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HLA Alloreactivity by Human Viral Specific Memory T-cells

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Do not consider it proof just because it is written in books…
Maimonides (1135-1204)

To my past, present and future...
...For my parents because I could not have arrived here without you
...Voor Frans omdat je me deze geweldige kans hebt gegeven
...Per Martina perche tu sei il sole al mio orizzonte

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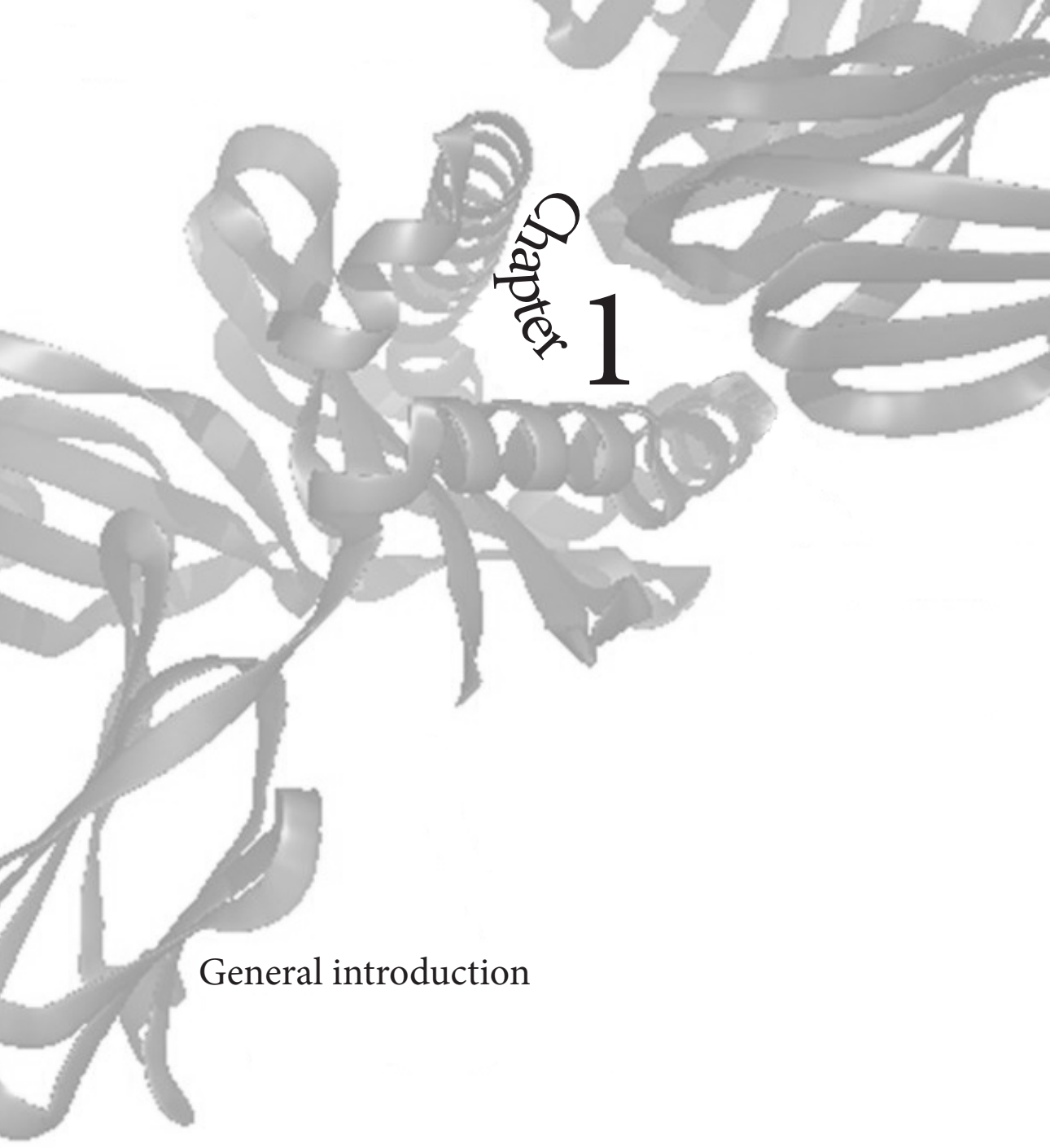

Chapter 1

General introduction

GENERAL INTRODUCTION - CONTENTS

INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end stage renal disease. However adaptive immune responses to donor HLA antigens are a potent barrier to successful transplantation and/or tolerance. Allograft rejection is initiated, and in many cases, executed by T-cells recruited into the graft (1). With current immunosuppressive regimens T-cell mediated rejection is less common than with previous regimens, but remains the dominant early rejection phenotype and is also associated with chronic allograft nephropathy. B-cells can make donor specific HLA antibodies which are associated with antibody mediated rejection.

The possible induction of specific tolerance towards the graft is the ultimate goal in clinical transplantation. The successful blockade of co-stimulatory pathways to induce prolonged graft survival in mice raised hopes for the successful transfer of tolerance inducing regimens into the clinic. However all these protocols rapidly failed in pathogen exposed mice (2-6).

Accumulating evidence suggests that graft rejection is a result of allo-HLA crossreactivity by self-HLA restricted T-cells. Furthermore memory T-cells that are generated as a result of previous infections may cross-react against allogeneic HLA molecules (2,7). These pre-existing memory T-cells may provide a potent barrier to transplantation tolerance because of their higher activation state, cytokine production, cytotoxicity and lower requirements for T-cell help and/or co-stimulation.

The aim of this thesis was therefore to determine if the high frequency of pre-existing alloreactive memory T-cells in non-sensitized individuals could be accounted for by allo-HLA crossreactivity by viral specific memory T-cells. Prior to discussing the current knowledge of mechanisms underlying T-cell alloreactivity, a review of the normal immune response against antigens is warranted.

1. GENERAL IMMUNOLOGY: THE HUMAN IMMUNE SYSTEM

Pathogens, such as viruses, represent a major threat to the human body and the immune system is the body's natural defence against these infections. The immune system can be divided into innate and acquired immunity. The innate immune system consists of physical barriers and a number of non-specific molecules, receptors and cells which provide immediate protection against invading organisms and initiate an adaptive acquired immune response.

The adaptive immune system comprises a repertoire of T-cells and B-cells that is generated upon antigenic challenge and thus depends on the individual's exposure to pathogens. These cells bear receptors on their surface that provide specificity. T-cells that have not yet encountered their cognate antigen are naïve T-cells. Upon encounter with their specific antigen these cells will expand and mature into effector and memory T-cells. The acquired immune system is specific and retains memory for pathogens that have been previously encountered.

Antigen presentation by the Major Histocompatibility Complex (MHC) initiates an antigen specific immune response by T-lymphocytes. In humans the MHC molecules are known as the human leukocyte antigens (HLA).

1.1 HUMAN LEUKOCYTE ANTIGENS

T-cells constantly survey tissue cells for the presence of pathogens. The T-cell receptor (TCR) recognizes foreign antigens in the form of peptides only when they are presented by specific molecules of the HLA complex. HLA molecules are expressed on all nucleated human cells and the phenomenon whereby T-cells recognize an antigenic peptide presented only by one self-HLA molecule is termed HLA restriction. There are two classes of HLA molecules, both with similar, yet distinct functions (Figure 1).

HLA Class I
The classical HLA class I molecules, HLA-A, HLA-B and HLA-C are constitutively expressed on all nucleated cells. HLA class I molecules consist of a transmembrane α-chain, a non-covalently associated light chain β2-Microglobulin and the peptide presented in the peptide binding groove of the α-chain. The α-chain is encoded on chromosome 6 and contains three extracellular domains (α1, α2, and α3). The α1 and α2 domains form the peptide binding groove, are the sites of most polymorphisms within the HLA class I molecule and are also the sites of TCR contact with the HLA molecule. The α3 domain contains a CD8 binding site which is necessary for presentation of intracellular peptides to CD8 T-cells. Peptides presented by HLA class I molecules are generally 8-13 amino acids in length.
In case of intracellular infection, e.g. virus infection, HLA class I molecules present pathogen derived peptides to CD8 cytotoxic T-lymphocytes (CTLs) which can then immediately and specifically eliminate the infected cell.

HLA Class II
HLA class II molecules are constitutively expressed on professional antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages, B-cells and activated T-cells. However inflammatory cytokines, such as IFNγ, can induce HLA class II expression on most cell types. HLA class II molecules are encoded by the HLA-DR, HLA-DQ and HLA-DP genes on chromosome 6. HLA class II molecules consist of two transmembrane chains (α and β) that both contribute to the peptide binding site, and also contain a CD4 binding site. The β chain of the HLA-DR molecule is the most polymorphic of the class II molecules. Peptides presented in HLA class II molecules are typically 12-25 amino acids long.

The function of the HLA class II molecules is to present extra-cellular peptides to CD4 T-cells for the initiation of immune reactions and recruitment of other effector mechanisms.

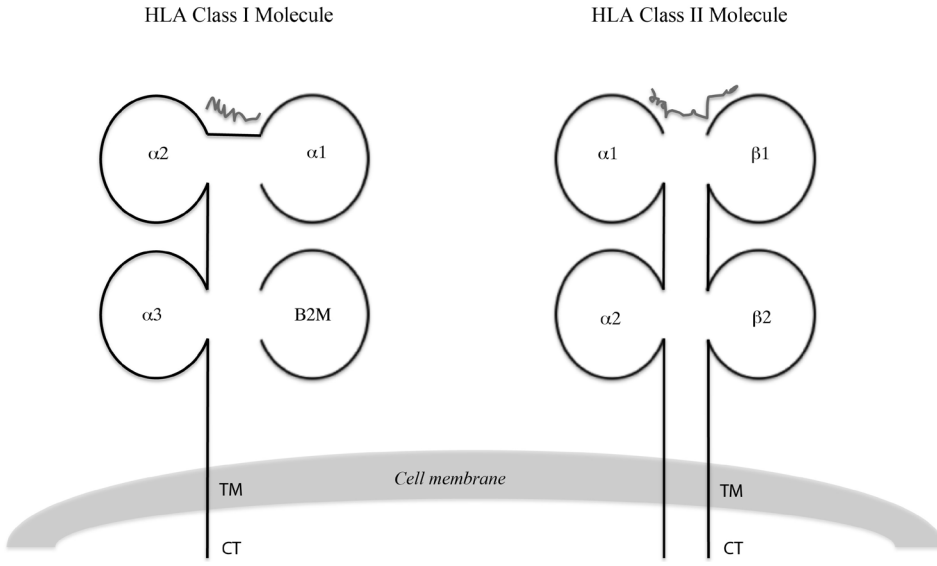

Figure 1. The structure of HLA class I and II molecules.
HLA class I consists of a heavy chain (α chain) and a non-covalently associated invariant light chain (β2-Microglobulin). HLA class II is a heterodimer consisting of α and β chains. The peptide binding groove is formed solely by the α chain in HLA class I molecules and by both the α and β chains in HLA class II molecules. HLA class II molecules bind and present longer peptides than HLA class I molecules. TM=Transmembrane region. CT=Cytoplasmic tail. B2M=β2-Microglobulin.

Antigen Processing and Peptide/HLA Restriction
In all cells proteosomes degrade cellular proteins that are poorly folded, damaged or unwanted. When a cell becomes infected, pathogen derived proteins in the cytosol are also degraded by the proteosome. Peptides are transported from the cytosol into the endoplasmic reticulum by a protein called transporter associated with antigen processing (TAP). Newly synthesized HLA class I molecules are also transported into the endoplasmic reticulum where they can now bind these peptides, before being transported to the cell surface in order to present these peptides to T-cells.

HLA class II molecules are prevented from binding peptides in the endoplasmic reticulum by the presence of the invariant chain bound in the groove. The invariant chain also targets class II molecules to endocytic vesicles where they bind proteins derived only from the extracellular space. When the HLA class II molecule has lost its invariant chain and has a tightly bound peptide it is carried to the cell surface.

It is known that HLA molecules can present both self and non-self peptides on the cell surface. The T-cell receptor specifically recognizes both the presented peptide and the HLA molecule.

1.2 THE T-CELL RECEPTOR AND THYMIC EDITING

Antigen recognition by T-cells is central to the generation and regulation of an effective immune response. The TCR recognizes antigen fragments (peptides) which are bound and presented by HLA molecules. T-cells do not recognize free antigen.

The T-cell Receptor (TCR)
The TCR is the highly variable recognition molecule used by T-cells. A typical TCR consists of an α and β chain, both embedded in the membrane. The diversity of the TCR is generated by gene rearrangement (Figure 2). The variable parts of the TCR are encoded by separate gene segments called V, D and J segments, each of which is present in the genome as a tandem array of polymorphic forms. For a functional TCR to be made one each of the different gene segments must be brought together by gene rearrangement with elimination of the intervening regions. The numerous combinations of V, D and J segments that can be brought together are the principal source of variable region diversity of the TCR. Each lymphocyte is clonal; a single TCR is expressed in each lymphocyte. An adaptive immune response is initiated when a naïve T-cell recognizes a pathogen specific peptide presented by an APC on a self-HLA molecule.

Antibodies are the receptors for antigen specific B-cells and are formed by very similar gene rearrangements to that used in formation of the TCR. Adaptive B-cell responses are not discussed in this thesis.

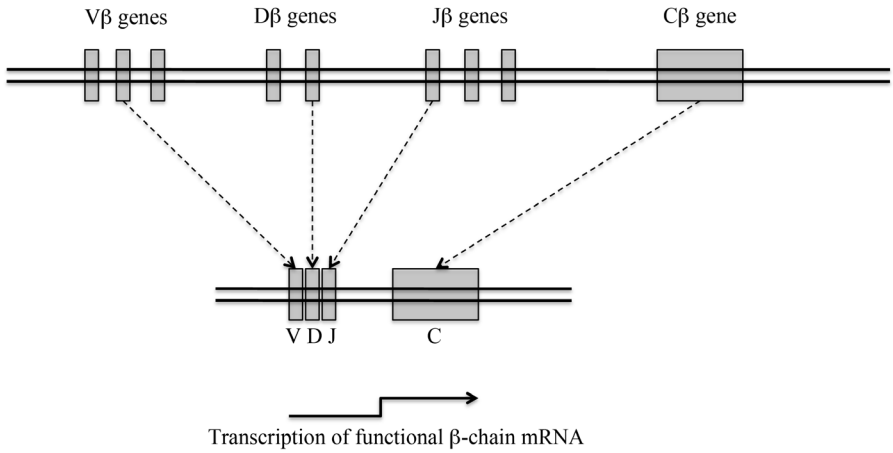

Figure 2. Synthesis of T-cell receptor β-chain.
Rearrangements of different V, D and J segments result in the formation of a unique β-chain. Productive β-chain gene rearrangement commits the T-cell to the α:β lineage. The T-cell receptor α-chain genes then commence comparable gene rearrangements except that they do not have D segments. Following successful α:β TCR generation the double-positive cell is signaled to survive and can proceed to positive and negative selection. V=Variable gene segment. D=Diversity gene segment. J=Joining gene segment. C=Constant region. TCR= T-cell receptor.

