avidity for donor cells, the latter mainly have a low avidity for donor cells. This observation made us reason that high avidity CTL are implicated in transplant rejection whereas low avidity CTL are not. In the present study, we analyzed whether both CTL subsets were distinct with respect to their IL-2, IL-4, IL-6, and IFN-γ secretion pattern. CTL clones with either a high or a low avidity for donor antigens (Ag) were stimulated with donor cells, third party cells, or immobilized anti-CD3 monoclonal antibody (mAb) and the amount of cytokine released was measured. High and low avidity CTL clones were found to differ with respect to their IFN-γ production profile. Stimulation with donor cells resulted in IFN-γ secretion by high avidity CTL clones but not by low avidity CTL clones. CD3 stimulation, on the contrary, led to secretion of equivalent amounts of IFN-γ by both CTL subsets. These observations indicate that low avidity CTL are fully capable of producing IFN-γ but, in contrast to high avidity CTL, fail to do so when they encounter donor cells. As IFN-γ favours the occurrence of transplant rejection, this observation emphasizes the relevance of high avidity CTL in the rejection process. Additionally, the data show that the cytokine production profile of CTL depends on the nature of the stimulus.

5.1. Introduction

The relevance of donor specific CTL in acute allograft rejection is still a matter of debate. Support for the involvement of CTL as terminal effector cells is provided by experiments showing the exquisite Ag specificity of the alloresponse (Sutton et al, 1989; Mintz and Silvers, 1967) and the ability of CTL clones grown from rejecting allografts to destroy allogeneic tissue when injected into appropriate hosts (Snider et al, 1986). In contrast, experiments showing the occurrence of graft rejection in $CD8^+$lymphocyte depleted mice have been interpreted as evidence against a critical role for CTL in transplant rejection (Bishop et al, 1993). In such modified animals, eosinophils were assumed to be responsible for the observed graft damage (Chan et al, 1995). This recruitment of eosinophils into the graft was ascribed to a decrease in the Th1 to Th2 cytokine ratio which in turn was assigned to the depletion of Th1-promoting, Th2-suppressing donor reactive $CD8^+$CTL. Although this model demonstrates that other cells besides donor specific CTL can function as terminal effector cells, these cells may only become relevant when the immune system is aberrant.
Also the presence of donor specific CTL within stable transplants (Dallman et al, 1987; Weber et al, 1989) argues against a critical role of CTL within the rejection process. However, we found that donor specific CTL propagated from rejecting and stable allografts were distinct (Ouwehand et al, 1993; Vaessen et al, 1994; Van Emmerik et al, 1997). As assessed by their sensitivity to CD8 or CD4 blocking, the former CTL mainly had a high avidity for donor cells whereas the latter CTL generally had a low avidity for donor cells (MacDonald et al, 1982; Marrack et al, 1983; Biddison et al, 1984; De Vries et al, 1989). The avidity of a T lymphocyte is a term that reflects the relative strength with which this T cell binds an Ag-bearing cell. According to current concepts, the avidity of a T cell portrays the overall strength of T cell receptor (TCR)-MHC/peptide interactions and hence depends on the intrinsic affinity of the TCR for its ligand and the number of TCR-MHC/peptide complexes that associate (Maryanski et al, 1988; Auphan et al, 1994; Kim et al, 1996; Al-Ramadi et al, 1995).
The selective presence of high avidity, donor specific CTL within rejecting allografts made us reason that this particular CTL subset is important in acute rejection. A relationship between the avidity of donor specific CTL and the rejection status of the allograft was indeed found by subsequent longitudinal studies within individual heart transplant recipients. It appeared that high avidity CTL populated the cardiac allograft immediately preceding and during acute rejection whereas low avidity CTL populated the cardiac allograft outside a rejection episode (Van Emmerik et al, 1996). In the same period, the in vivo efficiency of virus specific CTL was reported to depend on their avidity. Despite the fact that both high and low avidity CTL effectively killed virus infected cells in vitro, only high avidity CTL could do so in vivo (Alexander-Miller et al, 1996A). Yet, in the transplant model, only CTL with a high avidity for donor cells might exhibit their effector function in vivo.
In the present study, we further characterized the response of high and low avidity CTL towards donor cells. Since increasing evidence has indicated that T-lymphocytes producing

different cytokine profiles have different cytolytic potentials (Romagnani et al, 1996), we assumed that it might be informative to analyze both high and low avidity CTL clones for their cytokine secretion pattern. Previous analysis of solid endomyocardial biopsies (EMB) obtained from heart transplant recipients showed that EMB with and without histological signs of clinical cardiac rejection differed with respect to their cytokine gene expression (Baan et al, 1994). The IL-2, IL-4 and IL-6 genes were considerably more often expressed in the former EMB than in the latter EMB. However, the source as well as the specificity of the observed response remained undefined. In the present study, we evaluated the ability of high and low avidity CTL clones to release these cytokines after donor, third party, and polyclonal (CD3) stimulation. Additionally, we analyzed their ability to release IFN-γ as the gene expression and production of this cytokine within the allograft (Kupiec-Weglinski et al, 1993; Mottramm et al, 1995; Egawa et al, 1995) and the draining lymph nodes (Fallarino et al, 1996) repeatedly have been found associated with rejection.

5.2. Materials and Methods

All experiments described in this paper have been performed with donor specific CTL clones established from graft infiltrating lymphocyte (GIL) cultures or peripheral blood of 6 heart transplant patients (KU, BE, MI, ZA, PO, FO).

Generation of CTL clones. GIL and PBMC were obtained from endomyocardial biopsies and blood samples of heart transplant recipients, respectively as described before (Van Emmerik et al, 1996). Donor specific T cell clones were generated from GIL or PBMC by limiting dilution at 0.3 cells/well in culture medium expanded with 0.5-1% phytohaemagglutinin (PHA; Difco, Detroit, MI). Culture medium consisted of RPMI-1640 Dutch Modification (Gibco, Paisley, Scotland) supplemented with 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% pooled human serum, and 10% lectin free lymphocult-T-LF (Biotest GmbH, Dreieich, Germany) as a source of IL2. Irradiated (30 Gy) donor B-lymphoblastoid cell lines (B-LCL; $5x10^4$ cells/ml) and PBMC of healthy blood donors ($5x10^5$ cells/ml) were added as feeder cells. After 2 weeks, wells with visible cell growth were restimulated with irradiated donor B-LCL and PBMC of healthy blood donors. 7-14 days later, clones were phenotyped as described below and assayed for donor specific cytotoxicity. Clones used in the present study totally consisted of either $CD4^+WT31^+$cells or $CD8^+WT31^+$cells and exhibited cytotoxicity towards donor cells but not towards third party cells or the NK sensitive cell line, K562.

Target cells. B-LCL originated from infection of fresh PBMC or spleen cells with Epstein Barr Virus obtained from the marmoset cell line B95-8. These cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, Utah).

Phenotypic analysis. T cell clones were analyzed for WT31, CD8 (Becton Dickinson, San Jose, CA), and CD4 (Immunotech, Marseille, France) expression by three-color flow cytometry on a FACScan after staining with mAb directly conjugated for fluorescein, peridinin chlorophyll protein, and phycoerythrin, respectively.

Cell mediated lympholysis (CML). The cytotoxic capacity of T cell clones was tested against donor B-LCL, third party B-LCL (B-LCL that do not share MHC antigens with the donor nor with the acceptor), and the K562 cell line. Briefly, effector cells (cloned cells) were incubated with 2500 ^{51}Cr-labelled target cells at different effector:target (E:T) ratios in 0.2 ml culture medium without Lymphocult-T-LF. After 4 hours of incubation (37°C, 5%CO_2), supernatants were harvested using a Skatron harvesting system (Skatron-AS, Lierse, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard, Downers Grove, USA). Spontaneous and maximum release were defined by incubation of target cells with culture medium (without Lymphocult-T-LF) in the absence or presence of Triton X-100 detergent (5% v/v solution in 0.01 TRIS buffer), respectively. Microcultures were considered cytolytic when the experimental lysis percentages exceeded 10%. The cytotoxicity of all clones was directed towards MHC molecules of the donor.

CTL avidity. As CD8 and CD4 molecules interact with the same ligand (MHC class I and II molecules on Ag bearing cells, respectively) as the TCR, it is believed that both CD8 and CD4 molecules serve to enhance the overall strength of TCR-MHC/peptide interactions. Accordingly, by blocking the CD8 or CD4 molecules on a T cell, one can define whether the avidity of this T cell for an Ag-bearing cell is sufficiently high to overcome the need for these molecules. To block CD8 molecules a 1:500 dilution of FK18 (2μg/ml), a mouse anti-human CD8 mAb of the IgG3 subclass, was used (a kind gift of Dr F Koning, Dept of Immunohematology and Bloodbank, University Hospital Leiden, the Netherlands). To block CD4 molecules a 1:250 dilution of RIV6 (2μg/ml), a mouse anti-human CD4 mAb of the IgG2A subclass, was used (a kind gift of Dr. M.F. Leerling, RIVM, Bilthoven, The Netherlands).
To analyze the avidity of a CTL clone, CML experiments were performed in the absence and presence of FK18 or RIV6. For this, clones were preincubated with FK18 or RIV6 for 30 minutes at 37°C before ^{51}Cr-labelled target cells were added. As a control for TCR mediated lysis, CML experiments were performed in the presence of 1μg/ml anti-CD3 mAb (RIV9, RIVM).