Thymic Selection

The first phase of T-cell development is the production of a functional TCR, irrespective of antigen specificity. The TCR repertoire that actually exits the thymus is then the product of "positive" and "negative" selection based on self-peptide/HLA recognition in the thymus. Only a small percentage of the T-cells with successful TCR gene rearrangements have a TCR that can interact with one of the HLA class I or II isoforms expressed by the individual, these T-cells are positively selected for further development. T-cells that are positively selected by HLA class I molecules become CD8 T-cells and T-cells that are positively selected by HLA class II molecules become CD4 T-cells. Thus both CD4 and CD8 T-cells develop from a common precursor in the thymus.

Tissue-specific proteins are expressed in the thymus and T-cells that bind self-peptides presented on self-HLA molecules are removed in the thymus by negative selection. For example the TCR that uses the VB6 gene segment is specific for the EBV FLRGRAYGL peptide presented by HLA-B*08:01 (7,8). This TCR also binds the EEYLQAFTY self-peptide from the ABCD3 gene presented on HLA-B*44:02 (9). In HLA-B8 B44 heterozygous individuals this TCR is negatively selected in the thymus to avoid auto-immunity (10).

Thus during T-cell development any T-cells having receptors that respond to complexes of self-peptide and MHC class I and II molecules of healthy cells are eliminated. However this quality control mechanism encompasses only HLA isoforms expressed by that individual (autologous HLA), and not other HLA isoforms (allogeneic HLA). Accordingly T-cells that can respond to complexes of self-peptide and allogeneic HLA class I and II molecules are theoretically able to exit the thymus as they are not negatively selected. T-cells that have survived positive and negative selection leave the thymus and enter the circulation as mature naïve T-cells. Mature naïve T-cells exhibit a high frequency (10%) of crossreactivity against allogeneic HLA to which they have not been previously exposed (11,12).

1.3 T-CELL EFFECTOR MECHANISMS

T-cell mediated immunity is critical to the control and eradication of infectious agents. The first part of an adaptive immune response occurs when a naïve T-cell encounters its specific antigen and undergoes T-cell activation in a germinal centre reaction, and is stimulated to differentiate into an effector T-cell. Effector CD8 T-cells are long lived and travel to the sites of infection where they can kill any type of cell whose HLA class I molecule are presenting antigens to which the T cells are specific. Effector CD4 T-cells recognize their specific antigen presented via HLA class II molecules and via cell-cell contact and cytokine production can make macrophages more proficient at killing pathogens and can activate B-cells to make antigen specific antibodies.

Naïve T-cells and T-cell activation
Naïve T-cells have not yet encountered their specific antigen and are characterized by surface expression of CD45Ra, the lymph node homing receptor CCR7 and the presence of costimulatory molecule CD28 (Table 1).

Dendritic cells are adept at capturing and processing antigens from pathogens. Dendritic cells travel to the afferent lymph node that drains from the site of infection, where naïve T-cells first encounter their specific antigen presented by the dendritic cells. The intracellular signal generated by ligation of the T-cell receptor with a specific peptide/HLA complex is necessary to activate a naïve T-cell, but is not sufficient. Participation of the CD4 or CD8 co-receptor is essential for effective naïve T-cell activation. Activation of naïve T-cells also requires a co-stimulatory signal delivered by an APC. The co-stimulatory signal is delivered by the CD80/CD86/CD28 and CD40/CD40L co-stimulatory molecules delivered only by the professional APCs – dendritic cells, macrophages and B-cells.

In the absence of infection the APCs do not express co-stimulatory signals and thus the capacity of APCs to activate naïve T-cells is acquired only during infection.

Memory T-cells
Immunological memory is the result of clonal selection of antigen specific T-cells. When naïve T-cells are activated by antigen and co-stimulatory signals they are driven to proliferate and differentiate into memory T-cells, a process driven by the cytokine interleukin-2. The activation of naïve CD8 T-cells generally requires stronger co-stimulatory signals than is needed to activate naïve CD4 T-cells. Memory T-cells express the marker CD45Ro and thereby the cells gain an increased survival potential.

Naïve CD8 T-cells are activated to become cytotoxic effector memory CD8 T-cells. Effector memory CD8 T-cells lose expression of the CCR7 receptor and therefore leave the lymph node and enter the circulation where they can home to sites of inflammation. Effector function is turned on when the TCR bind to specific peptide/HLA complexes on a target cell, however effector T-cells have major functional differences versus their naïve counterparts as their responses to infection do not depend on co-stimulatory signals. Once generated, CD8 memory T-cells persist in high frequency and have lower activation requirements with novel co-stimulatory pathways that may be constitutively expressed (5,13). Upon activation, memory T-cells produce a wide variety of cytokines including IL-2, IL-4, IFNγ, TNFα and are capable of rapid up-regulation of cytolytic effector function without the need for CD4 T-cell help (14) (Table 1).

Effector memory CD8 T-cells are selective and specific serial killers of target cells at sites of infection. Therefore if viral specific memory T-cells do indeed crossreact against allogeneic HLA to which they have never been exposed they may be a major barrier to successful transplantation.

On activation CD4 T-cells acquire distinctive helper functions. Activated CD4 T-cells synthesize cell-surface molecules and cytokines that activate and help other types of cells, particularly macrophages and B-cells, to participate in the immune response. Antigen specific regulatory CD4 T-cells can limit the activities of effector CD4 and CD8 T-cells via production of inhibitory cytokines such as IL-4, IL-10 and TGF-β.

Table 1. Properties of CD8 T-cell subsets (14).

	Naïve	Effector memory
Genes Expressed		
CD45Ra	+	-
CD27	+	+/-
CD28	+	+/-
CD95	-	+
CCR7	+	-
Fas ligand	-	+
IL-2	+	+
IL-4	-	+/-
IFNγ	-	+
TNFα	-	+
Perforin	-	+
Granzyme	-	+
Functions		
Cytotoxicity	-	+
B-cell differentiation	-	+/-

2. ALLORECOGNITION

Alloreactive T-cells are recruited to the transplanted graft and initiate and execute organ rejection. A series of recent studies have characterized the frequency and cytokine profiles of T-cells responding to allogeneic grafts (12). Naïve and memory T-cells are capable of responding with similar frequency against allogeneic cells, even in non-sensitized transplantation recipients. Furthermore CD4 and CD8 memory populations mount similar proliferative responses and contain comparable frequencies of alloreactive precursors, even though effector molecule expression is significantly higher among CD8 T-cells. These alloreactive memory T-cells are a major barrier to successful transplantation because of their lower activation thresholds, absent requirements for T-cell help and immediate cytotoxic function.

2.1 DIRECT ALLORECOGNITION

Direct allorecognition occurs when recipient T-cells directly recognize donor cells expressing intact mismatched HLA molecules, and is usually associated with acute T-cell mediated rejection. It is generally accepted now that direct allorecognition is dependent on donor derived self-peptide presentation by the allogeneic HLA molecule (1). Direct allorecognition from pre-existing viral specific CD8 T-cells is the topic of this thesis.

2.2 INDIRECT ALLORECOGNITION

Indirect allorecognition involves donor antigen uptake by recipient APCs. Allopeptides can be derived from allogeneic HLA molecules or minor histocompatibility antigens that differ between donor and recipient. After processing and peptide presentation in the context of autologous HLA class II molecules, antigen specific CD4 T-cells are activated and can initiate an alloimmune response. The frequency of T-cell clones involved in indirect allorecognition is about 100 fold lower than in the direct pathway. Indirect allorecognition is not investigated as part of this thesis.

2.3 NON-SENSITIZED TRANSPLANTATION RECIPIENTS HAVE STRONG "MEMORY" RESPONSES FOR ALLO-HLA

Transplantation recipients can be sensitized against alloantigen by pregnancy, blood transfusion or previous transplantation. B-cell sensitization is revealed by the presence of HLA specific antibodies, which are not detectable in non-sensitized individuals. However, even in non-sensitized individuals a substantial portion of the pre-existing memory T-cell repertoire is already alloreactive (12,15-17), which is far greater than the proportion of T-cells that respond to any individual pathogen. The origin of these high-frequency pre-existing alloreactive memory T-cells in non-sensitized individuals was previously unclear, but has been hypothesized to relate to crossreactive allo-HLA responses from viral specific memory T-cells (7,18-19).

3. ALLOREACTIVITY BY VIRAL SPECIFIC MEMORY T-CELLS

In humans, acute rejection has been associated with varying viral infections, and CMV prophylaxis with oral ganciclovir is associated with improved long-term renal graft survival (20). Mismatched donor HLA antigens have differential impact on graft survival depending on the HLA phenotype of the recipient (21), and one possible explanation for the occurrence of these harmful HLA combinations may be that patients have had previous immunological contact with pathogens that elicit T-cell responses which crossreact against the HLA mismatches (7,19,21). The fact that cord blood T-cells are less able to mediate graft vs. host disease (GvHD) than marrow derived T-cells because of their naïve status supports this theory (22-23).

In-vivo, the presence of virally induced alloreactive T-cell memory is a potent barrier to transplantation tolerance in mice (2-3,5,24-26). Many strategies have been used to successfully induce tolerance to transplanted tissue in mice, most of which primarily block the CD80/CD86/CD28 and/or CD40/CD154 co-stimulatory pathways. For example, donor specific transfusion and anti-CD154 antibody readily induce tolerance to solid organ grafts in pathogen free mice; however, all these protocols fail in pathogen exposed mice as viral infections induce alloreactivity associated with the development of memory cells, which abrogate the induction of transplant tolerance (1,6,27-29). Furthermore, Adams clearly demonstrated a viral dose effect whereby mice previously exposed to multiple viral infections were refractory to tolerance induction and rejected their allografts, whereas naïve mice or single pathogen exposed mice were susceptible to tolerance induction (2). Evidence for virally induced alloreactive T-cell memory in mice is already extensively documented in the literature (2-3,5,24).

Taken together this evidence provides strong support for the ability of viral specific memory T-cells to directly elicit acute rejection, and for viral memory having a negative influence on graft survival and/or tolerance induction.

3.1 HUMAN EBV SPECIFIC CLONES ARE CROSSREACTIVE AGAINST ALLO-HLA-B*44:02 VIA MOLECULAR MIMICRY

Burrows and colleagues demonstrated the dual specificity of EBV EBNA3A specific T-cell clones for the immunodominant peptide FLRGRAYGL presented on HLA-B*08:01 and the alloantigen HLA-B*44:02, to which the individual had never been exposed (7). In fact the HLA-B8/FLR restricted response in a HLA-B8^{+} B44^{-} individual gives rise to a public BV6S2 TCR which always cross-reacts against allogeneic HLA-B*44:02 (8). This finding has been reproducibly found in different individuals from different genetic backgrounds using different techniques (7-8,12,18). HLA-B44 mismatching has been identified as higher risk among HLA-B8^{+} renal transplant recipients (30).

The EBV EBNA3A T-cell allo-HLA-B*44:02 crossreactivity is dependent on presentation of the EEYLQAFTY self-peptide derived from the ABCD3 gene (9). Molecular mimicry, as revealed by crystallography studies, is the mechanism for this human T-cell alloreactivity from

a viral specific memory T-cell (figure 3). Despite extensive amino acid differences between HLA-B*08:01 and HLA-B*44:02, and the disparate sequences of their bound viral and self peptides respectively, the HLA-B8/FLR restricted TCR engages these peptide-HLA complexes identically. The viral and allopeptides adopted similar conformations after TCR ligation, revealing that molecular mimicry is associated with TCR specificity. Structural studies confirm the exquisite specificity of the TCR and the self-peptide dependence of the T-cell alloreactivity.

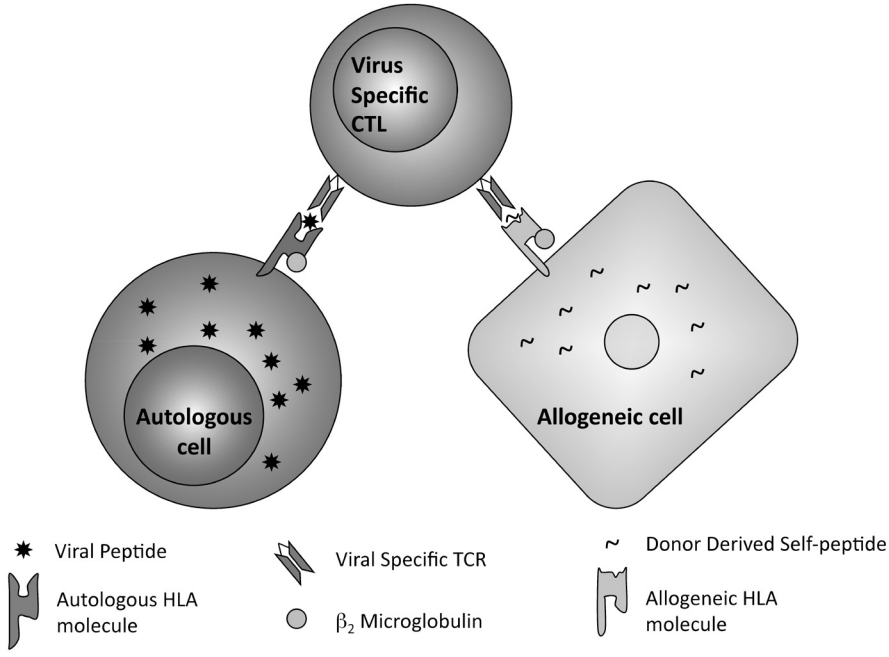

Figure 3. Allo-HLA crossreactivity by viral specific memory T-cells.
Viral specific memory T-cells target virus infected autologous cells presenting viral peptides in a self-HLA restricted fashion. Alternatively, the same viral specific TCR may crossreact against an allogeneic HLA molecule presenting a self-peptide.