Cytokine production. Clones to be tested for cytokine release were used at least 1 week after the last restimulation. The day before the cytokine-release assay, the clones were washed and seeded (5x10^4 cells/well) in V-bottom 96-well plates (Greiner, Alphen aan de Rijn, The Netherlands) in culture medium without lymphocult-T-LF. The next day, cells were incubated with irradiated (60 Gy) donor B-LCL or irradiated third party B-LCL (5x10^4

cells/well) in a final volume of 0.2 ml culture medium without lymphocult-T-LF. For polyclonal stimulation by immobilized anti-CD3 mAb, cells were transferred to 96-well round bottom culture plates (Costar, Cambridge, MA) that had been preincubated with 5μg/ml RIV9 (90 min, 37°C). Controls were wells containing clones incubated with medium alone and wells containing stimulator cells incubated with medium alone. Plates were centrifuged for 5 minutes at 400 g and incubated for the indicated time at 37°C. After incubation, supernatants were removed and analyzed for the amount of cytokine released. IL-2, IL-4, and IL-6 release was assessed using commercially available ELISA (IL-2, Immunotech; IL-4, IL-6, CLB, Amsterdam, The Netherlands).

IFN-γ release was determined according to the following protocol. IFNγ-binding mAb 350B10G6 (Medgenix, Fleurus, Belgium) was covalently coupled to microtiterplates with a hydrazide surface (carbohydrate binding plates, Costar) as described by Brillhart and Ngo (1991). In situ oxidation and coupling was performed by addition of 50 μl of mAb 350B10G6 (2 μg/ml) in 50 mM acetate buffer (pH 5.0) to each well. After 30 minutes of incubation at room temperature, 50 μl of 5 mM $NaIO_4$ in acetate buffer was added to each well. The plates were incubated again for 30 minutes, washed with washing buffer (Tris buffered saline + 0.05% Tween 20, pH 7.4), and blocked with 100 mM Tris + 0.05% Tween-20 + 1% BSA (pH 7.5). After 1 hour, plates were decanted and stored at -20°C until use.

Plates were washed and standards (3000 - 4.1 pg/ml; diluted in cell culture medium without Lymphocult-T-LF) prepared from a stock solution of recombinant human IFN-γ (R&D Systems, Minneapolis, MN, USA) or cell culture supernatants were added to the wells (100 μl/well). After 2 hours, wells were washed and incubated for another 2 hours with 100 μl (0.25 μg/ml) of biotin conjugated anti-human IFN-γ (Medgenix, clone 67F12A8) diluted in High Performance ELISA buffer (CLB, Amsterdam, The Netherlands). Plates were washed and 100 μl poly HRP-labelled streptavidin (1:10000, CLB) was added to each well. After 30 minutes, wells were washed again and 100 μl substrate solution (3,5,3',5',tetramethylbenzidine, 0.1 mg/ml in 0.11 M acetate buffer, pH 5.5 + 0.003% H_2O_2) was added. The reaction was stopped after 15 minutes by addition of 50 μl/well of 2N H_2SO_4 and the optical density was measured at 450 nm.

Statistical analysis. Differences between groups were analyzed using the Mann-Whitney U test of the statistical program INSTAT (Graphpad Software, San Diego, CA).

5.3. Results

Cytotoxic capacity and avidity of T cell clones. T cell clones obtained from peripheral blood or GIL cultures of heart transplant recipients were tested for their cytotoxic capacity in a standard 4-hour ^{51}Cr release assay. Clones that specifically lysed B-LCL of donor origin were selected and subsequently examined for their avidity for donor Ag. For this analysis, $CD8^+$CTL clones were tested for their capacity to lyse donor B-LCL in the absence or

presence of anti-CD8 mAb (figure 1A). Likewise, $CD4^+$CTL were tested for donor directed cytotoxicity in the absence or presence of anti-CD4 mAb (figure 1B). As depicted by figure 1, 2μg/ml of anti-CD8 or anti-CD4 mAb is sufficient to distinguish between clones that bind donor cells with a high (+) or a low (♦) avidity. As a control for TCR mediated cytotoxicity, blocking experiments were performed with anti-CD3 mAb. All clones used in the present report showed a significant reduction of donor target cell lysis after preincubation with anti-CD3 mAb.

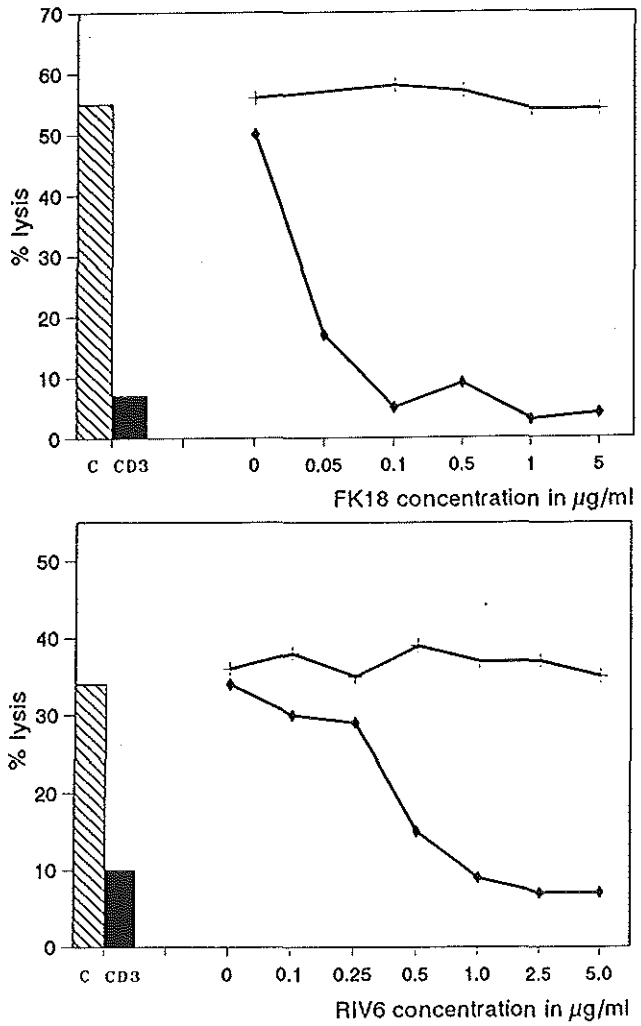

Figure 1: The susceptibility of $CD8^+$CTL (top) and $CD4^+$CTL (bottom) clones to inhibition by different doses of anti-CD8 mAb (FK18) and anti-CD4 mAb (RIV6), respectively. Clones whose cytotoxic capacity is not affected by FK18 or RIV6 (+) are considered to bind their target cells with a high avidity. Clones that fail to lyse their target cells in the presence of FK18 or RIV6 (♦; lysis <10%) are considered to bind these cells with a low avidity. Alternatively, FK18 was added to CD4-bearing cells and RIV6 to CD8-bearing cells to control for the specificity of the response (c). Anti-CD3 mAb was added as a control for TCR mediated lysis.

Cytokine profile of high and low avidity CTL clones. Fifteen donor specific CTL clones obtained from peripheral blood samples or GIL cultures of heart transplant patients were characterized for their IL-2, IL-4, IL-6, and IFN-γ release after donor, third party, or CD3 triggering. The results are shown in Table I. The number, name and source of each clone (either PBL or GIL) are depicted by the first column (Clone). The phenotype (either CD4 or CD8) of the clones is depicted by the second column (Type). The cytotoxic capacity of the clones to lyse donor target cells in the absence and presence of anti-CD4 or anti-CD8 mAb are shown in the third (%Lysis-mAb) and fourth (%Lysis+mAb) column, respectively. Addition of anti-CD4 or anti-CD8 mAb to ^{51}Cr release assays almost completely reduced donor directed cytotoxicity exhibited by clone 1 to 7 (Lysis $< 10\%$) but hardly effected donor target cell lysis by clone 8 to 15. Hence, the former 7 clones bind donor cells with a low avidity whereas the latter 8 clones bind donor cells with a high avidity. The remaining columns show the cytokine production profile of both high and low avidity CTL clones. For this analysis, CTL clones were stimulated for 6 and 20 hours with irradiated B-LCL of donor origin (do), with irradiated B-LCL that did not share HLA antigens with the donor or acceptor (3P), and with immobilized anti-CD3 mAb afterwhich the amount of cytokine released was assessed by ELISA.

As a control, unstimulated CTL clones and irradiated stimulator cells were tested for their cytokine production profile. Unstimulated clones did not secrete IL-2, IL-4, IL-6 nor IFN-γ indicating that these cytokines are not constitutively produced by CTL (data not shown). Irradiated stimulator cells, on the contrary, occasionally produced IL-6 but did not produce detectable amounts of IL-2, IL-4, or IFN-γ (data not shown). This observation implies that the amount of IL-6 detected in the supernatant of stimulated CTL clones may also partly be the product of the stimulator cells added.