3.2 MECHANISMS OF TCR CROSSREACTIVITY

A very high level of crossreactivity is an essential feature of the T-cell receptor (31). While the human immune system does generate a vast number of clonotypically unique T-cell receptors, it is not possible to generate a unique TCR for every immunogenic peptide. Crossreactivity of the TCR ensures that the number of T-cells that can recognize an individual pathogenic peptide presented on a HLA molecule is sufficiently large to elicit a rapid response, and that no pathogenic peptides go unrecognized.

Crossreactivity by pathogen specific memory T-cells may help protect against subsequent unrelated infections, however, in the transplantation setting such crossreactivity may give rise to harmful alloresponses.

Induced Fit
Structural adjustments in the TCR binding site can allow a single receptor to recognize different peptide/MHC ligands. Usually such flexibility is observed in the CDR loops of the TCR. For example the TCR BM3.3 is able to recognize three distinct peptides bound to H-2Kb through changes in the conformation of the flexible complementarity-determining region loops, especially the CDR3 loop (32).

Differential TCR docking
Disparate docking orientations can allow the same TCR to engage different peptide-MHC ligands. The 2C TCR utilizes a different binding strategy to recognize its allogeneic ligand H-2Ld-QL9 and the self-ligand H-2Kb-dEV8 by which it was positively selected (33).

Structural Degeneracy
TCR cross-reactivity can also occur when there is a paucity of peptide-MHC interactions. The TCR 3A6 recognizes a self-peptide from myelin basic protein presented on HLA-DR2a, but is also able to recognize many other peptides presented on HLA-DR2a because of absence of hydrogen bonds between the TCR and the peptides (34).

Molecular Mimicry
Molecular mimicry, whereby the TCR engages the allogeneic ligands and viral ligands with the same overall docking topology, has long been proposed to explain TCR crossreactivity. This can occur despite disparate sequences of the allo and viral peptides (9). It is also suggested that molecular mimicry operates in other alloreactions (35-39).

Antigen-Dependent Tuning of Peptide-MHC Flexibility
Conformational flexibility of peptide-MHC can also allow recognition of different ligands by the same TCR. Recognition of Tax-HLA-A2 antigen (from HTLV-1 virus) by TCR A6 proceeds without substantial adjustments in the ligand, whereas the same TCR recognizes the Tel1p-HLA-A2 antigen (from S. Cerivisae) only following large conformational changes in both the peptide and MHC (40).

4. AIM OF THIS THESIS

The aim of this thesis is to determine if the presence of alloreactive T-cells in non-sensitized individuals can be explained by allo-HLA crossreactivity by viral specific memory T-cells. If true, a further aim is to determine the frequency of allo-HLA crossreactivity by viral specific memory T-cells. The ability of viral specific T-cells to exert HLA alloreactivity could have especially serious consequences as memory T-cells lack the requirement for costimulation and therefore could be efficiently triggered by nonprofessional antigen-presenting cells after HLA-mismatched stem cell transplantation or solid organ transplantation. In order to detect allo-HLA crossreactivity from viral specific memory T-cells, viral specific T-cell clones were generated using single cell sorting based on viral peptide/HLA tetrameric complex staining. The viral specific T-cell clones were then tested for alloreactivity by stimulating with various tissue cells expressing allogeneic HLA molecules.

Chapter 2 of this thesis describes a new tool to detect allo-HLA crossreactivity from viral specific memory T-cell clones using K562 cells transfected with single HLA molecules. The appendix to chapter 2 extensively describes the methodology used in chapter 2. Chapter 3 uses multiple different viral specific T-cell clones to address the frequency of allo-HLA cross-reactivity from viral specific memory T-cells. An example of how self-peptide presentation can alter the tissue specificity of allo-HLA crossreactivity from viral specific T-cell clones is described in chapter 4. In chapter 5 it is shown that anti-viral vaccination, not just viral infection, can also induce alloreactive T-cells. The current evidence for alloreactivity by human viral specific memory T-cells is reviewed in chapter 6. In chapter 7 it is confirmed that allo-HLA stimulation of non-sensitized blood cells can conversely elicit a viral specific cytolytic T-cell response, and the possible clinical implications are discussed. Chapter 8 provides a general conclusion and discussion to summarize all findings and put them into clinical perspective. Included in the general discussion are unpublished results describing how proteosomal digestion could generate or destroy allopeptides.

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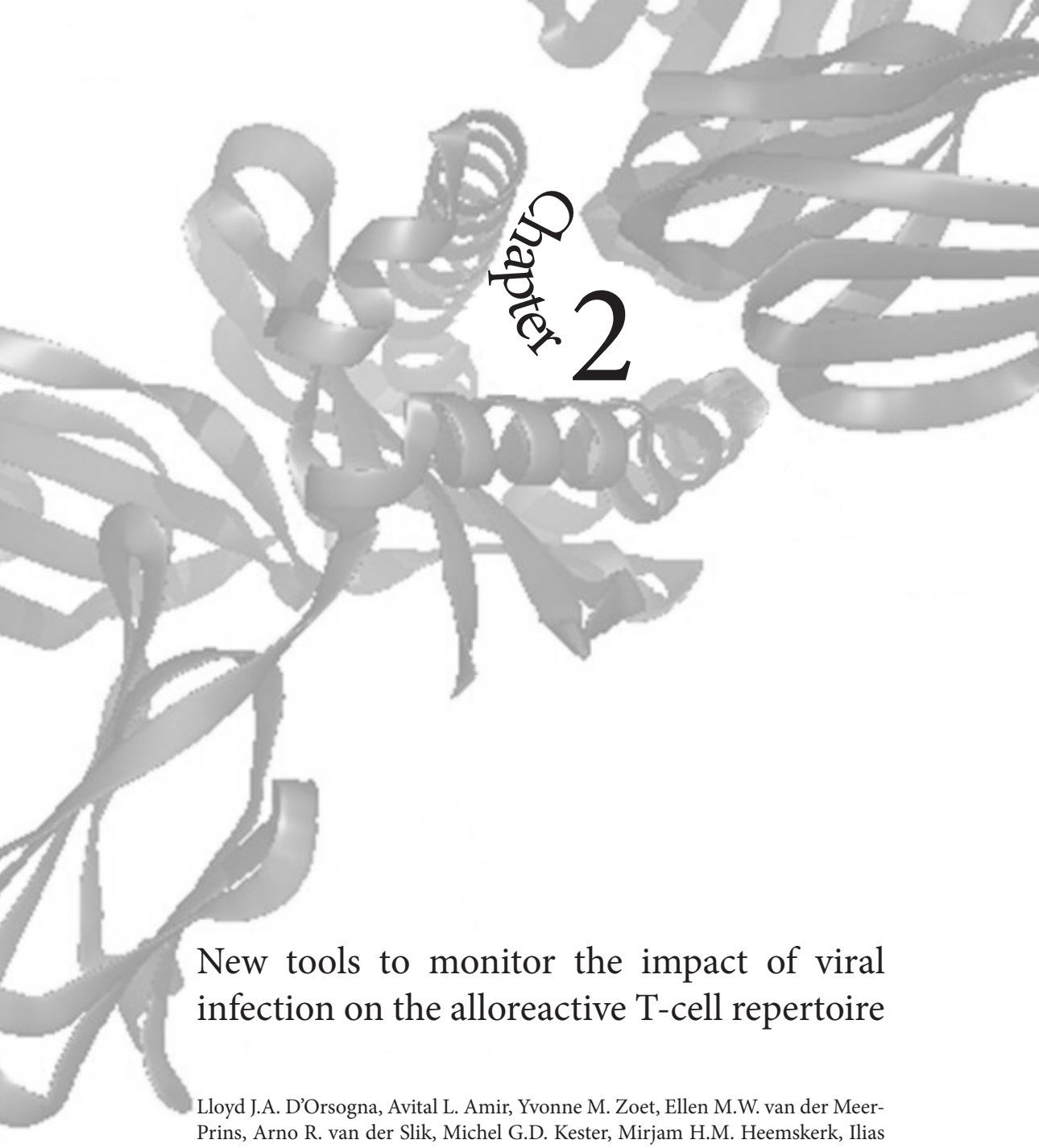

Chapter 2

New tools to monitor the impact of viral infection on the alloreactive T-cell repertoire

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ABSTRACT

Accumulating evidence suggests that alloreactive memory T-cells may be generated as a result of viral infection. So far a suitable tool to define the individual HLA cross-reactivity of virus-specific memory T-cells is not available. We therefore aimed to develop a novel system for the detection of cross-reactive alloresponses using single HLA antigen expressing cell lines (SALs) as stimulator. Herein we generated EBV EBNA-3A specific CD8 memory T-cell clones (HLA-B*08:01/FLRGRAYGL peptide restricted) and assayed for alloreactivity against a panel of SALs, using IFNγ Elispot as readout. Generation of the T-cell clones was performed by single cell sorting, based on staining with viral peptide/MHC complex specific tetramer. Monoclonality of the T-cell clones was confirmed by TCR PCR analysis. Firstly, we confirmed the previously described alloreactivity of the EBV EBNA3A specific T-cell clones against SAL expressing HLA B*44:02. Further screening against the entire panel of SALs also revealed additional cross-reactivity against SAL expressing HLA B*55:01. Functionality of the cross-reactive T cell clones was confirmed by chromium release assay using PHA blasts as targets. SALs are an effective tool to detect cross-reactivity of viral specific CD8 memory T-cell clones, against individual class I HLA molecules. This technique may have important implications for donor selection and monitoring of transplant recipients.

INTRODUCTION

Previous immunological exposures and resultant T cell memory can influence the course of future immune responses to unrelated pathogens (1,2). Less is known about the effect of an individuals immune history on the response to an allogeneic tissue transplant. However the presence of memory alloreactive T cells in humans who have never been exposed to alloantigens has been attributed to past viral infections (3-5). It is hypothesized that these viral specific memory T-cells are able to recognize cross-reactive allogeneic MHC with lower affinity because of lower activation thresholds (4). However, a reproducible in-vitro system for the detection of cross-reactive alloresponses from viral specific T-cells is currently not available.

Burrows et al have shown that the cytotoxic T-lymphocyte (CTL) response against the human HLA-B*44:02 alloantigen may actually be due to cross-reactivity against a previously primed viral antigen (3). Limiting dilution analysis of the alloresponse to HLA-B*44:02 in eight healthy individuals revealed that HLA-B*08:01, EBV seropositive donors had significantly higher CTL precursor frequencies for alloantigen HLA-B*44:02 than HLA-B8 positive, EBV seronegative control donors (3). The cytotoxic T-cell response against the immunodominant EBV peptide FLRGRAYGL presented on the HLA-B*0801 molecule also recognized the HLA-B*44:02 molecule (presumably presenting a self-peptide) to which the T-cells had never been exposed.

This study of Burrows demonstrates that the allospecific T-cell repertoire overlaps with the repertoire which recognizes a single viral epitope in the context of self-MHC. This theory is also supported by other groups that have reported similar cross-reactivity between environmental pathogens and allogeneic MHC molecules (6-9). Although the frequency of naïve T-cells available to respond to any given pathogen is relatively small (approx. 1:200,000), the proportion of memory T cells that can directly recognize foreign MHC represents a substantial fraction of the total T-cell repertoire (10,11). Analysis of cloned T-cell populations has demonstrated that between 20-60% of antigen specific, MHC restricted T-cell clones crossreact with alloantigens (12-13). It has also been shown that approximately half of a "primary" alloresponse is contributed by previously primed MHC-restricted T-cells (14-15).

Therefore accumulating evidence suggests that the CD8 T-cell alloresponse could, at least in part, result from molecular mimicry by an environmental antigen which induces an alloreactive memory T-cell response (3-5,8,16). It is therefore not surprising that increased alloreactivity is found following viral infection in experimental models (2, 17-19). These cross-reactive memory T-cell responses not only affect allograft survival but also prevent the induction of transplantation tolerance (4,20).

Human memory CD8 T-cells can be defined based on phenotypic and functional characteristics (21). Memory CD8 T-cells express CD8, CD45Ro, CD27, CD28, CD11a, CD49d, CD95 and can secrete IL-2, IL-4, IFNγ and TNFα. This memory subset contains virus-specific cytotoxic T lymphocyte (CTL) precursors that can have cytotoxic function including expression of perforin and granzyme B. Memory CD8 T-cells have less stringent requirements for activation, with a reduced requirement for co-stimulation, and have the potential to secrete a more

extensive array of cytokines (22-24). As primed (cross-reactive) memory T-cells may have lower activation thresholds than their naive counterparts, their presence before transplantation may increase the risk of a poor outcome of an allograft.

In our laboratory, cell lines have been established expressing a single MHC class I antigen on the cell surface. These cells, named single HLA antigen lines (SAL's), have originally been developed for humoral tests (25,26), as their expression of a single HLA antigen, instead of the 3-6 of usual peripheral blood lymphocytes, facilitates the definition of HLA antibody specificities in patients sera. Similarly the use of a SAL as target will allow the determination of the exact HLA specificity of the alloreactive T-cells.

The purpose of this study was to develop a reproducible in-vitro system for the detection of CD8 T-cell cross-reactive alloresponses by viral specific CD8 T-cell clones. We used EBNA3A specific CD8 memory T-cell clones to confirm the previously described cross-reactive alloresponse against HLA-B*44:02 and check whether additional crossreactivities can be observed using a panel of different SALs as stimulators. SALs proved to be the basis of an effective screening system for heterologous alloreactivity that lead to the definition of additional cross-reactive HLA-alloantigens.

METHODS

Generation of viral specific CD8 memory T-cell clones
EBNA3A-FLR/B8 CD8 T-cell clones were derived from healthy donor (x.x0116x) with HLA typing HLA-A*01:01,02:01; B*08:01,-; DRβ1*03:01,-. HLA-B8/FLR tetramer positive CD8 T-cells accounted for 1.7% of the peripheral blood CD8 T-cells (Figure 1a). The EBV-specific T cells were isolated from the peripheral blood as previously described (27). Briefly, PBMCs were harvested and labeled with HLA-B8/FLR tetrameric complexes for 30 minutes at 4 0C in RPMI without phenol, supplemented with 2% FCS, washed three times and single cell sorted at 4 0C using the FACS vantageTM (Becton Dickinson). Tetramer+ CD8 T-cells were non-specifically stimulated every 2 weeks with feeder cell mixture containing irradiated allogeneic PBMCs (3500 Rad), irradiated EBV transformed B-cells (5000 Rad), 800ng/ml phytohaemagglutinin (PHA), 100 IU/ml IL-2 in IMDM medium supplemented with glutamine, human serum (5%) and fetal calf serum (5%). Multiple clones for testing were generated from the same healthy donor.