Some general remarks can be made with respect to the data in Table I. Comparing the cytokine profile after donor and third party stimulation reveals that the production of IL-2, IL-4, and IFN-γ is donor specific. CTL clones fail to produce these cytokines after stimulation by third party B-LCL but may do so when stimulated by donor B-LCL. IL-6, on the contrary, can be found in the supernatant after both donor and third party stimulation, indicating that the production of this cytokine is aspecific. Alternatively, as stated above, the IL-6 detected may be the product of the stimulator cells used.

Comparing the cytokine profile after stimulation with donor cells and immobilized RIV9 shows that the release of IL-2, IL-4, and IFN-γ but not of IL-6 can be induced or significantly increased by CD3 stimulation. For example, only 3 clones (20%) produce detectable amounts of IL-2 after 20 hours of donor stimulation whereas 9 clones (60%) do so after 20 hours of CD3 stimulation. This observation indicates that care should be taken with ascribing functions to T cells that are simply grounded on their cytokine profile as such a profile can easily be modulated by the stimulus chosen.

Table 1: Cytokine profile of CTL clones that either have a low or a high avidity for donor Ag.

				IL-4						IL-6					
Clone	Type	%Lysis[1] -mAb	%Lysis[2] +mAb	do[3] 6 hr	do 20 hr	3P[4] 6 hr	3P 20 hr	CD3[5] 6 hr	CD3 20 hr	do 6 hr	do 20 hr	3P 6 hr	3P 20 hr	CD3 6 hr	CD3 20 hr
1 KU7PBL	CD4	19	0	21	46	-	-	19	118	-	20	-	-	-	-
2 BE3GIL	CD8	51	0	n.d	-	n.d	-	n.d	80	n.d	-	n.d	-	n.d	-
3 KU5PBL	CD8	44	3	-	-	-	-	41	265	-	-	-	-	-	-
4 MI6PBL	CD4	23	5	-	58	-	-	-	50	-	19	-	-	-	-
5 ZA21GIL	CD4	29	9	-	-	-	-	>450	>450	-	-	-	20	-	25
6 PO9PBL	CD4	29	9	-	44	-	-	-	69	-	35	-	-	-	22
7 ZA26GIL	CD4	20	7	-	50	-	-	>450	>450	-	-	-	19	-	26
8 PO27PBL	CD4	61	40	69	280	-	-	>300	>300	-	-	-	-	-	-
9 KU11PBL	CD8	69	46	-	30	-	-	-	80	-	21	-	-	-	-
10 FO4GIL	CD8	90	65	-	-	-	-	40	42	-	-	-	-	-	-
11 BE32GIL	CD8	63	47	n.d	365	n.d	-	n.d	412	n.d	-	n.d	-	n.d	-
12 FO110GIL	CD4	55	53	-	-	-	-	-	-	90	269	-	33	-	-
13 ZA12GIL	CD8	16	18	-	-	-	-	-	-	-	-	-	-	-	-
14 ZA1GIL	CD8	44	52	-	-	-	-	-	-	23	26	30	27	-	-
15 FO6GIL	CD8	71	78	54	57	-	-	52	50	-	-	-	26	-	-

				IL-2						IFN-γ					
Clone	Type	%Lysis -mAb	%Lysis +mAb	do 6 hr	do 20 hr	3P 6 hr	3P 20 hr	CD3 6 hr	CD3 20 hr	do 6 hr	do 20 hr	3P 6 hr	3P 20 hr	CD3 6 hr	CD3 20 hr
1 KU7PBL	CD4	19	0	-	-	-	-	-	-	-	-	-	-	-	63
2 BE3GIL	CD8	51	0	n.d	-	n.d	-	n.d	133	n.d	-	n.d	-	n.d	1766
3 KU5PBL	CD8	44	3	-	-	-	-	16	185	-	-	-	-	23	144
4 MI6PBL	CD4	23	5	-	26	-	-	-	-	-	-	-	-	5	9
5 ZA21GIL	CD4	29	9	-	-	-	-	322	325	10	33	-	8	1044	>3000
6 PO9PBL	CD4	29	9	-	-	-	-	-	-	-	121	-	-	45	307
7 ZA26GIL	CD4	20	7	-	-	-	-	335	248	28	60	-	8	>1000	>1000
8 PO27PBL	CD4	61	40	-	-	-	-	74	132	192	861	-	-	1252	2657
9 KU11PBL	CD8	69	46	-	-	-	-	-	40	131	483	-	-	47	451
10 FO4GIL	CD8	90	65	-	-	-	-	189	106	590	723	-	-	>3000	>3000
11 BE32GIL	CD8	63	47	n.d	59	n.d	-	n.d	259	n.d	281	n.d	-	n.d	>1000
12 FO110GIL	CD4	55	53	-	-	-	-	-	-	58	290	-	-	76	122
13 ZA12GIL	CD8	16	18	-	-	-	-	26	-	214	231	-	-	>1000	897
14 ZA1GIL	CD8	44	52	-	-	-	-	-	-	110	206	-	-	860	904
15 FO6GIL	CD8	71	78	140	159	-	-	95	41	>1000	>1000	-	-	>1000	>1000

[1]Donor target cell lysis at an effector/target ratio of 5:1. Cultures were considered cytolytic when the experimental lysis percentage exceeded 10%. [2]Effect of anti-CD8 or anti-CD4 mAb addition on the donor directed cytotoxicity of $CD8^+$ or $CD4^+$CTL clones, respectively. Addition of anti-CD8 or anti-CD4 mAb inhibited donor target cell lysis by low avidity CTL (lysis <10%; nr 1-7) but hardly effected cytotoxicity by high avidity CTL clones (nr 8-15). Both high and low avidity CTL clones were stimulated with irradiated [3]donor B-LCL, [4]third party B-LCL, and [5]immobilized anti-CD3 mAb. After 6 and 20 hours supernatants were harvested and assayed for cytokine content by ELISA. The amount of cytokine released is expressed as pg/ml. Values below the detection level of the ELISA kits (IL-2: 15 pg/ml; IL-4: 9 pg/ml; IL-6: 6 pg/ml: IFN-γ: 4 pg/ml) are depicted as negative (-). n.d = not done. Unstimulated clones did not produce detectable amounts of IL-2, IL-4, IL-6, or IFN-γ (data not shown).

Comparing the amount of cytokines produced after 6 and 20 hours of stimulation, it can be stated that the amount of cytokine secreted and the number of clones producing detectable levels of cytokine is increased after 20 hours of stimulation.
Despite these common characteristics, the data in table I show that CTL clones are rather diverse in their cytokine profile. We analyzed whether the various cytokine patterns observed could be explained by differences in the source (PBL versus GIL), phenotype (CD4 versus CD8), or avidity (high versus low) of the clones. This analysis lead to one striking observation. It appeared that low avidity CTL and high avidity CTL were distinct with respect to their IFN-γ cytokine profile. Low avidity CTL barely produced IFN-γ after donor B-LCL stimulation whereas high avidity CTL produced significant amounts of IFN-γ both after 6 and 20 hours of donor B-LCL stimulation ($P<0.001$ and $P<0.0003$, respectively). No correlation was found between the IFN-γ profile and the phenotype or the source of the clones demonstrating that the avidity of CTL clones is the only variable associated with the observed IFN-γ production profile. The results are shown in figure 2.

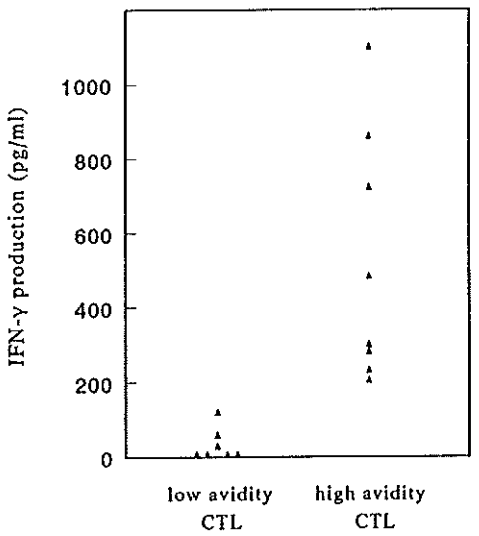

Figure 2: The amount of IFN-γ released by low and high avidity CTL clones after 20 hours of Ag-specific stimulation by donor B-LCL. Detection level is 4 pg/ml.