Confirmation of T-cell clonality
TCRα and TCRβ rearrangements were analyzed on 4 separate EBNA-3A T-cell clones.
Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Oligo dT primed first-strand cDNA was synthesized from 1 μg RNA template using AMV reverse transcriptase (Promega, Madison, WI, USA). First RT-PCR was performed to determine the TCR AV and BV usage, using primers that cover the complete TCR repertoire. Sequencing templates were obtained performing high fidelity PCR using Pfx50 DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Each reaction contained forward primers targeting the

Va4S1 or Vb6S2 variable region and reverse primers specific for the alpha and beta chain constant region. Amplicons spanning the variable, CDR3 and joining regions were purified using illustra S-400 HR microspin columns (GE Healthcare, Buckinghamshire, UK) according to manufacturers' protocol. Thermo sequenase primer cycle sequencing (GE healthcare) reactions were performed using a CY5 labeled m13 sequencing primer (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturers' protocol. Sequencing reactions were run on an ALFexpress DNA sequencer (GE healthcare), and analyzed with sequence analyser 2.10 software (GE healthcare).

Generation of single HLA antigen expressing cell lines (SALs)
Plasmid constructs (pLNCX, ampicillin and neomycin resistant) containing various MHC class I heavy chain genes were obtained from the 13th International Histocompatability Working Group and were transfected in K562 cells, obtained from the American Type Culture Collection (Manassas, VA, order number CLL-243) (28) by electroporation using the Genepulser (Biorad, Hercules, CA) with instrument settings of 230V and 960μF. Electroporation was performed with 107 cells and 10μg of plasmid DNA. On day 2 after transfection, selection was started with G418 (neomycin derivative, final concentration: 200μg/ml; Invitrogen, Groningen, the Netherlands). The antibiotic-resistant transfectants were expanded for at least two weeks. Major histocompatability complex class I positive cells were enriched by cell sorting using w6/32 coated antimouse immunoglobulin (Ig) magnetic beads (Dynal, Oslo, Norway). Sorted cells were expanded using G418, tested for class I expression with HLA specific monoclonal antibodies (25,26) and cryopreserved in multiple aliquots. The full list of available transfected SAL cells is available in reference 25.

Elispot
Ninety six well ELISPOT plates (NUNC) were coated with capture antibody for IFNγ (Mab 1-D1K – Mabtech) in PBS overnight at 4 0C. The plates were then washed with PBS three times. 10000 responder EBNA-3A T-cell clone were added to each well in 100μL of IMDM supplemented with 10% FCS (without IL-2), together with 1.105 stimulator SALs (non-irradiated). Control wells contained responder EBNA-3A specific T-cell clone with medium, non-transfected K562 cells or FLRGRAYGL peptide (10μg/ml positive control). The plates were washed after 24 hours and biotinylated detection antibody (Mab 7-B6-biotin – Mabtech) was added to the wells for 2 hours at room temperature, followed by further washing step. Extravidin Alkaline Phosphatase conjugate (E2636 - Sigma) was then added for 1 hour at room temperature and plates were washed again. The spots were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT plus B-5655 – Mabtech) and counted using a computer assisted ELISPOT image analyzer Immunospot.

Chromium release assay and generation of PHA blasts
EBNA-3A specific CD8 T-cell clones were evaluated for cytotoxicity by incubating 5000 PHA blast target cells with serial dilutions of the T-cell clone for 4 hours in a chromium release assay. PHA blasts were generated by stimulating PBMC with PHA (800ng/ml) and IL-2 (150IU/ml) for 7 days (Growth medium 15% human serum/RPMI), and were incubated with chromium for 60 minutes. Supernatants were harvested for gamma counting: *percent specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.* An

inhibition assay was also performed with and without the presence of HLA-B8/FLR tetramer or control HLA-B35/IPS tetramer (1μg/ml). Results are expressed as the mean of triplicate samples.

Statistics
Values for Elispot and specific lysis are presented as the mean of triplicate wells, with standard deviation. Comparative analyses are non-parametric (unpaired) t-tests, $p<0.05$ is considered to be significant. Statistics are derived using Graph Pad Prism 4 for Windows (Version 4.02, 2004).

RESULTS

Confirmation of monoclonality and TCR repertoire analyses of the EBNA3A-FLR/B8 specific CD8 T-cell clones
EBNA3A-FLR/B8 CD8 T-cell clones were all confirmed to bind viral peptide/HLA-B8 tetramer complexes (Figure 1b). Burrows et al have reported that persistent EBV infection in a HLA B*08:01 positive, B*44 negative individual gives rise to a public AV4S1, BV6S2 TCR (29). We therefore performed RT-PCR and sequencing to determine the TCR usage of the EBNA3A specific CD8 T-cell clones we have isolated. As shown in table 1, all clones analyzed

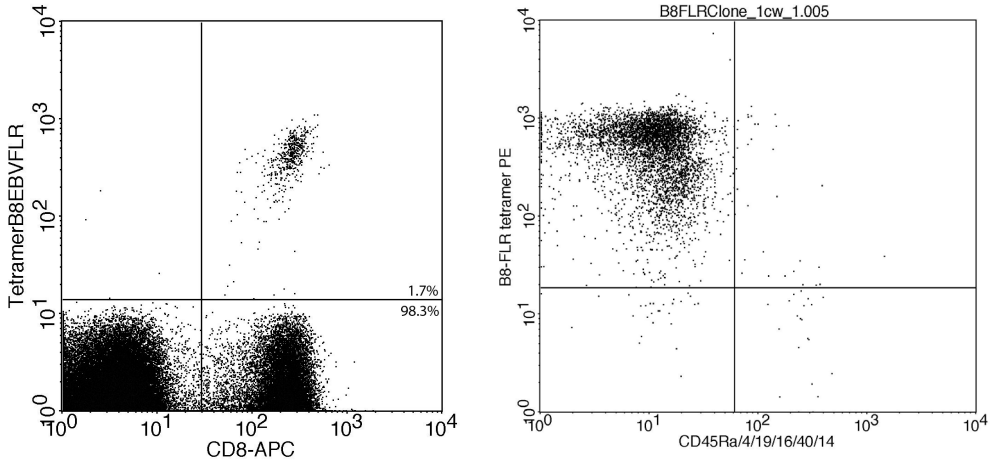

Figure 1. EBNA-3A CD8 memory T-cell clone.
Generation of the T-cell clone was performed by single cell sorting based on HLA-B*0801/FLRGRAYGL specific tetramer staining. (a): HLA-B8/FLR specific T-cells amounted to 1.7% of peripheral CD8 T-cells in the healthy donor from whom the EBNA3A-FLR/B8 T-cell clone was sorted. (b): T-cell clone is >99% HLA-B8/FLR tetramer binding and clonality was confirmed with TCR PCR (table 1). T-cell clone is of memory immunophenotype (CD45Ra-ve) and did not stain with markers specific for CD4 T-cells, B-cells, NK cells nor monocytes.

expressed the AV4S1 and the BV6S2 TCR. Three clones (#1,#8,#19) were identical, however differed from the clones described by Burrows et al at one amino acid located in the CDR3 region of the AV4S1 chain (29). Clone #2 was identical to the LC13 clone described by Burrows (29) (Table 1). Monoclonality of the T-cell clones was confirmed using TCR PCR analysis. The DNA and amino acid sequences of the TCR gene segments is given in table1, with comparison to clones reported by Burrows (29).

SAL cell lines are a suitable tool to detect "cross-reactive"alloresponses of viral specific memory CD8 T-cells

To confirm that SALs are an effective tool to detect cross-reactive alloresponses we tested the EBNA3A-FLR/B8 specific clones against SAL expressing HLA B*44:02. Strong IFNγ production was elicited, as measured by detection of the number of IFNγ producing cells ($p<0.0001$) (Figure 2). FLR peptide (positive control), medium, K562 cell and HLA matched SALs all gave appropriate control results. In addition to cross-reactivity against SAL expressing HLA-B*44:02, screening against the entire panel of SAL cells identified that our EBNA3A specific T-cell clones also cross-reacted with SAL expressing HLA B*55:01 ($p=0.0019$, Figure 2). Cross-reactivity was also confirmed with PHA blasts expressing HLA B*44:02 and HLA-B*55:01, in the same Elispot assay (data not shown).

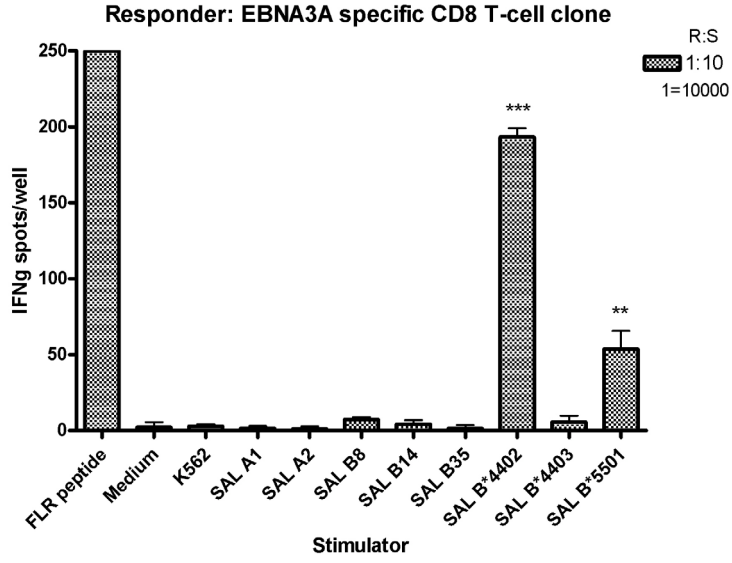

Figure 2. SALs are an effective tool to detect cross-reactive alloresponse(s) from a viral specific CD8 memory T-cell clone. EBNA3A-FLR/B8 T-cell clone recognized K562 cell transfected with either HLA-B*44:02 or HLA-B*55:01 (***$p<0.0001$ and **$p=0.0019$ respectively) (comparison to non-transfected K562). Remaining panel of SALs were not recognized, including SAL B14 and SAL B35. All available HLA-A and HLA-B SALs were tested, while HLA-C SALs were not tested (The full list of available transfected SALs is available in reference 25). HLA typing of donor from whom T-cell clone was sorted is HLA-A1:2; B*08:01,-; DR17,-.

*EBNA-3A viral specific CD8 T-cell clones exert cytolytic activity against HLA B*44:02 and HLA-B*55:01 expressing PHA blasts*

To confirm that the EBNA3A specific CD8 T-cell clones were cytolytic against allogeneic PBMCs expressing the cross-reactive HLA molecules, we performed a chromium release assay using PHA blasts as target cells. The EBNA-3A specific clones specifically lysed PHA blasts expressing either HLA B*44:02 or HLA-B*55:01 in proportion to the E/T ratio ($p<0.0001$ and $p=0.0054$ respectively), whereas HLA-B*44:03 expressing PHA blasts were not lysed (Figure 3).

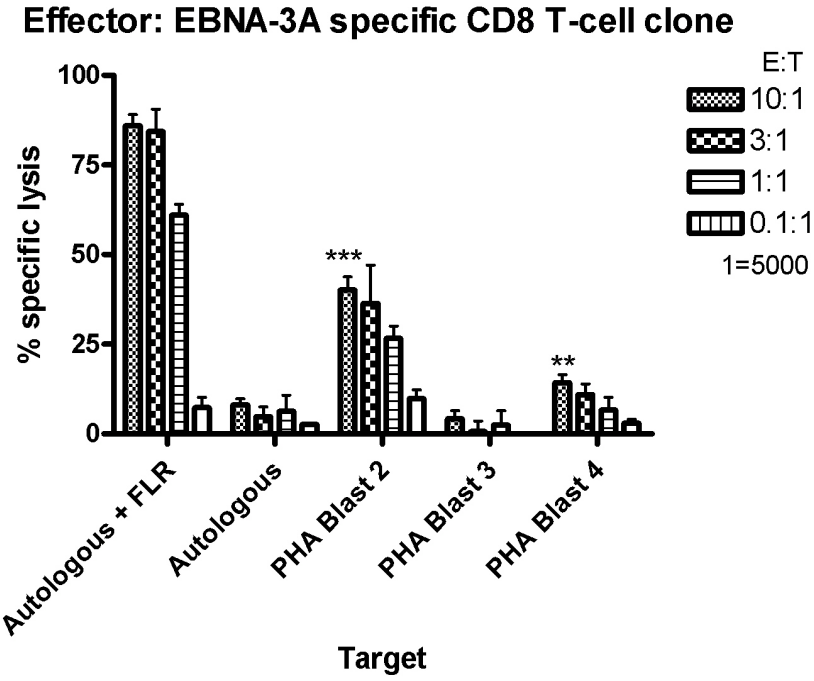

Figure 3. Cytolytic effector function of EBNA-3A clone against allogeneic target cells.
Chromium release cytotoxicity assay using effector EBNA-3A T-cell clone demonstrates functional activity against PHA blasts expressing either HLA B*44:02 or HLA-B*55:01 (***$p<0.0001$ and **$p=0.0054$ respectively) (comparison to PHA Blast 3 ratio 10:1). Autologous PHA blasts are from the same donor used to sort the EBNA3A-FLR/B8 T-cell clone.
Autologous: HLA-A1:2, B*08:01, DR17.
PHA Blast 2: A2,32; B7,**B*44:02**; DR9,11.
PHA Blast 3: A23,31; B39,**B*44:03**; DR4,7.
PHA Blast 4: A24,30; B41,**B*55:01**; DR7,13.

Cytotoxicity against the cross-reactive HLA molecules is specifically inhibited by the presence HLA-B8/FLR tetramer

To confirm that the crossreactive potential of the EBNA-3A specific T-cell clones was mediated by the same T-cell, a cytotoxicity assay was performed in the presence of HLA-B8/FLR tetramer or control tetramer. The results demonstrated that cytotoxicity against both HLA-B*44:02 and HLA-B*55:01 allogeneic molecules was specifically inhibited by the presence of HLA-B8/FLR tetrameric complexes, but not irrelevant tetrameric complexes ($p<0.0001$ and $p=0.0026$ respectively) (Figure 4). Thus confirming that a single viral specific memory T-cell can indeed simultaneously recognize autologous HLA molecules loaded with viral peptide, as well as allogeneic HLA molecule(s) to which it has never been primed.

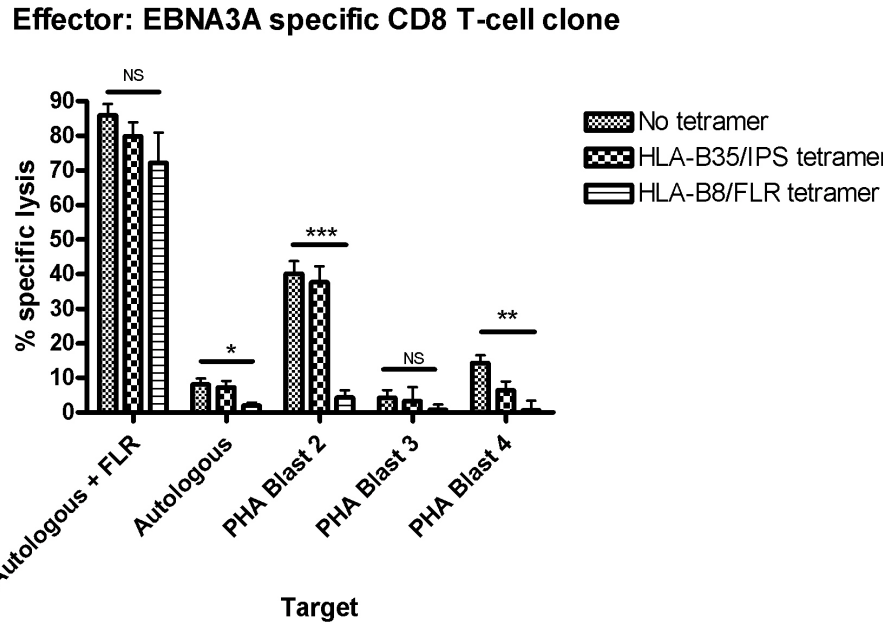

Figure 4. Alloreactivity and viral specificity are mediated by the same T-cell receptor.
Cytotoxicity of the EBNA3A-FLR/B8 CD8 T-cell clone against HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts is specifically inhibited by the presence of HLA-B8/FLR tetramer. An irrelevant tetramer does not suppress the cross-reactivity. Responder:target ratio 10:1, targets 5000. ***$p<0.0001$, **$p=0.0026$, *$p<0.05$. Note: Cytotoxicity against autologous PHA blast loaded with FLR peptide can be significantly inhibited with higher amounts of HLA-B8/FLR tetramer (data not shown).
Autologous: HLA-A1:2, B*08:01, DR17.
PHA Blast 2: A2,32; B7,**B*44:02**; DR9,11.
PHA Blast 3: A23,31; B39,**B*44:03**; DR4,7.
PHA Blast 4: A24,30; B41,**B*55:01**, DR7,13.

Table 1. Sequence analysis of TCR CDR3 regions

	AV4S1	N	Ja52	BV6S2	NDN	Jb2.7	Reported alloreactivity
EBNA3A(#2)	**C I** **tgc atc**	**L P L** **ctg ccc ct**	**A G G** **t gct ggt ggt**	**C A S S L** **tgt gcc agc agc tta g**	**G Q A** **gg cag g**	**Y E Q Y** **cc tac gag cag tac**	HLA-B*4402 & HLA-B*5501
EBNA3A(#1,8,19)	**C I** **tgc atc**	**L P L** **cta ccc tta ga**	**D G G** **t ggt ggt**	**C A S S L** **tgt gcc agc agc tta g**	**G Q A** **ga cag g**	**Y E Q Y** **cc tac gag cag tac**	HLA-B*4402 & HLA-B*5501
IM6 (29)	C I tgc atc	L P L cta ccc ct	A G G t gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
SC17 (29)	C I tgc atc	L P L ctt cct ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
AS1 (29)	C I tgc atc	L P L ctc cct ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A ga cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
AS7 (29)	C I tgc atc	L P L ctt ccc ctc	A G G gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A gc cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
LC13 (29)	C I tgc atc	L P L ctg ccc ct	A G G t gct ggt ggt	C A S S L tgt gcc agc agc tta g	G Q A gg cag g	Y E Q Y cc tac gag cag tac	HLA-B*4402
DD1 (29)	C I tgc atc	L P L ctc cct ctt a	S G G ct ggt ggt	C A S S tgt gcc agc agc	I G Q A atc ggt caa g	Y E Q Y cc tac gag cag tac	HLA-B*4402

For each EBNA-3A clone (in bold) the α chain sequence, β chain sequence and alloreactivity is shown left to right. The one-letter amino acid code is shown above the first nucleotide of the codon. The borders between TCR V, N (DN) and J regions are displayed according to previously reported sequences (29). The EBNA-3A clones sequenced here are all single sorted from the same individual. Variable gene segments are depicted according to Arden nomenclature.

CONCLUSION AND DISCUSSION

We have shown for the first time that T-cell alloresponses from viral specific CD8 memory T-cell clones are reliably detectable in-vitro using transfected K562 cells expressing a single HLA molecule. Using this technique we have confirmed that EBNA3A-FLR/B8 CD8 memory T-cell clones exhibited an alloresponse against HLA-B*44:02, as reported by Burrows (3). In addition, our viral specific clones also exhibited an alloresponse against HLA B*55:01, demonstrating the power of our technique as a screening tool.

The EBNA-3A specific CD8 T-cell clones recognized SAL cells transfected with HLA-B*44:02 but not B*44:03. These two HLA molecules differ only by a single amino acid at position 156, a position critical for interaction with the TCR (30). However, HLA B*55:01 does not share this same amino acid and in fact has the same amino acid (L) at this position as does HLA B*44:03. Furthermore sequence alignment of these HLA molecules reveals there is no common amino acid between HLA B*08:01, B*44:02 and B*55:01 that is not present on HLA B*44:03 (31). Key amino acids within the MHC α2 helix may be critical for these cross-reactive alloresponses (32), however our work suggests that additional factors must also be necessary. In fact, alloreactivity between disparate cognate and allogeneic pMHC class I complexes is likely the result of highly focused, peptide dependent structural mimicry (33).

Our EBNA3A specific T-cell clones recognize HLA-B*55:01, in addition to the previously described HLA B*44:02. The EBV antigen FLRGRAYGL presented on HLA-B8 selects for a public TCR (29), a fact confirmed by sequencing of our own EBNA3A specific T-cell clones (Table 1). It is possible that the single amino acid difference within the CDR3 region of the TCR of our clones (EBNA3A #1,#8 and #19), as compared to the clones reported by others, retains alloreactivity against HLA B*44:02 but in addition enables alloreactivity against HLA B*55:01. Complex structural studies are required to determine if this is indeed the case.

However since clone #2 also exhibited alloreactivity against HLA-B*55:01 and this T-cell clone expressed an identical TCR compared to the EBNA3A specific T-cell clones of Burrows, the most likely explanation is that the EBNA3A specific clones reported by Burrows also exhibit alloreactivity against HLA-B*55:01, but this may not have been detectable without the use of single HLA expressing cell types. This demonstrates the sensitivity of our technique. EBV EBNA3A specific T-cell clones have never been reported not to recognize HLA-B*55:01.

Contrary to a previous report the EBNA3A specific T-cell clones described in this study did not cross-react with HLA-B14 nor HLA-B35 alloantigens (34). The HLA-B14 or HLA-B35 crossreactive T-cell clones however did not express an AV4S1, BV6S2 TCR, and did not recognize HLA-B*44:02 (34). Therefore we would indeed predict that our clones should not recognize HLA-B14 nor HLA-B35, thus demonstrating that our detection technique is not only sensitive but also specific. We propose that subtle amino acid differences of the α and/or β chains within the CDR3 accounts for the various patterns of cross-reactivity, even if these T-cell clones were all restricted by the same viral peptide presented on HLA-B8.

The possibility that the T-cell clones cross-reacted against a HLA class II molecule in this

assay (instead of the transfected HLA class I molecule) can be excluded. The single HLA molecule expression of the SALs has been confirmed against a panel of 84 human HLA-specific monoclonal antibodies (25,26), there is no surface expression of HLA class II. Furthermore, K562 cells lack IFNγ mediated induction of the class II transactivator (35).

The importance of our findings are reinforced by functional studies showing that our EBNA3A specific T-cell clones are able to specifically lyse both HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts, as determined by chromium release assay. Furthermore this dual alloreactivity was specifically inhibited by the HLA-B8/FLR tetramer complex, confirming that a single viral specific memory T-cell can indeed simultaneously recognize autologous HLA molecules presenting viral peptide, as well as allogeneic HLA molecule(s) to which it has never been primed.

The lower percentage of specific lysis of the HLA-B*44:02 and HLA-B*55:01 expressing PHA blasts, versus the positive control (autologous PHA blast loaded with exogenous FLRGRAYGL peptide) is not unexpected. The HLA-B8 expressing PHA blast was exogenously loaded with excess amount of viral peptide, while the cross-reactive alloresponses are dependent on presentation of endogenous self-peptide. Furthermore, it has been suggested by others that cross-reactive alloresponses may be of lower affinity to the original viral specificity against which the T-cell was selected (2). Nevertheless, this cross-reactivity is clearly detectable using our novel technique.

The clinical relevance of our findings are re-enforced by the fact that a HLA-B*44:02 mismatch has been identified as higher risk amongst HLA-B*08:01 renal transplant recipients (36). A HLA-B*55:01 mismatch has not been identified as high risk within EBV positive, HLA-B*08:01 recipients, however further database studies may be warranted in light of our findings.

Results presented here support evidence that virally activated memory T-cells could play a major role in human alloresponses. EBNA3A specific T-cells amounted to 1.7% of peripheral CD8 T-cells in the healthy donor from whom the EBNA3A specific T-cell clone was derived. The frequency of memory CD8 T-cells is highest for the chronically persistent viruses such as human herpes viruses EBV and CMV, infections that are common and persistent in the general population. To our knowledge, this is the first report of a viral specific T-cell clone that can simultaneously cross-react with more than one allogeneic HLA class I molecule. This tool is not only useful for this particular clone but also for detecting cross-reactive alloresponses from other different viral specific clones (manuscript in preparation). The allo-MHC/self-peptide target antigen is presumably sufficiently similar to the MHC/viral-peptide complex involved in activating the T-cell, in three dimensions, to allow crossreactivity.

These findings may have important future implications for donor selection and monitoring. The immune response against the EBNA3A-FLR peptide presented on HLA-B8 selects for a public TCR, with alloreactivity against HLA-B*44:02. The ability to detect the viral specific memory T-cells giving rise to cross-reactive alloresponses may lead to better transplantation matching and/or monitoring. Assay of alloantigen specific T-cells in-vitro for renal trans-

plantation monitoring is not new (37-40), but does not have adequate sensitivity or specificity to enter routine clinical practice. However, the concept of producing tailor-made cells for analysis of cellular reactivity against individual HLA molecules is novel and may have advantages over these previous assays. If in vitro tools can more specifically predict which renal transplant patients are at risk for rejection and which patients are predisposed to tolerance, the immunosuppressive regimen could be adjusted accordingly (37). In theory, using our technique (un)acceptable mismatches might be partially determined based, not only on HLA typing, but also on immunological history.

If our technique can define pathogen driven clonal expansions of T-cells that are involved in initiating allograft rejection then it is possible that immunomodulating techniques could be used to inhibit these harmful T-cell clonotypes, as suggested by Burrows (41). We also hypothesize that anti-viral therapy or viral eradication may decrease the proportion of these cross-reactive alloresponsive T-cells. For example, it has already been shown that CMV prophylaxis post-transplantation is associated with less acute rejection episodes and better one year graft survival (42).

The significance and characteristics of memory CD8 T-cells in viral infections have been extensively studied. In many studies of T-cell memory and transplantation tolerance many experimental immunologists go to great lengths to ensure their animal colonies are pathogen free. Although these studies can be enlightening, humans are not immunologically naive. We have shown here that a single EBV specific memory T-cell can indeed cross react with two alloantigens, thereby possibly influencing the success of tissue transplantation. The presence of Tm correlates with both acute and chronic rejection and may be responsible for the failure to induce tolerance in human clinical settings (4-5). Our results support the conclusion that transplantation/tolerance studies using pathogen free models could be flawed, as has also been suggested by others (4-5,16).

In conclusion, we have shown that SAL cell lines are an effective tool to detect functional immune responses from CD8 T-cell clones, specifically; SALs are an effective tool to screen for cross-reactive alloresponses from viral specific memory T-cells. This technique to define cross-reactive T-cells may also lead to important future improvements in donor selection and monitoring. Cross-reactive alloresponses should be further defined (in-vitro) using CD8 T-cell clones directed against other common persistent viral infections.

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Appendix to chapter 2

Detection of allo-HLA crossreactivity by viral specific memory T-cell clones using single HLA transfected K562 cells

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SUMMARY

The ability to directly measure viral specific lymphocytes using fluorochrome labeled tetrameric complexes has proven a great advancement for the transplantation field. Viral peptide/HLA tetrameric complexes allow the rapid generation of viral specific clones using single cell sorting apparatus, permitting the determination of alloreactivity from a single TCR with known specificity. When combined with new target "detector" cells called single HLA antigen transfected K562 cells (SALs) the human alloresponse can for the first time be examined specifically and reliably. Here we describe a method for detection of "heterologous immunity" from viral specific memory T-cells using single HLA expressing cell lines as allogeneic targets.

1. INTRODUCTION

The mechanisms by which alloreactive memory T-cells are generated in non-sensitized individuals have begun to be elucidated. It is generally accepted that a very high level of crossreactivity is an essential feature of the T-cell receptor, for example, memory T-cells that have been generated as a result of a previous viral infection can subsequently respond to a second unrelated infection. However, it has only recently been shown that alloreactivity from viral specific memory T-cells is far more common than predicted, 45% of viral specific T-cell clones were found to be allo-HLA crossreactive (1).

Detection of alloreactivity from viral specific memory T-cells should first be performed by screening against a panel of cells expressing most common HLA molecules, such as a panel of EBV LCLs as described elsewhere (1). However crossreactivity should be confirmed using a single HLA transfected cell type. This will not only confirm the individual HLA molecule recognized but also exclude the possibility of the clone recognizing viral peptides presented on allo-HLA.