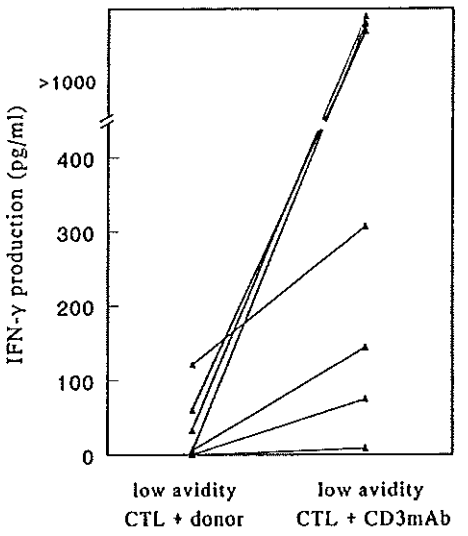

Figure 3: The amount of IFN-γ released by low avidity CTL after 20 hours of stimulation by donor B-LCL (Ag-specific; left) and immobilized anti-CD3 mAb (right), respectively.

In contrast to donor stimulation, CD3 stimulation resulted in the production of equivalent amounts of IFN-γ by both high and low avidity CTL at 6 and 20 hours of stimulation ($P<0.10$ and $P<0.40$, respectively). This observation indicates that low avidity CTL are fully capable to produce IFN-γ but, in contrast to high avidity CTL, fail to do so when they encounter donor cells (figure 3). This observation also emphasizes that the cytokine profile

of cells can easily be influenced by the nature of the stimulus used.
No distinction between high and low avidity CTL could be made with respect to their IL-2, IL-4, or IL-6 production pattern. Likewise, no differences in IL-2, IL-4, or IL-6 profile were found between CD4- and CD8-expressing CTL clones and between peripheral blood and graft derived CTL clones.

5.4. Discussion

Although numerous studies have demonstrated the existence of T cells that do not require CD8 or CD4 co-receptors to exhibit their function, hardly any attention has been paid to the immunological significance of these cells. Our data in this field refer to donor specific CTL propagated from graft biopsies or blood samples of heart transplant patients. Kinetic studies showed that CD8/CD4-dependent (low avidity) donor specific CTL were prominent within the cardiac graft until 2 weeks preceding acute rejection. From that point onwards, CD8/CD4-independent (high avidity) donor specific CTL rapidly accumulated within the graft and became predominant at the time of acute rejection. Successful anti-rejection therapy resulted in the destruction of these CTL and the graft was repopulated again by low avidity donor specific CTL (Van Emmerik et al, 1996). As intragraft accumulation of donor specific CTL with a high avidity precedes myocyte destruction (i.e. acute rejection) we believe that these CTL, as opposed to low avidity donor specific CTL, are involved in graft rejection. In a subsequent study, the kinetics of circulating donor specific CTL was analyzed and their avidity was compared with the rejection status of the graft. Obtained data showed that donor specific CTL with a high avidity were most prominent in blood samples taken during a rejection episode (submitted for publication). Analysis of the maturation status of peripheral donor specific CTL revealed that these cells were precursor CTL and not fully mature CTL such as found within the graft. Together these data suggest that, prior to rejection, peripheral donor specific CTL with a high avidity selectively home to the graft and differentiate into mature CTL which mediate graft destruction.
In the present study, donor specific CTL clones obtained from the graft and peripheral blood of heart transplant patients were analyzed for their IL-2, IL-4, IL-6, and IFN-γ production profile and the results were compared with their avidity for donor Ag. It appeared that CTL clones that differed in the avidity with which they interacted with donor cells were distinct with respect to their IFN-γ production profile. CTL clones that interacted with donor cells with a high avidity produced significant amounts of IFN-γ after donor stimulation while CTL clones that associated with donor cells with a low avidity did not.
IFN-γ is considered to play a potential role in allograft rejection as it is believed to recruit macrophages into the graft, activate macrophages and lymphocytes, and increase the immunogenicity of the allograft by enhancing MHC expression on donor tissue (Halloran et al, 1993). In support of this concept, proteins and/or transcripts for intragraft IFN-γ have consistently been shown to correlate with transplant rejection (Kupiec-Weglinski et al, 1993;

Mottramm et al, 1995; Egawa et al, 1995). The fact that high avidity CTL produce IFN-γ when they encounter donor cells whereas low avidity CTL do not, therefore, underscores our concept that high avidity CTL are involved in allograft rejection (Ouwehand et al, 1993; Vaessen et al, 1994; Van Emmerik et al, 1996, 1997; Roelen et al, 1995).

Stimulation of high avidity CTL clones with third party B-LCL did not result in IFN-γ production demonstrating that the release of this cytokine after donor stimulation is specific and hence is based on TCR-MHC/peptide interactions. To explain a relationship between the avidity of T cells and their ability to produce IFN-γ, we should bear in mind that the avidity of a T cell as defined by CD4/CD8 inhibition studies actually reflects the overall strength of TCR-MHC/peptide interactions (Al-Ramadi et al, 1995; Kim et al, 1996). Most likely, the overall strength of TCR-MHC/peptide interactions determines the intensity of TCR triggering and consequently whether thresholds of intracellular signal pathways leading to IFN-γ production are reached. In support of this view, low avidity CTL clones were fully capable of producing IFN-γ after stimulation by immobilized anti-CD3 mAb, a stimulus which bypasses the requirement for TCR-ligand association.

The above theory suggests that the IFN-γ production profile of T cells is governed by the avidity with which these cells interact with Ag-bearing cells. Some other studies support this view. It has been reported that antigenic peptides that bind well to MHC molecules and/or TCR favour the generation of IFN-γ producing cells whereas peptides that bind less well do not (Pfeiffer et al, 1995, Kumar et al, 1995). Additionally, it has been shown that TCR transgenic $CD4^+$ T cells differentiate into IFN-γ producing T cells only when high Ag doses are used for priming (Constant et al, 1995). In these studies, the extent of TCR-MHC/peptide interactions is enhanced and hence the avidity of the T cell-antigenic cell interaction.

Also the production of IL-2 by $CD8^+$T cells (Heath et al, 1993; Cai and Sprent, 1994) and IL-4 by $CD4^+$T cells (Pfeiffer et al, 1995; Kumar et al, 1995; Constant et al, 1995) has been reported to depend on the avidity with which these cells bind Ag-bearing cells. We, however, did not find any distinction between high and low avidity CTL with respect to their IL-2 or IL-4 production profile. The reported association between the production of IL-4 and the avidity of the interaction was found to depend upon the use of naive cells as the starting population (Constant et al, 1995). Accordingly, the observed disparity in data might be explained by the differentiation status of the T cell population examined. While all above mentioned studies were performed on naive T cell populations, we used mature T cells. Additionally, above mentioned studies were performed on murine T cell subsets (either $CD4^+$ or $CD8^+$T cells) with unknown cytolytic potential whereas we used human CTL clones as the starting population. These variables (human versus murine T cells; CTL versus T cells which may not be cytolytic; and T cell clones versus T cell subsets) might also have contributed to the diversity in data.

An additional point we would like to emphasize in this manuscript is that the type of stimulus used to assess the cytokine production profile of cells has an important impact on their response. More clones produced a particular cytokine after CD3 stimulation than after Ag-

specific stimulation. Low avidity donor specific CTL clones, generally unable to produce IFN-γ after stimulation with donor cells, did produce significant amounts of IFN-γ after CD3 stimulation. Likewise, CD3 stimulation led to IL-2 and IL-4 production by clones that failed to produce these cytokines after Ag-specific stimulation. Our data are in line with those of Maccalli et al. (1994) who demonstrated that the cytokine pattern in response to the Ag-specific stimulus was different from the one induced by CD3 stimulation. Hence, it is clear that the cytokine profiles of T-lymphocytes can be easily modulated by the type of activation signals delivered to the T cells.

In conclusion, our in vitro studies show that the avidity of donor specific CTL determines their ability to produce IFN-γ when they encounter donor cells. If a similar situation exists in vivo, this may be the explanation for the earlier observed association between the avidity of graft infiltrating CTL and the rejection status of the transplanted human heart.

Chapter 6

C1.7 monoclonal antibody designates high avidity $CD4^+$cytotoxic T lymphocytes (CTL) involved in clinical heart rejection

It is assumed that not all donor specific CTL but only those with a high avidity for donor antigens (Ag) can function as terminal effector cells in transplant rejection. In the present study we searched for markers that would exclusively designate these high avidity CTL. FACS analysis of donor specific CTL clones obtained from heart transplant patients revealed that high and low avidity CTL varied in their expression of p38, a surface molecule involved in signal transduction that is stained by the antibody C1.7. High and low avidity $CD8^+$CTL and high avidity $CD4^+$CTL expressed p38 whereas low avidity $CD4^+$CTL did not. Non-cytotoxic and naive $CD4^+$lymphocytes also lacked p38 surface expression. Therefore, we conclude that p38 is a marker for $CD4^+$lymphocytes with the potency to damage the transplanted heart. Accordingly, p38 might be used to analyze the contribution of $CD4^+$CTL in immune responses such as transplant rejection.

During the last years we have focused our research on the significance of donor specific CTL in clinical cardiac rejection. Donor specific CTL could be recovered both from rejecting and stable cardiac allografts. However, donor specific CTL propagated from rejecting allografts could lyse donor target cells in the presence of anti-CD8 or anti-CD4 monoclonal antibodies (mAb) whereas those recovered from stable allografts could not (Vaessen et al, 1994; van Emmerik et al, 1996;1997). As CD8 and CD4 co-receptors enhance the overall strength of T cell receptor (TCR)-MHC/peptide interactions (De Vries et al, 1989; Auphan et al, 1994; Al-Ramadi et al, 1995), these data imply that CTL present within rejecting cardiac allografts bind donor cells with sufficient high avidity to overcome their need for CD8 or CD4 molecules while CTL present within stable allografts bind donor cells with a low avidity.
In the same period, Alexander-Miller and co-workers (1996A) reported that the avidity of virus specific CTL is critical for their in vivo efficiency. Although both high and low avidity CTL were able to kill viral infected cells in vitro, only high avidity CTL could do so in vivo. Combining these observations, we assume that CTL with a high avidity for donor cells are responsible for the observed myocyte damage associated with clinical cardiac rejection whereas low avidity donor specific CTL are not functional in vivo.
The avidity of heart infiltrating CTL as assessed by CD8/CD4 blocking assays requires the time-consuming culturing of these cells from cardiac transplant biopsies. In the present study we searched for a surface marker specific for high avidity CTL which could directly inform us about the immunological status of the transplanted heart.
One explanation for the observed differences in susceptibility to inhibition by anti-CD8 or anti-CD4 mAb exhibited by CTL could be a heterogeneous expression of CD8 or CD4 molecules. Alternatively, as the requirement for CD8 or CD4 co-receptors is linked to the number of TCR and MHC/peptide complexes that can associate (De Vries et al, 1989), high and low avidity CTL might vary in their TCR density. In the present study we investigated these possibilities. Kane et al. (1989) showed that CTL that did not require CD8 molecules to lyse whole target cells (high avidity CTL) became very sensitive to anti-CD8 mAb when tested against immobilized MHC class I molecules (where only the TCR and CD8 can interact). This observation implies that the CTL's requirement for CD8 might depend on the extent with which other receptor-ligand pairs compensate for CD8-MHC class I interactions. Therefore, we examined whether high and low avidity CTL differed in CD11a (LFA-1) and CD2 (LFA-2) surface expression since both molecules are implicated in adhesion and co-stimulation of T cells (De Vries et al, 1989). Additionally, we analyzed whether high and low avidity CTL clones differed in activation status (CD45RO expression) or in p38 expression.
The expression of p38 on different leucocyte subsets has extensively been studied by Valiante and Trinchieri (1993). As shown by their data, p38 is a signal transduction molecule expressed on NK cells and approximately half of $CD8^+$T cells. On $CD8^+$T lymphocytes, p38 expression correlates with higher cytotoxic activity. As high avidity CTL are reactive to cells expressing low levels of Ag whereas an increase in Ag density is required to induce lysis by

low avidity CTL (Shimonkevitz et al, 1985; Mentzer et al, 1990), high avidity CTL seem more competent to lyse target cells than low avidity CTL. Accordingly, on the basis of p38 expression, high avidity CTL might be distinguished from low avidity CTL.

In the present study, donor specific CTL clones generated from peripheral blood and endomyocardial biopsies of heart transplant patients were stained for the above mentioned molecules and their phenotype was compared with their avidity. Briefly, cloned T cells were incubated with 2500 ^{51}Cr-labelled donor cells, third party cells (cells that did not share MHC antigens with the donor and acceptor), and NK-sensitive K562 cells at different effector:target (E:T) ratios in 0.2 ml culture medium. After 4 hours of incubation (37°C, 5%CO_2), supernatants were harvested and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA). This study included 26 T cell clones that lysed donor target cells specifically. These clones were assessed for their avidity by performing also ^{51}Cr-release assays in the presence of 2 μg/ml mouse anti-human CD8 (FK18; a kind gift of Dr F Koning, University Hospital Leiden, the Netherlands) or CD4 mAb (RIV6; a kind gift of Dr MF Leerling, RIVM, Bilthoven, the Netherlands). Addition of anti-CD8/CD4 mAb totally reduced donor directed lysis by 14 CD8$^+$CTL and CD4$^+$CTL clones (low avidity CTL) but did not affect the cytolytic activity of 12 other donor specific CD8$^+$CTL and CD4$^+$CTL clones (high avidity CTL).

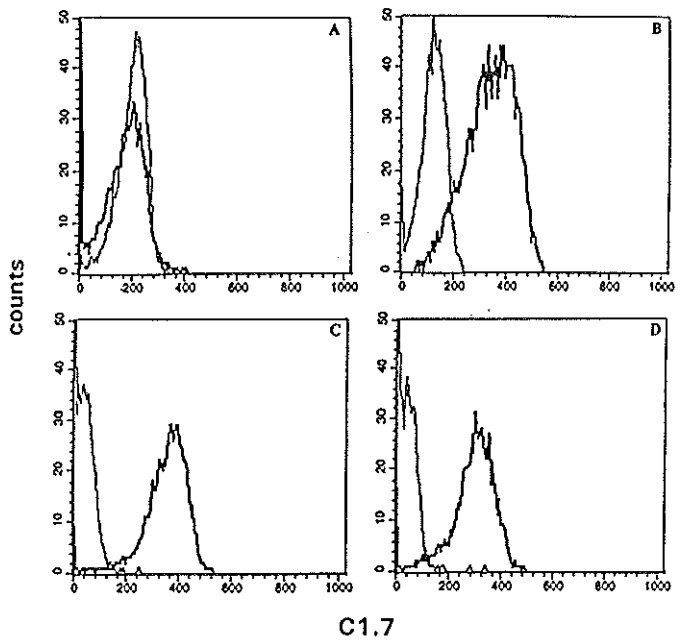

Figure 1: Donor specific CTL clones that differ in their avidity for donor antigen were examined for their p38 surface expression by FACS analysis using PE-conjugated C1.7 mAb (thick lines). Thin lines indicate staining with PE-goat anti-mouse IgG alone. Low avidity CD4$^+$CTL (A) lack p38 surface expression while high avidity CD4$^+$CTL (B), low avidity CD8$^+$CTL (C), and high avidity CD8$^+$CTL (D) express p38 on their cell surface.

The cell surface phenotype of both donor specific CTL subsets was successively analyzed by flow-cytometry after staining with a panel of mAb directly conjugated with fluorescein (FITC), peridinin chlorophyll protein (PERPC), or phycoerythrin (PE). Aliquotes of $1x10^5$ cells were incubated at 4°C for 30 minutes with the relevant fluorescent mAb (mAb specific for TCRαß (WT31), CD8, CD4, and CD45RO were obtained from Becton Dickinson, California, USA; mAb specific for CD2, CD11a, and p38 were obtained from Immunotech, Marseille, France) in 50µl Hanks' balanced salt solution (Gibco, Paisley, Scotland) supplemented with 1% bovine serum albumin and 0.1% sodiumazide. Subsequently, cells were washed and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Non-specific staining of the clones was assessed by incubating the cells with irrelevant isotypically matched murine mAb.

FACS analysis demonstrated that all CTL clones studied expressed comparable levels of TCRαß, CD8 (or CD4), CD2, CD11a, and CD45RO despite the fact that they differed in avidity (data not shown). Staining with PE-conjugated C1.7 mAb, however, revealed that these CTL clones varied in their p38 surface expression level. Low avidity $CD4^+$CTL (n=8) did not react with C1.7 mAb and thus lack p38 surface expression (figure 1A) while high avidity $CD4^+$CTL (figure 1B; n=6), low avidity $CD8^+$CTL (figure 1C; n=6), and high avidity $CD8^+$CTL (figure 1D; n=6) expressed p38 on their cell surface. The level of p38 did not alter after restimulation demonstrating that this molecule, when present, is constantly expressed. According to these results, the p38 molecule delineates high avidity $CD4^+$CTL from low avidity $CD4^+$CTL.

Table 1: p38 expression by naive and stimulated $CD4^+$ and $CD8^+$T cells

Experiment	I		II	
	CD4	CD8	CD4	CD8
$p38^+$T cells (%):				
- no stimulus	0	36	0	54
- PHA stimulus	34	57	28	53
- RIV9 stimulus	15	78	24	64

Peripheral $CD4^+$ and $CD8^+$T lymphocytes of 2 healthy blood donors were analyzed for their p38 surface expression before and 5 days after stimulation with either PHA or immobilized anti-CD3 (RIV9). The percentage of p38-expressing lymphocytes is shown.

We then questioned whether p38 is expressed on other $CD4^+$T cells besides high avidity $CD4^+$CTL. To analyze the p38 expression on naive T lymphocytes, fresh peripheral blood lymphocytes (PBL) of 2 healthy volunteers were stained simultaneouly with PE-conjugated C1.7 mAb and PERCP-conjugated anti-CD4 mAb or FITC-conjugated anti-CD8 mAb. The FACScan results are shown in table 1. None of the $CD4^+$T cells expressed the p38 surface molecule whereas 36% and 54% of the $CD8^+$T cells present within both PBL samples stained

positive. A considerable proportion of the CD4⁺T cells, however, did become p38-positive after 5 days of stimulation with either phytohaemagglutinin (PHA; Difco, Detroit, MI, USA) or immobilized anti-CD3 (RIV9; RIVM). Hence, naive CD4⁺T cells lack p38 but may become positive after priming.