Single HLA transfected K562 cells (SALs) are a new sensitive and specific tool to detect alloresponses from viral specific T-cells (2). SALs express only the transfected HLA class I molecule (3), unlike C1R cells that may have low expression of other HLA molecules. For example, the previously described alloreactivity of the EBV EBNA3A specific T-cell against HLA-B*4402 has been confirmed using SALs (2). Furthermore, the same T-cell clone was also found to recognize HLA-B*5501 expressing SALs (2). Suggesting that SALs may be a more sensitive target than other cells expressing multiple HLA molecules.

Therefore detection of HLA-specific alloresponses from viral specific T-cells in-vitro is now feasible in the routine laboratory.

This technique to define cross-reactive T-cells may lead to important future improvements in donor selection and monitoring. The ability to define public TCR responses that give specific allo-HLA crossreactivity may assist in the definition of (un)acceptable mismatches. If in vitro tools can more specifically predict which transplant recipients are at risk for rejection and which patients are predisposed to tolerance, the immunosuppressive regimen could be adjusted accordingly. We also hypothesize that anti-viral therapy may decrease the proportion and activation status of these alloreactive T-cells.

2. MATERIALS

2.1 Viral specific T-cell cloning using single cell sorting
1. Viral peptide/HLA tetrameric complex of interest
2. PBMCs from donor known to have viral peptide/HLA complex binding T-cells in the peripheral blood (or at least known to be serologically positive for virus of interest)
3. Iscoves Modified Dulbecco Medium (IMDM) with L-glutamine
4. Fetal Calf Serum (FCS)
5. Human Serum (HS)
6. Phytohaemagglutinin (PHA) 800ng/mL
7. Interleukin-2 (IL-2)
8. FACS sorting apparatus e.g. FACSAriaII

2.2 Generation of single HLA transfected K562 cells lines (SALs)
1. K562 cell line
2. plasmid (pLNCX, pCDNA3.0, resistant for neomycin (G418), pEAK10 resistant for puromycin, with HLA-cDNA construct (10 µg per transfection)
3. IMDM supplemented with L-glutamine
4. Fetal Calf Serum (FCS)
5. G418 (200 µg/ml)
6. Pen/strep
7. Gene pulser (Biorad)
8. Dynabeads Sheep anti Mouse Ig
9. w6/32 (anti MHC class I antibody), w6/32-PE, w6/32-FITC
10. Goat anti Mouse FITC
11. Puromycin (0.5 µg/ml)
12. Hygromycin (50 µg/ml)
13. Sterile Gen pulser 0.4 cm cuvettes (Biorad)
14. FACS calibur flow cytometer
15. FACS tubes
16. Solution of 0.1% BSA in PBS
17. HLA-specific Human Monoclonal Antibodies
18. Rabbit anti Human IgG FITC
19. Rabbit anti Human IgM FITC
20. DMSO
21. Incubator
22. PBS/1% paraformaldehyde
23. 15 ml tubes
24. Dynal magnet

2.3 IFNγELISA
1. Viral specific T-cell clone
2. SALs transfected with the HLA molecule of interest

3. Non transfected K562 cells
4. Viral peptide for which the clone is specific (positive control), and a control viral peptide
5. IMDM supplemented with L-glutamine
6. FCS
7. HS
8. PHA 800ng/mL
9. Interleukin-2 (IL-2)
10. Sterile 96 well round bottom plates
11. Human IFN-γ Elisa kit.

2.4 Cytotoxicity assay

1. Viral specific T-cell clone
2. SALs transfected with the HLA molecule of interest
3. Non transfected K562 cells
4. Viral peptide for which the clone is specific (positive control), and a control viral peptide
5. IMDM supplemented with L-glutamine
6. FCS
7. HS
8. Phytohaemagglutinin (PHA) 800ng/mL
9. Interleukin-2 (IL-2)
10. Sodium Chromate (1 mCi/ml (370mBq))
11. 1% Triton X-100
12. 96-well round bottom plates

3. METHODS

3.1 Viral Specific T-cell Cloning using single cell sorting

1. Perform all steps at 4 degrees
2. Ensure person for single cell sorting is serologically positive for virus and has a population of the relevant tetramer binding T-cells
3. Wash PBMCs twice in medium containing 1% FCS
4. Add tetramer-PE at high concentration e.g. 1:100
5. Add negative markers CD45Ra/CD4/CD14-FITC
6. Incubate 30 minutes
7. Wash PBMCs twice in medium containing 1% FCS
8. Place 5 million cells for sorting in 1mL 10% FCS/IMDM without phenol red
9. Prepare T-cell Medium - 5% FCS/5% Human serum in IMDM medium with 1% Penicillin/Streptamycin, 3mg L-Glutamine and IL-2 100 IU/mL
10. Prepare T-cell feeder mix – To the T-cell medium add 0.5 million irradiated feeder cells, 0.05 million EBV LCLs and 2μL PHA per mL of required feeder mix.
11. Prepare 96 well round bottom plate(s) with 100μL feeder mix per well.
12. Perform single cell sorting based on tetramer positive gate using single cell sorting ap-

paratus
13. Add further 100µL T-cell medium per well on day 7
14. At day 14 select expanding wells and restimulate in 24 well plate with 1mL feeder mix.
15. After day 6 confirm tetramer positivity of T-cell clones
16. Determine Vβ TCR usage using Vβ kit or TCR PCR.
17. Freeze interesting clones after expansion.
18. 1 million defrosted T-cell clone can be restimulated in T-cell medium with 5 million feeder cells, 0.5 million EBV LCLs and 2uL/mL PHA

3.2 Generation of single HLA transfected K562 cells lines (SALs)
1. Culture K562 until you have 10*106 cells per transfection in IMDM supplemented with 10% FCS and pen/strep
2. Spin down the cells and wash two times in IMDM/10%FCS
3. Count the cells and resuspend the cells in IMDM/10%FCS at a concentration of 10*106 cells per ml
4. Mix 1 ml of the cells with 10µg of the plasmid DNA
5. Put 1 ml of the cell/DNA mixture in a 0.4 cm cuvette and store on ice for a minimum of 5 minutes
6. Mix and pulse (960 µF, 230 V)
7. Store on ice for 15 minutes
8. Add a few drops of medium to the cuvette and transfer the cells to a culture flask containing 9 ml medium (IMDM/10%FCS/pen/strep)
9. Culture the cells for two days
10. Add selection antibiotic at the correct final concentration
11. Culture for another week
12. Perform a flow cytometry test to see whether there are HLA-positive cells (incubate 105 cells with w6/32-PE for 30 minutes on ice, wash twice, take up in 100 µl PBS/1% paraformaldehyde, read in FACS calibur and analyze)
13. If positive cells are present, separate positive cells from negative cells
14. Take 20-25 ml of your cultured cells (leave some in the flask and continue culturing them)
15. Spin down, remove supernatant and resuspend the cell pellet
16. Add 0.5 ml un-labeled-w6/32 and mix
17. Incubate on ice for 30 minutes
18. Wash 3 times with ice cold medium (IMDM/10%FCS) in a 15 ml tube
19. Resuspend the cells and add 30 µl Sheep anti Mouse dynabeads
20. Incubate on a rollerbench in 4°C room for 30 minutes
21. Put the tube in the magnet, add 10 ml icecold medium
22. Wait for 5 minutes
23. Remove the medium with the non bound cells
24. Repeat from step 21 twice
25. Add 10 ml ice cold medium and resuspend the cells bound to the beads, put them in a small culture flask
26. Wait for the cells to grow
27. When the cells expanded well, remove the beads as follows

28. Put the cells in a 15 ml tube in the magnet
29. Wait for five minutes
30. Collect the medium with the cells in a culture flask, leave the beads in the tube
31. Add 10 ml fresh medium
32. Repeat twice from step 28
33. After a few days growing test the class I expression of the cells (step 12)
34. If there still are cells with no expression at all, repeat the bead sorting
35. If the cells all have a good expression, freeze several samples
36. Test the cells with a flow cytometry test against a panel of Human monoclonal antibodies to confirm the HLA-type of the cell

3.3 IFNγ ELISA using responder viral specific T-cell clones and SAL stimulator cells

1. Harvest the T-cell clone
2. Make a T-cell clone solution of 0.1*106/ml in T-cell medium
3. Dilute the SAL's and the K562 line in T-cell medium to a concentration of 0,25.106/ml.
4. Add 50µl /well of the T-cel clone in a sterile 96 well plate
5. Add 100µl /well of the different SAL's in duplicate or triplicate to the clone
6. Use a SAL transfected with the restricting HLA molecule loaded with the viral peptide that is recognized by the clone as a positive control
7. Use a SAL transfected with the restricting HLA molecule loaded with the control peptide that is not recognized by the clone as a negative control
8. Use non transfected K562 as a negative control
9. As background control also add only T-cell medium to some of the wells without SALs
10. Incubate 24 hours at 37°C, 5% CO2
11. Transfer 120µl supernatant to a new 96 well plate and store the samples at -20°C till use in IFNγ ELISA
12. Thaw the supernatants and use in an IFN-γ ELISA according to the manufacturer's protocol.

3.4 Cytotoxicity assay using effector viral specific T-cell clones and SAL target cells

1. Harvest the SALs and the K562 line and spin down the cells
2. Use a SAL transfected with the restricting HLA molecule loaded with the viral peptide that is recognized by the clone as a positive control
3. Use a SAL transfected with the restricting HLA molecule loaded with the control peptide that is not recognized by the clone as a negative control
4. Use non transfected K562 as a negative control
5. Add the appropriate amount of sodium chromate to the cell pellets
6. Incubate the cells in a 37°C water bath for one hour
7. Meanwhile harvest the T-cell clone and make the necessary dilutions of the clone. Add 100µl/well in a 96 well plate.
8. Wash the chromium labeled cells 3 times with 4ml IMDM+1%FCS
9. Add the labeled target cells to the T-cell clone in a concentration of 5000cells/100µl per well.

10. Make a control plate for spontaneous and maximum release. For each target make 3 wells with 100 µl TCM and 3 wells 100 µl 1% Triton X-100. Add 100ul target suspension per well.
11. Spin the plates 1 minute 2000 rpm
12. Incubate the plates 4 hrs at 37°C, 5%CO2
13. Spin the plates 1 minute 2000 rpm
14. Harvest the supernatants
15. Measure the chromium release and calculate the specific lysis.

4. NOTES

4.1 Viral Specific T-cell cloning using single cell sorting
1. We recommend using PE-labelled tetrameric complexes
2. Prior to attempting sorting we recommend screening first for the presence of viral peptide/HLA tetramer complex binding T-cells within the peripheral blood of the donor. Ensuring the donor has a high proportion of the viral specific T-cells of interest in the peripheral blood increases the probability of successful sorting and cloning. Viral seropositivity does not guarantee the donor blood will contain T-cells with the viral specificity of interest.
3. Do not forgot control tubes for set up of single cell sorting apparatus
4. Viral specific T-cell clones are used to confirm that the alloreactivity and viral specificity are mediated by the same TCR. Viral specific T-cell lines can also be generated by sorting multiple tetramer complex binding T-cells per well (e.g. 25 cells/well). T-cell lines are useful for screening purposes and may contain T-cells with the same viral specificity but different TVR Vb usage to that of the single cell sorted clones.
5. Freshly defrosted PBMCs are suitable for single cell sorting.
6. Only freshly collected PBMCs are suitable as feeder cells. Previously frozen cells are not suitable.
7. EBV LCLs can be used to provide additional non-specific stimulation to the sorted T-cells however are not definitely required for successful stimulation.
8. We recommend using fresh T-cell medium for every T-cell sorting or stimulation (Maximum two weeks old).
9. Perform all steps prior to sorting at 4 degrees. When tetramers are used at room temperature they are capable of activating the viral specific T-cells for sorting.
10. For single cell sorted wells expect growth at day 12-14.
11. The TCR will often be absent from the cell surface for the first 5 days after sorting/stimulation.

4.2 Generation of single HLA transfected K562 cells lines (SALs)
1. Leaving SALs at 26°C for two nights increases HLA expression
2. Culturing SALs with HLA-leader peptides increases the HLA expression of HLA-A and HLA-C SALs
3. Flow cytometry tests for HLA should always be performed on ice to avoid capping
4. When you have a low number of HLA-positive cells (before sorting) use PE-labeled w6/32,

it is more sensitive than FITC-labeled w6/32
5. IFNγ stimulation probably does increase HLA expression.
6. K562 cells do not have functional CIITA gene product (4).

4.3 IFNγ production from viral specific T-cell clones using SAL stimulator cells
1. Viral specific T-cell clones should be used in IFNγ ELISA assays at day 9-10 after stimulation. Earlier use of the T-cell clone may be associated with ongoing IFNγ production from the T-cells.
2. For easy transfer of your supernatants, make the layout of your culture plate the same as the layout of the ELISA plate and leave wells open for your standard dilutions and blank.
3. When the supernatants have to be stored for a longer period (> 1 month) it is better to store them in small siliconized vials.

4.4 Cytotoxicity assay using SAL target cells
1. Cytotoxicity assays are best performed at day 7-8 after stimulation.
2. The effector target ratio's used are commonly 30:1, 10:1, 1:1 and 0.1:1.
3. The half life off sodium chromate is short, be sure to use the right amount.
4. The cells survive better if the wash medium contains protein (e.g. 1% FCS). Instead of IMDM you can also use other media for washing e.g. RPMI
5. % specific lysis= ((test release - spontaneous release) /(maximum release - spontaneous release))x100%.