As Valiante and Trinchieri demonstrated that cell mediated cytotoxicity exhibited by CD8⁺T lymphocytes is generally restricted to those bearing p38, we questioned whether the same would be true for CD4⁺T lymphocytes. For this analysis, five non-cytotoxic CD4⁺T cell clones of 3 heart transplant recipients were examined for their p38 expression. Additionally, we analyzed whether the cytotoxic capacity of p38-negative low avidity CD4⁺CTL differed from that of p38-positive high avidity CD4⁺CTL, low avidity CD8⁺CTL, or high avidity CD8⁺CTL. The results are depicted in figure 2. Non-cytolytic CD4⁺CTL totally lacked p38 surface expression. Low avidity CD4⁺CTL that are also negative for p38, lysed their specific target cells less efficiently than did p38-expressing CTL subsets ($p=0.02$; Mann Whitney U-test). The p38-expressing CTL subsets were comparable with respect to their lytic capacity (N.S; Mann Whitney U-test).

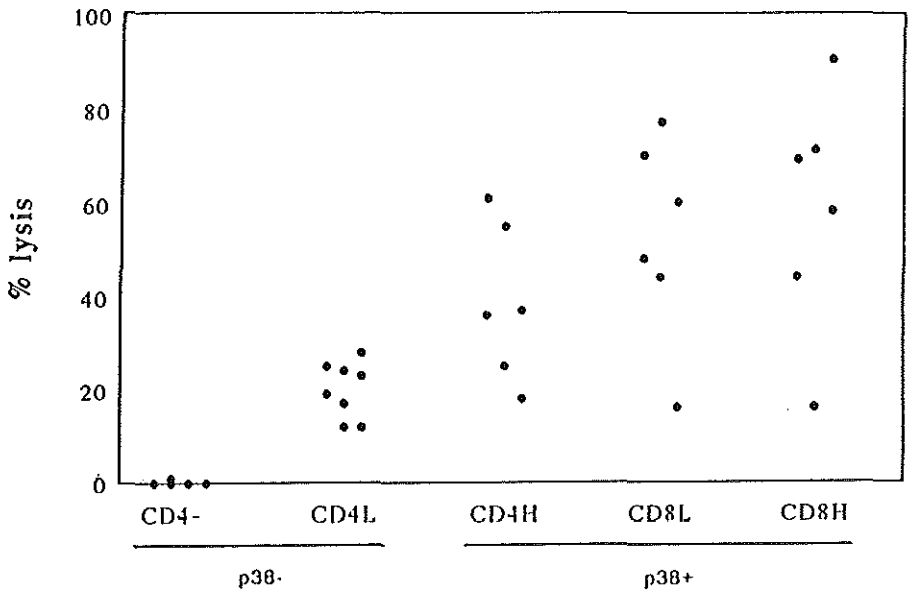

Figure 2: The ability of T cell clones obtained from heart transplant patients to lyse donor target cells in ^{51}Cr-release assays (E:T ratio = 5:1) is compared with their p38 surface expression. Non-cytotoxic T cells (CD4-) do not express p38. Low avidity CD4⁺CTL (CD4L), that also lack p38, are less destructive ($p<0.02$) towards donor target cells than p38-expressing high avidity CD4⁺CTL (CD4H), low avidity CD8⁺CTL (CD8L), or high avidity CD8⁺CTL (CD8H).

Although this observation implies that T cells lacking p38 surface expression are not cytolytic or less efficient CTL, the reverse is not always true. As shown in figure 2, p38-expressing CTL clones vary enormously in their ability to lyse their specific target cells (range: 16-90%

lysis). Four of these CTL clones lysed their target cells in the same range as low avidity CD4$^+$CTL. However, in contrast to low avidity CD4$^+$CTL, these clones did stain with C1.7 mAb.

In an attempt to analyze the significance of p38 on CTL, we examined whether blocking of this molecule would affect their cytolytic capacity. Concentrations of C1.7 mAb reported to modify non-HLA-restricted cytotoxicity of lymphocytes and NK cells (Valiante and Trinchieri, 1993) did not influence antigen-specific lysis by p38-expressing CTL clones in ^{51}Cr release assays demonstrating that CTL do not require this molecule to interact with their specific target cells (table 2).

Table 2: Effect of p38 blocking on the cytolytic capacity of CTL.

	%lysis					
	0	0.01	0.1	1	10	μg/ml C1.7
CD4 low	26	13	21	29	32	
CD4 high	52	51	47	49	53	
CD8 low	67	54	66	60	62	
CD8 high	57	51	40	51	59	

Donor specific CD4$^+$CTL (E:T = 5:1) and CD8$^+$CTL (E:T = 2.5:1) clones with either a high or a low avidity were tested for their capacity to lyse donor target cells in the presence of increasing concentrations of soluble C1.7 mAb.

Although the existance of CD4$^+$T cells with cytolytic potential has long been accepted, most studies on CTL still elaborate on the assumption that all cytotoxic T cells are contained in the CD8$^+$subset. Due to this prejudice, the contribution of CD4$^+$CTL in immune responses has largely been overlooked. According to our studies on cardiac transplant patients, approximately half of the graft derived cell cultures displayed cytotoxicity towards donor HLA class II antigens (Van Emmerik et al, 1997). As for donor HLA class I specific CD8$^+$CTL, the avidity of donor HLA class II specific CD4$^+$CTL correlated with the rejection status of the cardiac transplant. Donor HLA class II specific CD4$^+$CTL propagated from rejecting grafts mainly had a high avidity for donor Ag whereas those established from stable grafts mainly had a low avidity. According to the current concept, high avidity CTL are able to display their cytotoxic function in vivo whereas low avidity CTL are not. In the present study it is shown that high avidity CD4$^+$CTL are the only cells within the CD4$^+$lymphocyte population that express p38 on their cell surface. Accordingly, histological staining of high avidity CD4$^+$CTL by p38 might provide us with a tool to directly examine the contribution of in vivo functional CD4$^+$CTL in immune processes such as allograft rejection. It might reveal when CD4$^+$CTL come into play and which cells serve as their target.

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Summary and discussion

Although it is well established that T-lymphocytes are required for allograft rejection, there is still controversy over which cell subset is responsible for the tissue destruction. Support for the involvement of CTL as terminal effector cells is provided by experiments showing the exquisite antigen specificity of the alloresponse (Sutton et al, 1989; Mintz and Silver, 1967) and the ability of CTL clones grown from rejecting allografts to destroy allogeneic tissue when injected into appropriate hosts (Snider et al, 1986). However, donor specific CTL were also found within transplants in which no histological evidence of rejection was found. If allospecific CTL are implicated in graft rejection, the question arises why their presence does not automatically result in graft destruction. To explain these contradicting data, two speculations were made. the occurrence of graft damage may depend on the number of donor specific CTL present within the allograft. Alternatively, donor specific CTL that populate the allograft in the presence or absence of rejection may have different characteristics. In the present thesis, both possibilities were studied to analyze the relevance of donor specific CTL in the development of acute cardiac rejection in human.

Our first study (**chapter 2**) concerned the importance of CTL directed towards donor HLA class I antigen. Endomyocardial biopsies routinely taken for the diagnosis of acute cardiac rejection, were cultured to obtain graft infiltrating lymphocytes (GIL). The number of donor class I specific CTL present among serial GIL cultures was then estimated by a modified limiting dilution assay (LDA). The data of this longitudinal follow-up study demonstrated that donor class I specific CTL that had been activated in vivo (cCTL) and their precursors (pCTL) both accumulate within the graft during acute rejection. After successful rejection therapy, donor specific cCTL and pCTL frequencies recovered to levels found before rejection. This observation implies that the number of graft infiltrating, donor class I specific CTL might be relevant for the occurrence of acute cardiac rejection.
Subsequently, these graft infiltrating CTL were divided into CTL with a high or a low avidity for donor class I antigen based on their resistance or sensitivity to anti-CD8 mAb, respectively. The results revealed that donor specific cCTL and pCTL propagated from EMB taken during or immediately preceding acute cardiac rejection generally had a high avidity for donor antigen. In contrast, donor specific cCTL and pCTL grown from EMB obtained before or after the rejection period generally had a low avidity for donor antigen. According to these data we reasoned that high avidity CTL might be implicated for allograft rejection whereas low avidity CTL might not be involved. Support for this hypothesis was only recently provided by Alexander Miller et al (1996A). According to their data, the in vivo functionality of CTL depends on their avidity for antigen. High avidity CTL efficiently cleared antigen-bearing cells in vivo whereas low avidity CTL lines, though effective in vitro, were incompetent.

The relationship between the occurrence of graft rejection and the presence of high avidity CTL was then extended to donor HLA class II specific CTL (**chapter 3**). Lymphocyte cultures established from EMB exhibit cytotoxicity towards donor HLA class II determinants in 46% of the cases. We analyzed the frequency of donor HLA class II specific cCTL within these cultures and compared the results with the rejection status of the transplant. Frequencies of donor class II specific cCTL found during a rejection period were comparable to values found in the absence of rejection. Addition of anti-CD4 mAb to the LDA revealed how many of the class II specific cCTL enumerated could be scored as high (resistant to anti-CD4 mAb) or low avidity cCTL (sensitive to anti-CD4 mAb). During a rejection episode, the majority of the class II specific cCTL had a high avidity for donor antigen. In the absence of rejection, class II specific cCTL mainly had a low avidity for donor antigen. These data support the concept that CTL with a high avidity for donor antigen are implicated in the rejection process whereas CTL with a low avidity are not.

The accumulation of high avidity CTL within the cardiac allograft implies the migration of these cells towards the allograft before acute rejection is diagnosed by histology. Accordingly, the kinetics of high avidity CTL within the peripheral blood might inform us about the immunological status of the transplanted heart and by that provide a method to monitor for acute rejection (**chapter 4**). In order to assess this idea, donor specific CTL present within peripheral blood samples of heart transplant recipients obtained immediately preceding and at various timepoints after transplantation were enumerated by LDA. Subsequently, we analyzed how many of the donor specific CTL present could be scored as high avidity CTL. The fraction of donor specific CTL with a high avidity was then compared with the rejection status of the cardiac allograft.
Donor specific cCTL were rarely observed within the peripheral blood indicating that donor specific CTL circulate as pCTL. Since cCTL can be detected within the graft, this observation demonstrates that the final differentiation of pCTL into cCTL occurs at the graft site. The number of donor specific pCTL among the peripheral blood cells was found to fluctuate in time after transplantation and did not associate with the rejection status of the graft. During acute rejection, however, a significant higher fraction of these donor specific pCTL had a high avidity for donor antigen when compared to values found before the diagnosis of rejection or after rejection therapy. More than one year after transplantation, when all patients included in the study had achieved stable engraftment, high avidity pCTL were even absent. These observations imply that changes in the overall avidity of circulating donor specific pCTL reflect immunological processes occurring within the allograft. The application of this technic for non-invasive monitoring of acute cardiac rejection, however, is currently hindered by the duration of the experiments (10 days).

Combining the results of the graft and the peripheral blood, we assume that, prior to acute cardiac rejection, circulating donor specific pCTL with a high avidity selectively home to the

transplanted heart. At the graft site these pCTL differentiate into functional cCTL with a high avidity. Sufficient numbers of these cCTL then cause graft damage as observed by histology. Subsequent anti-rejection therapy terminates the process again by depleting lymphocytes both in the graft and in the circulation. The importance of circulating CTL with a high avidity for donor antigen for the initiation of the rejection cascade is supported by the observation that high avidity, donor specific pCTL have disappeared from the peripheral blood when stable engraftment is achieved.

The above data suggest that not all donor specific CTL but only those with a high avidity for donor antigen are important for the rejection process. Accordingly, the characterisation of this particular subset might shed light on pivotal events in allograft rejection and support the development of selective immunotherapeutic strategies. As the cytokine profile of cells often corresponds with their function we examined whether high and low avidity CTL were distinct with respect to their cytokine production profile (**chapter 5**). Obtained data demonstrated that both subsets differ with respect to their IFN-γ production profile. Stimulation with donor cells resulted in IFN-γ secretion by high avidity CTL but not by low avidity CTL. CD3 stimulation, on the contrary, led to secretion of equivalent amounts of IFN-γ by both CTL subsets. These observations indicate that low avidity CTL are fully capable to produce IFN-γ but, in contrast to high avidity CTL, fail to do so when they encounter donor cells. As IFN-γ favours the occurrence of transplant rejection, the production of this cytokine by high avidity CTL might explain the earlier observed association between the presence of these cells and the rejection status of the transplanted human heart.

Additionally, we analyzed whether high and low avidity CTL differed with respect to their surface phenotype. In **chapter 6**, we describe a surface molecule, p38, which delineates high avidity CTL within the $CD4^+$lymphocyte population. We believe that this marker provides us with a tool to analyse the contribution of $CD4^+$CTL in cardiac rejection and to define their target cells (as human myocytes may not serve as target cells as they lack MHC class II). Additionally, staining of routinely taken endomyocardial biopsies for both p38 and CD4 expression, might predict the occurrence of acute rejection. Kinetic studies of donor specific, high avidity CTL demonstrated that these cells are absent within stable cardiac allografts, but start accumulating approximately 2 weeks prior to the diagnosis of acute rejection (chapter 2). As a result, increases in the number of high avidity CTL present within the cardiac allograft would predict the occurrence of acute rejection. As $CD4^+$CTL are found in a significant proportion of cardiac biopsies (chapter 3), immunohistochemical analysis of $CD4^+p38^+$lymphocytes would inform us about the number of high avidity, donor specific CTL among graft infiltrating $CD4^+$T cells and hence about the rejection status of the cardiac allograft.

Samenvatting

Hoewel het algemeen geaccepteerd is dat T cellen een essentiele rol spelen bij transplantaatafstoting blijft de bijdrage van de afzonderlijke T cel subsets en met name die van de cytotoxische T cellen (CTL) een punt van discussie. Zoals vermeld in hoofdstuk 1 van dit proefschrift doet de specificiteit waarmee het afstotingsprocces verloopt sterk vermoeden dat CTL functioneren als terminale effector cellen wiens specifieke binding aan donorcellen leidt tot de destructie van de laatsten. Echter, donorspecifieke CTL worden ook aangetroffen in transplantaten welke geen tekenen van afstoting vertonen. Wanneer CTL daadwerkelijk verantwoordelijk zijn voor de weefselschade die een afstoting karakteriseren dan rijst de vraag waarom de aanwezigheid van deze cellen in het transplantaat niet altijd gepaard gaat met afstoting. Van de mogelijke verklaringen werden er twee onderzocht: I. Het optreden van afstoting zou afhankelijk kunnen zijn van het *aantal* in het transplantaat aanwezig zijnde donorspecifieke CTL. II. Het optreden van afstoting zou afhankelijk kunnen zijn van het *type* donorspecifieke CTL dat in het transplantaat aanwezig is.

Het eerste onderzoek waarvan de data beschreven staan in **hoofdstuk 2** betreft CTL die donor HLA klasse I antigenen herkennen. Endomyocardbiopten, op verschillende tijdstippen na transplantatie afgenomen voor de diagnose van afstoting, werden in kweek gebracht om zo de transplantaat infiltrerende lymfocyten (TIL) te verkrijgen. Vervolgens werd met behulp van limiting dilution analysis (LDA) het aantal donor HLA klasse I specifieke CTL dat zich onder deze TIL bevond bepaald. Deze longitudinale studie toonde aan dat in vivo geactiveerde donor HLA klasse I specifieke CTL (cCTL) en hun naive precursors (pCTL) beide aanwezig waren in het transplantaat ongeacht de afstotingsgraad van de laatste maar dat hun aantal drastisch toenam kort voor en tijdens afstoting. Na een succesvolle afstotingsbehandeling daalden de donorspecifieke cCTL en pCTL frequenties weer tot waarden die voor de aanvang van afstoting gevonden werden. Deze waarneming impliceert dat het aantal in het transplantaat aanwezig zijnde donor HLA klasse I specifieke CTL van belang is voor het optreden van acute afstoting.
Vervolgens werden deze donor HLA klasse I specifieke CTL onderverdeeld in CTL met een hoge of een lage aviditeit voor donor-antigeen al naar gelang hun (on)gevoeligheid voor CD8-blokerende antilichamen. De verkregen data onthulden dat donor HLA klasse I specifieke pCTL en cCTL gekweekt uit afstotingsbiopten of uit biopten afgenomen kort voor (1-2 weken) afstoting een hoge aviditeit voor donor-antigeen hadden terwijl CTL afkomstig uit EMB die op andere tijdstippen afgenomen waren juist een lage aviditeit voor donor-antigeen hadden. Deze resultaten leidden tot de hypothese dat alleen hoog avide donorspecifieke CTL betrokken waren bij transplantaatafstoting. Deze hypothesis werd onlangs gesteund door data van Alexander-Miller en medewerkers (1996A) welke aantoonden dat het in vivo functioneren van virus specifieke CTL afhing van hun aviditeit.

De gevonden relatie tussen het optreden van transplantaatafstoting en de aanwezigheid van hoog avide CTL werd nu uitgebreidt naar donor HLA klasse II specifieke CTL, een CTL subset wiens impact in immunologische processen vaak verwaarloosd wordt. Onze studies lieten echter zien dat deze subset uit 46% van de endomyocardbiopten gekweekt kon worden, een percentage dat niet overeenstemt met een onbeduidende rol (**hoofdstuk 3**). Het aantal donor HLA klasse II specifieke cCTL in deze biopt cultures werd bepaald met behulp van LDA en vergeleken met de afstotingsgraad van het transplantaat. In tegenstelling tot de data gevonden voor donor HLA klasse I specifieke cCTL (hoofdstuk 2) werd er geen correlatie gevonden tussen de frequentie aan donor HLA klasse II specifieke cCTL en de mate van afstoting.
Toevoeging van anti-CD4 antilichamen aan de LDA onthulde welk percentage van de aanwezige HLA klasse II specifieke cCTL een hoge aviditeit (i.e. resistent tegen CD4-blokerende antilichamen) en welk percentage een lage aviditeit (i.e. gevoelig voor CD4-blokerende antilichamen) voor donor-antigeen had. Deze studie toonde aan dat donor HLA klasse II specifieke cCTL die aanwezig zijn in het getransplanteerde hart ten tijde van afstoting nagenoeg allen een hoge aviditeit voor donor-antigeen hadden. Dit in tegenstelling tot diegene die in het hart aangetroffen werden gedurende een periode zonder afstoting en met name een lage aviditeit hadden.