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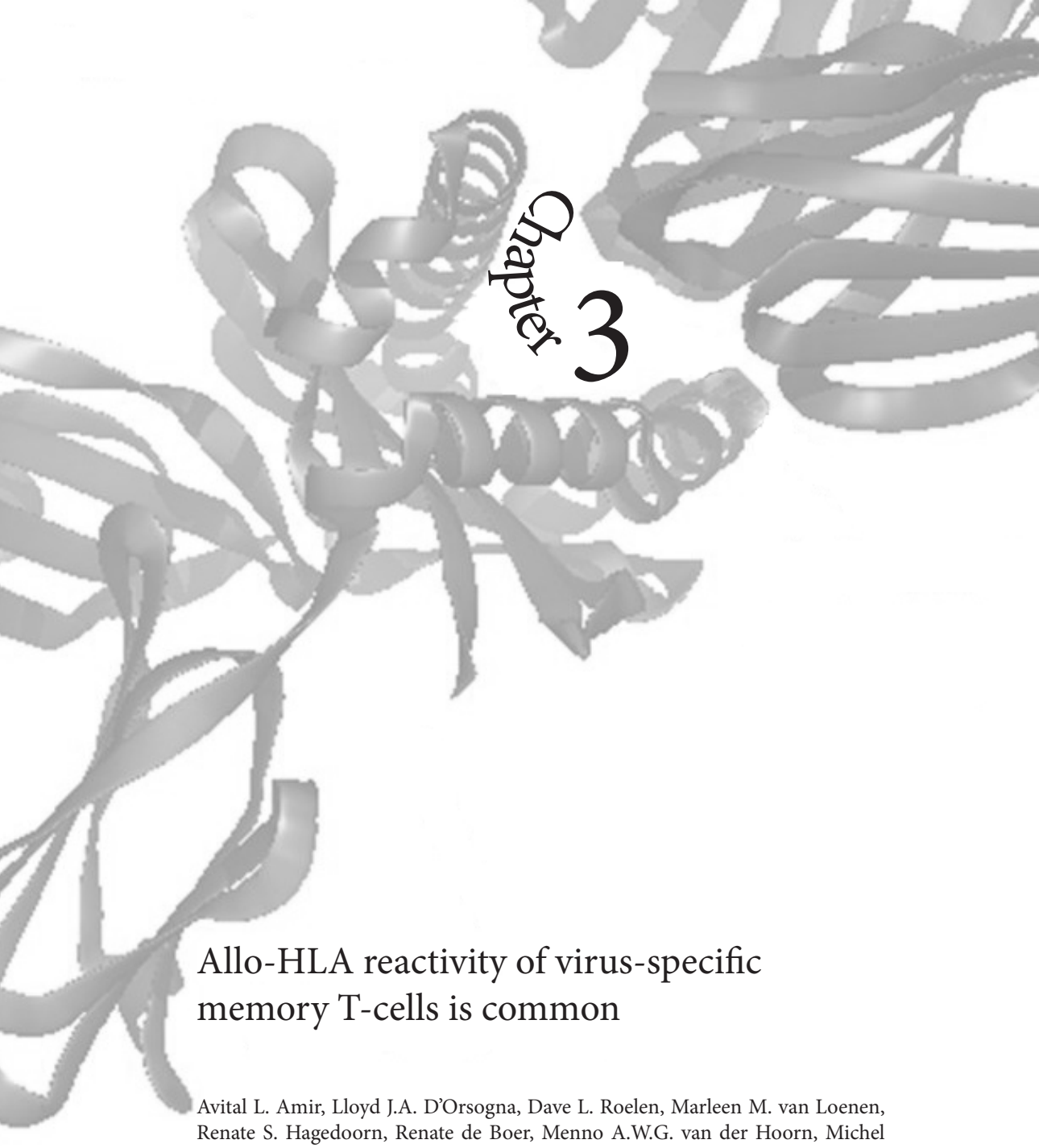

Chapter 3

Allo-HLA reactivity of virus-specific memory T-cells is common

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ABSTRACT

Graft-versus-host disease and graft rejection are major complications of allogeneic HLA-mismatched stem cell transplantation or organ transplantation that are caused by alloreactive T cells. Because a range of acute viral infections have been linked to initiating these complications, we hypothesized that the cross-reactive potential of virus-specific memory T cells to allogeneic (allo) HLA molecules may be able to mediate these complications. To analyze the allo-HLA reactivity, T cells specific for Epstein-Barr virus, cytomegalovirus, varicella zoster virus, and influenza virus were tested against a panel of HLA-typed target cells, and target cells transduced with single HLA molecules. Eighty percent of T-cell lines and 45% of virus-specific T-cell clones were shown to cross-react against allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was directed primarily against HLA class I and II, respectively. However, a restricted number of CD8 T cells exhibited cross-reactivity to HLA class II. T-cell receptor (TCR) gene transfer confirmed that allo-HLA reactivity and virus specificity were mediated via the same TCR. These results demonstrate that a substantial proportion of virus-specific T cells exert allo-HLA reactivity, which may have important clinical implications in transplantation settings as well as adoptive transfer of third-party virus-specific T cells.

INTRODUCTION

HLA disparity between donor and recipient increases the risk and the severity of graft-versus-host disease (GVHD) after stem cell transplantation (SCT). The risk of graft rejection is also significantly increased in HLA-mismatched compared with HLA-matched SCTs and solid organ transplantations. The negative effect of HLA disparity on the clinical outcome of transplantations is the result of high frequencies of alloreactive T cells. In HLA-mismatched mixed lymphocyte reactions, the frequency of reactive T cells was demonstrated to be a 1000-fold higher than the frequency of T cells reactive in HLA-identical mixed lymphocyte reactions (1,2). By testing alloreactive T cells against panels of third-party target cells expressing different HLA molecules (1,3–5) and against target cells blocked with different HLA antibodies (6–8), it was determined that the recognition exhibited by alloreactive T cells is directed against non–self-HLA (allo-HLA) molecules, and that the frequency of allo-HLA–reactive T cells ranged from 1% to 10%.

During thymic development, T cells undergo an instruction process of positive and negative selection that results in the composition of a mature T-cell repertoire that is selected on the basis of tolerance for self-HLA molecules presenting self-peptides (9,10). However, during thymic development, T cells never encounter allo-HLA molecules, and therefore no selection based on tolerance for allo-HLA molecules occurs. We therefore hypothesize that every antigen-specific self-HLA–restricted T-cell could potentially cross-react with non–self-HLA molecules and exert allo-HLA reactivity.

Although it was shown that alloreactivity is equally presented in the naive and memory T-cell populations (11), the ability of T cells to exhibit allo-HLA reactivity could have especially serious consequences when exerted by memory T cells. Memory T cells lack the requirement for costimulation (12,13), and therefore allo-HLA reactivity of memory T cells can be efficiently triggered by nonprofessional antigen-presenting cells after HLA-mismatched SCT or solid organ transplantation. Based on the restricted T-cell receptor (TCR) repertoire of virus-specific memory T cells (14–17), the number of different virus-specific T cells will be limited, but the total number of virus-specific T cells with an identical TCR will be much higher in the memory pool compared with the naive compartment. T cells directed against latent viruses, such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV), are present at high frequencies in blood of healthy persons and patients (18–21). Therefore, if certain virus-specific T cells with cross-reactive potential against the mismatched allo-HLA molecule are triggered by viral activation and expanded in the memory pool, these T cells will react against the mismatched HLA molecule, and may induce severe GVHD or graft rejection.

Studies by Burrows et al first illustrated that virus-specific T cells exert allo-HLA reactivity by demonstrating that EBV-EBNA3A–specific HLA-B8–restricted T cells cross-react with HLA-B44 (22,23). T cells specific for HSV-VP13/14 presented in HLA-A2 were also found to cross-react with HLA-B44 (24) and CD4 T cells specific for tetanus toxoid presented in HLA-DR3 were found to be cross-reactive against HLA-DR4 (25). In addition, the association between reactivation of viral infections during organ transplantation and increased graft rejection (26) supports the hypothesis that virus-specific T cells exhibit allo-HLA–reactive potential.

In this study, we investigate the ability of a large panel of virus-specific T cells to exert allo-HLA reactivity. We determined the cross-reactive potential of virus-specific T cells to allo-HLA molecules by screening single viral antigen-specific T-cell lines and clones against a panel of EBV-transformed B cells (EBV-LCLs), together covering almost all prevalent HLA class I and II molecules, as well as single HLA-transduced target cells. The tested CD8 and CD4 virus-specific memory T cells were specific for Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV), and influenza virus (Flu). Most virus-specific T-cell lines and 45% of the virus-specific T-cell clones were demonstrated to be cross-reactive against allo-HLA molecules. TCR gene transfer demonstrated that the virus specificity and the cross-reactivity to allo-HLA molecules were mediated by the same TCR. These results demonstrate that T cells specific for different viruses exert cross-reactivity to allo-HLA molecules, and illustrate the high frequency of T cells able to exert allo-HLA reactivity.

METHODS

Cell collection and preparation

After informed consent was given in accordance with the Declaration of Helsinki, peripheral blood (PB) was obtained from different persons. All experiments were approved by the Leiden University Medical Center ethics committee. Mononuclear cells (MNCs) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable EBV-LCLs were generated using standard procedures. EBV-LCLs and K562 cells were cultured in Iscove modified Dulbecco medium (IMDM; Lonza) and 10% fetal bovine serum (FBS; Lonza). Phytohemagglutinin (PHA) blasts were generated by stimulation of PB-MNCs with PHA (0.8 μg/mL; Murex Biotec Limited) in IMDM, 5% FBS, 5% human serum, and interleukin-2 (IL-2; 120 IU/mL). K562 and EBV-LCLs expressing single allo-HLA molecules were generated by transduction with retroviral vectors encoding for the allo-HLA molecules or by transfection of allo-HLA molecules (27,28). For the isolation of T cells, B cells, and monocytes, peripheral blood mononuclear cells of healthy donors were stained with either anti-CD3, anti-CD19, or anti-CD14 magnetic-activated cell sorting beads (Miltenyi Biotec), respectively, and isolated according to the manufacturer's instructions. CD40 ligand (CD40L)–activated B cells were generated by culturing the $CD19^+$ fraction for 3 days on CD40L-transduced murine fibroblasts (29) in medium containing CpG (10 μg/mL) and IL-4 (500 IU/mL; Schering-Plough). Monocyte-derived DCs were generated by culturing the CD14+ fraction in medium containing activating cytokines as described previously (30). Fibroblasts were cultured from skin biopsies in Dulbecco modified Eagle medium (Lonza) with 1 g/L glucose (BioWhittaker) and 10% FBS.

Generation of virus-specific T-cell lines and clones

PB-MNCs from healthy persons were stained with tetramer and anti-CD8 monoclonal antibody (mAb) for 1 hour at 4°C and washed once. The tetramers used were constructed as described previously (31) and are shown in Table 1. Tetramer-positive, $CD8^+$ T cells were sorted at 50 cells per well for the generation of lines or a single cell per well for the generation of clones into U-bottom microtiter plates containing 100 μL of feeder mixture. Sorting was performed at 4°C using the FACSVantage (BD). The feeder mixture consisted of IMDM, 5%

FBS, 5% human serum, IL-2 (120 IU/mL), PHA, and 30 Gy irradiated allogeneic third-party PB-MNCs (0.5 x 106/mL). Proliferating T-cell clones were selected and further expanded by nonspecific stimulation every 14 days using the previously mentioned feeder mixture. The viral specificity of the expanded lines and clones was confirmed by tetramer staining, cytotoxicity, and cytokine production assays. Polyclonality or monoclonality of the T-cell lines and clones was analyzed by TCR Vβ analysis using the TCR Vβ kit (Beckman Coulter).

Table 1. Tetramers used for the generation of virus specific T-cell lines and clones

Virus	Viral antigen	HLA	Epitope
CMV	pp50	HLA-A*0101	VTEHDTLLY
CMV	pp65	HLA-A*0101	YSEHPTFTSQY
CMV	pp65	HLA-A*0201	NLVPMVATV
CMV	pp65	HLA-B*0702	RPHERNGFTVL
CMV	pp65	HLA-B*03501	IPSINVHHY
CMV	pp65	HLA-DRB1*0101	KYQEFFWDANDIYRI
CMV	IE1	HLA-B*0801	ELRRKMMYM
EBV	EBNA3A	HLA-A*0301	RLRAEAQVK
EBV	EBNA3A	HLA-B*0702	RPPIFIRRL
EBV	EBNA3A	HLA-B*0801	FLRGRAYGL
EBV	BMLF1	HLA-A*0201	GLCTLVAML
EBV	BRLF1	HLA-A*0301	RVRAYTYSK
EBV	BZLF1	HLA-B*0801	RAKFKQLL
EBV	LMP2	HLA-A*0201	CLGGLLTMV
FLU	IMP	HLA-A*0201	GILGFVFTL
FLU	HA	HLA-DRB1*0401	PKYVKQNTLKLAT
VZV	IE62	HLA-A*0201	ALWALPHAA

CMV indicates cytomegalovirus; EBV, Epstein-Barr virus; FLU, influenza virus; and VZV, varicella zoster virus.

Allo-HLA reactivity of the virus-specific T-cell lines and clones

In the interferon-γ (IFNγ) production assays, 5000 T cells were cocultured with 20 000 stimulator cells in a final volume of 150 μL IMDM culture medium supplemented with 100 IU/mL IL-2. After 18 hours of incubation, supernatants were harvested and IFNγ production was measured by standard enzyme-linked immunosorbent assay (ELISA; CLB). In the cytotoxicity assays, the virus-specific T-cell clones were tested in a standard 6-hour ^{51}Cr-release assay (32) against EBV-LCLs at an effector to target ratio of 10:1.

TCR gene transfer

The TCRAV and TCRBV gene usage of the BRLF1/HLA-A3 clone 19 and the VZV-IE62/HLA-A2–specific T-cell clone 7 was determined using reverse transcriptase–polymerase chain reaction and sequencing (27). Retroviral vectors were constructed that encoded the TCRα chain in combination with green fluorescent protein and the TCRβ chain in combination with the marker gene NGF-R (27). CMV-IE1/HLA-A1–specific T cells derived from an HLA-A*0301–negative healthy person were transduced with the TCRα and TCRβ of the BRLF1/HLA-A3 clone 19. CMV-pp50/HLA-A1–specific T cells derived from peripheral blood of a healthy person negative for HLA-A*0201 and HLA-A*0205 were transduced with the retroviral vectors encoding for the TCRα and TCRβ chain of the VZV-specific T-cell clone (27). The TCR-transduced T cells were sorted on basis of double positivity for green fluorescent protein and NGF-R, and after expansion the T cells were tested for viral specificity and allo-HLA reactivity in stimulation assays.