De accumulatie van hoog avide CTL in het getransplanteerde hart suggereert de migratie van deze cellen naar het transplantaat voordat acute afstoting gediagnostiseerd wordt. Dit gegeven impliceert dat de kinetiek van hoog avide donor specifieke CTL in het bloed informatie zou kunnen verstrekken over de immunologische status van het getransplanteerde hart zonder dat daarvoor biopten afgenomen behoeven te worden. Om deze optie te verifieren werd in bloedmonsters die op verschillende tijdstippen na transplantatie afgenomen waren de frequentie en aviditeit van donor HLA klasse I specifieke CTL bepaald. Vervolgens werden deze gegevens vergeleken met de afstotingsgraad van het getransplanteerde hart op het moment van bloedafname. De resultaten van deze studie staan beschreven in **hoofdstuk 4**.
Donor specifieke cCTL werden zelden aangetroffen in de bloedmonsters wat aangeeft dat donorspecifieke CTL circuleren als pCTL. Voorgaande studies hebben echter aangetoond dat cCTL aanwezig zijn in het getransplanteerde hart. Deze waarneming demonstreert dat de uiteindelijke differentiatie van pCTL naar cCTL plaatsvindt in het transplantaat. Het aantal donor specifieke pCTL in het perifere bloed vertoonde fluctuaties in tijd na transplantatie welke niet associeerde met de rejectie status van het transplantaat. Wanneer men echter de aviditeit van de aanwezige donor specifieke pCTL in beschouwing nam dan bleek dat, net als in de voorgaande studies, het percentage donor specifieke pCTL met een hoge aviditeit steeg tijdens acute afstoting; i.e. hoog avide donor specifieke pCTL waren prominenter aanwezig in het bloed van harttransplantatie patienten ten tijde van afstoting.
Meer dan 1 jaar na transplantatie bleek de "rust" in het transplantaat wedergekeerd daar geen van de onderzochte harttransplantatie patienten nog afstoting ondervond. Hoewel het aantal

donor specifieke pCTL dat in deze periode in het bloed aanwezig was niet afweek van eerdere waardes bleken hoog avide pCTL verdwenen te zijn. Deze down-regulatie van hoog avide donor specifieke pCTL (maar niet van laag avide donor specifieke pCTL) ten tijde van "immunologische rust" gecombineerd met hun toename ten tijde van afstoting impliceert het belang van deze subset bij afstoting. Bovendien suggereert het dat de kinetiek van circulerende donor specifieke hoog avide pCTL ons kan informeren over de welstand van het transplantaat, een gegeven dat nieuwe deuren opent voor non-invasieve monitoring van acute cardiale afstoting.

Wanneer we de tot nu toe verkregen data bundelen komen we tot het volgende concept. Kort voor acute afstoting optreedt migreren pCTL met een hoge aviditeit voor donor-antigeen selectief naar het getransplanteerde hart waar zij accumuleren en uitdifferentieren tot functionele cCTL. Een voldoende aantal van deze cCTL leidt nu tot histologisch waarneembare myocytschade (i.e. acute afstoting). De hierop volgende anti-rejectie therapie beeindigt het process door de depletie van T-lymfocyten in de periferie en het transplantaat. Het belang van circulerende CTL met een hoge aviditeit voor donor-antigeen voor het initiëren van de afstotingscascade wordt ondersteund door het gegeven dat deze subset na 1 jaar, wanneer afstoting nog zelden optreedt, uit het bloed verdwenen is. Dit concept houdt in dat niet alle donor specifieke CTL maar slechts diegene met een hoge aviditeit for donor-antigeen van belang zijn voor het afstotingsproces. De karakterisering van deze subset zou dan ook informatie kunnen verstrekken over de immunologische fasen die leiden tot transplantaat afstoting. Daarnaast zou de karakterisering van deze subset een potentiële waarde kunnen hebben voor de ontwikkeling van selectieve immunotherapeutische strategieën.

Daar het cytokine profiel van cellen vaak informatie verschaft over hun functie werd onderzocht of hoog en laag avide CTL verschilden in cytokine productie profiel. De resultaten van dit onderzoek staan beschreven in **hoofdstuk 5**. Beiden CTL subsets bleken te verschillen in hun IFN-γ productie profiel. Een encounter met donor cellen induceerde de productie van IFN-γ door hoog avide CTL maar niet door laag avide CTL. CD3-stimulatie daarintegen induceerde de produktie van gelijke hoeveelheden IFN-γ door beide CTL subsets. Deze waarneming impliceert dat laag avide CTL volledig capable zijn om IFN-γ te produceren maar dat zij in tegenstelling tot hoog avide CTL dit niet doen wanneer zij donor cellen tegen komen. Het is bekent dat IFN-γ secretie het optreden van afstoting begunstigd. De produktie van dit cytokine door hoog avide CTL ondersteunt dan ook de eerder gevonden relatie tussen de aanwezigheid van hoog avide CTL in het humane getransplanteerde hart en het optreden van afstoting.

Een volgend onderzoek richtte zich op het fenotype van hoog en laag avide donor specifieke CTL. Onderzocht werd of dat beide subsets verschilden in de expressie van oppervlakte

moleculen. In **hoofdstuk 6** beschrijven we p38, een oppervlakte molecuul dat herkend wordt door het C1.7 antilichaam en dat alleen op hoog avide CTL binnen de $CD4^+$T cel populatie tot expressie komt. Gezien dit resultaat geloven wij dat deze marker gebruikt kan worden om de bijdrage van $CD4^+$CTL in cardiale afstoting te onderzoeken en indirect om de target cellen van $CD4^+$CTL te definieren. Op dit moment is het namelijk nog onduidelijk ofdat humane myocyten herkent kunnen worden door $CD4^+$CTL daar zij geen HLA klasse II moleculen tot expressie brengen. Met enig optimisme zou het aantal $p38^+CD4^+$T cellen dat in opeenvolgende endomyocardbiopten aanwezig is ook het optreden van acute afstoting kunnen voorspellen.
Kinetiek studies demonstreerden dat donor HLA klasse I specifieke hoog avide CTL tot 2 weken voor afstoting afwezig zijn in het getransplanteerde hart waarna zij in groten getale in het transplantaat accumuleren (hoofdstuk 2). Dit geeft aan dat een toename van hoog avide CTL binnen het getransplanteerde hart gezien kan worden als een voorbode van acute afstoting. Daar $CD4^+$CTL in een significant percentage van de endomyocardbiopten aanwezig zijn (hoofdstuk 3), zou immunohistochemische analyse van $CD4^+p38^+$lymfocyten ons kunnen informeren over het aantal hoog avide donor specifieke CTL binnen de transplantaat infiltrerende $CD4^+$T cel populatie en daarmee over de rejectie status van het getransplanteerde hart.

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*nominated for the Caves Award 1996.

Curriculum Vitae

De auteur van dit proefschrift werd op 9 september 1968 te Goirle geboren. In 1986 behaalde zij het Atheneum B diploma aan de Rijksscholengemeenschap Koning Willem II te Tilburg. In dat zelfde jaar begon zij aan de studie Medische Biologie aan de Rijksuniversiteit Utrecht. Als student werkte zij gedurende 8 maanden bij de afdeling Oncologie van het AZU waar zij onder leiding van Dr. G. Westerhof en Dr. G. Jansen participeerde in onderzoek naar het membraantransport van folaten en folaat-analogen in tumorcellen. Deze bijvakstage werd opgevolgd door een hoofvakstage van 15 maanden bij de afdeling Experimentele Pathologie van het AZU waar onder leiding van Dr. R. Vandebriel en Dr. R. de Weger de betrokkenheid van $\gamma\delta$T cellen in de immuunrespons tegen allogene tumoren onderzocht werd. Het doctoraal diploma werd in Augustus 1992 afgelegd. Vervolgens werd zij als AIO aangesteld bij de afdeling Thoraxchirurgie (hoofd: Prof. Dr. E. Bos) en werkte onder supervisie van Prof. Dr. W. Weimar op het transplantatielab van de afdeling Interne Geneeskunde I van het Academisch Ziekenhuis Rotterdam aan het in dit proefschrift beschreven onderzoek. Vanaf 1 juli 1997 is zij als post-doc werkzaam bij de afdeling Cellulaire Immunologie van het NIMR instituut te Londen.

Dankwoord

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Nancy