RESULTS

Alloreactivity of virus-specific T-cell lines

To investigate the ability of virus-specific T cells to exert alloreactivity, virus-specific T-cell lines were tested against a panel of EBV-LCLs, together covering almost all frequently occurring HLA class I and II molecules. The HLA-typing of the EBV-LCLs used in the panel is listed in Table 2. The virus-specific T-cell lines were generated by isolation of tetramer-positive $CD8^+$ T cells by fluorescence-activated cell sorting and subsequent expansion. Tetramer and CD8 staining confirmed the purity of the virus-specific lines and showed that all T-cell lines were more than 98% tetramer positive (Figure 1A-G). In total, 11 virus-specific lines were tested, derived from 9 different donors, specific for 7 different antigens of CMV, EBV, and VZV and restricted to 3 different HLA molecules. In the figures in which EBV-specific T-cell lines were tested, the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted were not shown, to present only the alloreactivity of the T cells and not the virus specificity. All LCLs were tested for IFNγ production in the absence of T cells and did not show production of IFNγ (data not shown). In Figure 1A through G, 7 representative lines are shown. Of the 11 tested virus-specific T-cell lines, 9 were shown to be alloreactive, because these lines produced IFNγ upon stimulation with at least 1 of the EBV-LCLs of the panel. Of the 11 tested lines, 2 exerted no alloreactivity against the EBV-LCLs tested in our panel (including Figure 1C). The recognition pattern of the virus-specific lines that demonstrated alloreactivity ranged from recognition of almost all EBV-LCLs, as shown in Figure 1A and B by the CMV-pp50/HLA-A1–specific lines of patients MBX and UKL, to recognition of a limited number of EBV-LCLs, as shown by the IE1/HLA-B8–, BMLF1/HLA-A2–, VZV-IE62/HLA-A2–, and EBNA3A/HLA-B8–specific lines (Figure 1D, E, F, and G, respectively). The pattern of allorecognition of some of the T-cell lines suggested that the alloreactivity was directed against 1 or several of the allo-HLA molecules presented by the EBV-LCL panel. The BMLF1/HLA-A2–specific line showed high reactivity against 6 EBV-LCLs, of which 5 EBV-LCLs expressed HLA-A*1101. All HLA-A*1101–expressing EBV-LCLs within this panel were highly recognized by this T-cell line. The EBV-LCLs recognized by the EBNA3A/HLA-

Table 2. HLA expression of the EBV-LCL panel

	HLA class I			HLA class II		
ID	A	B	C	DR	DQ	DP
CEL	1101	1502, 4001	0401, 0801	0901	0202, 0303	0501
AAS	3101, 6601	2705, 4102	0202, 1703	1101, 1303	0301	0401, 0402
ABV	0301, 2902	0702, 4403	0702, 1601	0701, 1454	0502, 0202	0401, 1101
AHT	2402, 2501	5501, 1501	0303	0806, 1501	0501, 0602	0401
CVV	11, 31*	57*, 1501	3*, 0602	0401, 7*	0301, 0303	0201, 0401
EIR	0101, 2402	3502, 3701	0401, 0602	0404, 1104	0301, 0402	0301, 0401
FAQ	2301, 6802	1402, 3801	0802, 1203	1301, 1303	0301, 0603	0201
FRQ	0101, 1101	0801, 4402	0501, 0701	0301, 0401	0201, 0301	0101, 0401
GGT	2601, 3101	1401, 4901	0701, 0802	0101, 0701	0202, 0504	0402, 1101
JLX	0101, 6802	0801, 5301	0401, 0701	0301, 1302	0201, 0604	0101, 0401
LAJ	1101, 2402	1302, 5501	0303, 0602	0701, 1601	0202, 0502	0401, 1701
LMB	2902	4402, 5101	1402, 1601	0701, 0801	0202, 0402	0401, 1101
MBX	0101	0801, 1517	0701	1202, 0301	201, 0301	0101, 0401
NGI	1101, 2402	0801, 3906	0701	0301, 0801	0201, 0402	0101, 1401
RSW	3001, 6802	4201	1701	0302	0402	0101, 0402
ZIL	2402, 2601	5601, 5801	0101, 0701	0101, 0804	0402, 0501	0201, 0301
ESK	23, 66*	51, 72*	2, 18*	12, 13*	1, 3*	ND
LSR	3201, 6801	3503, 5201	1202, 1203	1502, 1602	0502, 0601	0401, 1401
EBM	2301	1401	0802	0401	0302	0201
PMT	0301, 3301	1402	0802	0102	0501	0301, '0401
TSJ	2, 24	1501, 75	4	12, 15	6, 0301	ND
IZA	0201, 2402	0801, 4001	0304, 0701	0301, 1301	0603	0401, 1401
GSL	2, 3*	47, 1501*	3, 6*	11, 13*	1*, 0301	ND
JPF	0201, 0205	4002, 1501	0202, 0304	0701, 1104	0301, 0303	0402, 1401
CSV	0201, 3101	5101, 5501	0303, 1402	0404, 1001	0501, 0301	0401, 0402
MHX	0101, 0205	1801, 5001	0602, 0701	0701, 0901	0201, 0303	0201, 0301
OMR	201	4501	1601	1301	0603	0101
TIS	0206, 0207	4601	0102, 0801	0901	0303	1301
CODI	2*, 8001	58, 70*	2, 6*	17, 11*	2, 7*	1, 4*
AKB	0101, 0201	3701, 3901	0602, 0702	0101, 1001	501	0201, 0401
PSJ	0201, 3001	1302, 4402	0501, 0602	0401, 0701	0202, 0301	0402, 1401
MWX	0101, 3401	1521, 3503	0403, 1203	0101, 1502	0501, 0601	0601, 1301
NGZ	1101, 2401	5201, 4002	0202, 1202	0101, 1101	0501, 0301	0201, 0301
RSB	0201, 0301	5701, 4402	0602, 0704	0701, 1101	0301, 0303	0201, 0401
WOH	1, 28	8, 27	2, 7	ND	ND	ND
FBV	0201, 1101	0702, 5501	0303, 0702	1454, 1501	0503, 0602	0401
RTN	0101, 1101	0801, 5701	0602, 0701	0301, 0701	0201, 0301	0401, 1401

The panel of EBV-transformed B cells (EBV-LCLs) was composed of HLA-typed EBV-LCLs, which together covered almost all frequently occurring HLA molecules. The HLA typing was determined mainly molecularly; however, some EBV-LCLs were only serologically typed. ND indicates that the HLA expression was not determined. * HLA expression was determined by serologic typing.

B8–specific line expressed either HLA-B*4402 or HLA-B*5501. TCR Vβ analysis of the lines demonstrated that the complexity of the TCR composition was correlated with the broadness of the alloreactivity. T-cell lines that did not show alloreactivity expressed maximally 2 different TCR Vβ chains. T-cell lines with limited alloreactivity expressed 1 to 4 different TCR Vβ chains, and lines that recognized almost all EBV-LCLs expressed at least 8 different TCR Vβ chains. This relation between the clonal composition and the recognition of the lines suggests that the alloreactivity of the lines is the sum of the alloreactivity of the various clonal populations present within the lines.

The results demonstrate that 80% of the tested virus-specific T-cell lines were able to exert alloreactivity. Some virus-specific lines showed a pattern of alloreactivity suggestive of allo-HLA reactivity. However, for most of the virus-specific cell lines, allo-HLA reactivity could not be determined because the exerted alloreactivity was very broad.

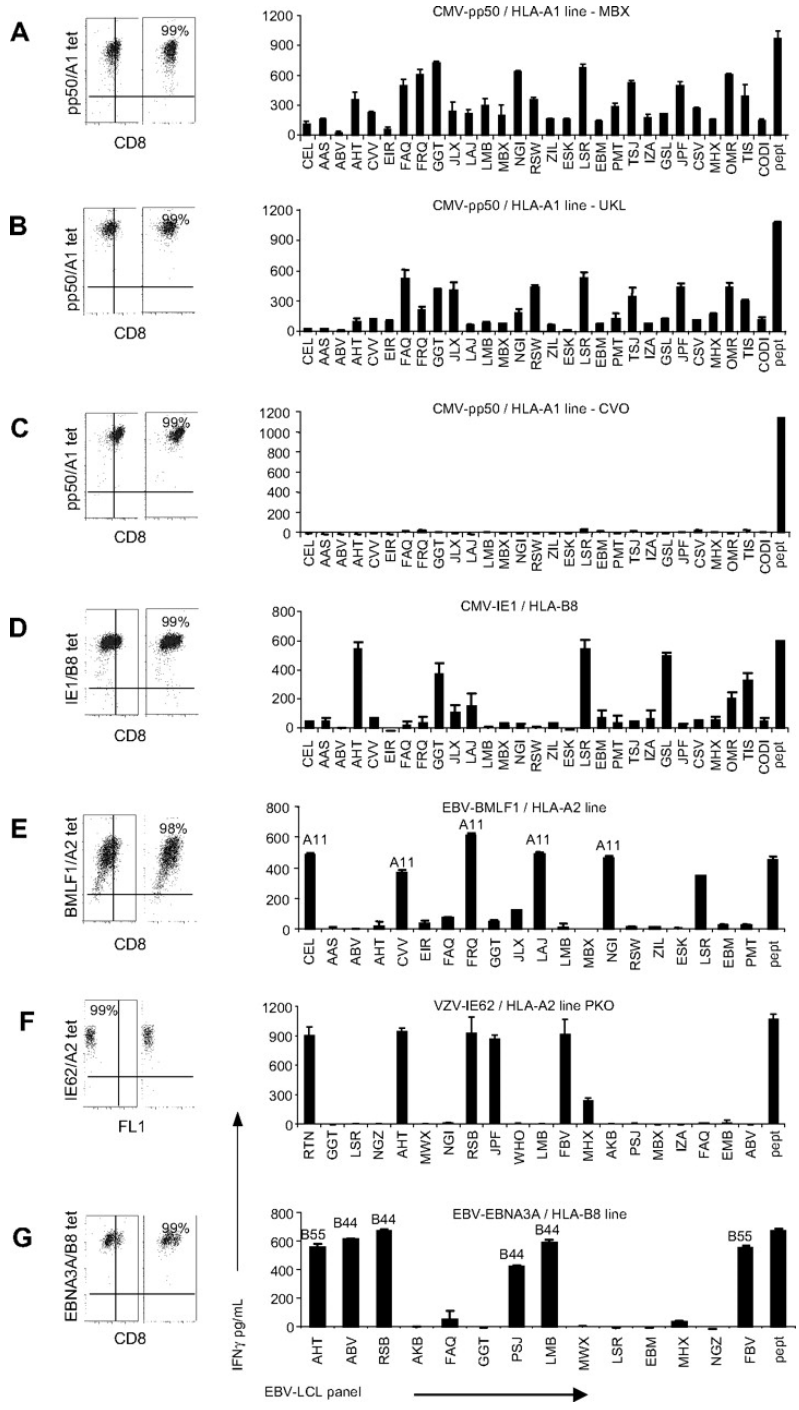
A
pp50/A1 tet
99%
CD8
CMV-pp50 / HLA-A1 line - MBX
B
pp50/A1 tet
99%
CD8
CMV-pp50 / HLA-A1 line - UKL
C
pp50/A1 tet
99%
CD8
CMV-pp50 / HLA-A1 line - CVO
D
IE1/B8 tet
99%
CD8
CMV-IE1 / HLA-B8
E
BMLF1/A2 tet
98%
CD8
EBV-BMLF1 / HLA-A2 line
A11
F
IE62/A2 tet
99%
FL1
VZV-IE62 / HLA-A2 line PKO
G
EBNA3A/B8 tet
99%
CD8
EBV-EBNA3A / HLA-B8 line
B55
B44
IFNγ pg/mL
EBV-LCL panel
CEL AAS ABV AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM PMT TSJ IZA GSL JPF CSV MHX OMR TIS CODI pept
RTN GGT LSR NGZ AHT MWX NGI RSB JPF WHO LMB FBV MHX AKB PSJ MBX IZA FAQ EMB ABV pept
AHT ABV RSB AKB FAQ GGT PSJ LMB MWX LSR EBM MHX NGZ FBV pept

Figure 1.

Figure 1. Alloreactivity of virus-specific T-cell lines.
Eleven virus-specific T-cell lines, of which 7 are shown in this figure, were stimulated with a panel of EBV-LCLs for 18 hours and IFNγ production was measured by ELISA. In experiments in which EBV-specific T-cell lines were tested, we excluded the EBV-LCLs expressing the HLA molecules to which the T-cell lines were restricted. The purity of the virus-specific lines was analyzed by tetramers and CD8 staining, and all T-cell lines proved to be more than 98% pure. As a positive control, the lines were tested against EBV-LCLs expressing the HLA-restricting molecule of the viral epitope, loaded with the viral peptide recognized by the T-cell lines (pept). (A) The CMV-pp50/HLA-A1–specific lines of patient MBX recognized almost all EBV-LCLs. (B) The CMV-pp50/HLA-A1–specific line of patient UKL showed broad alloreactivity. (C) Two of the 10 tested T-cell lines exerted no alloreactivity against the tested EBV-LCLs of which 1, the pp50/HLA-A1–specific line, is shown. (D) The CMV-IE1/HLA-B8 recognized a limited number of EBV-LCLs. (E) The BMLF1/HLA-A2–specific line showed high reactivity against all HLA-A11–positive EBV-LCLs and 1 HLA-A11–negative EBV-LCL. (F) The VZV-IE62/HLA-A2–specific line of patient PKO recognized a limited number of EBV-LCLs. (G) EBNA3A/HLA-B8–specific line recognized EBV-LCLs expressing either HLA-B44 or HLA-B55. Experiments were performed in duplicate, mean values are shown ± SD.

Allo-HLA reactivity of virus-specific T-cell clones

Because we were unable to determine allo-HLA reactivity with the oligoclonal virus-specific T-cell lines, further characterization of the allo-HLA reactivity of virus-specific T cells was performed with T-cell clones. For this purpose, tetramer-positive T cells were sorted at a single cell per well and expanded. The specificity of the T-cell clones was confirmed by tetramer staining, and the TCR Vβ usage of the clones was analyzed using the TCR Vβ kit. The T-cell clones as shown in Table 3 were different based on either their different origin, TCR Vβ usage, or recognition pattern. In total, 41 virus-specific CD8 and CD4 T-cell clones were tested against the EBV-LCL panel. These virus-specific T-cell clones were derived from 16 persons, specific for 13 different CMV, EBV, VZV, and Flu antigens and restricted to 8 different HLA molecules. The results demonstrated that 18 of the 41 virus-specific CD8 and CD4 T-cell clones were alloreactive, as shown by recognition of at least 1 of the EBV-LCLs from the panel. Most alloreactive T-cell clones exhibited cross-reactivity against EBV-LCLs that shared an HLA molecule, suggesting allo-HLA reactivity. To confirm that the reactivity of a T-cell clone was directed against a specific allo-HLA molecule, the virus-specific T-cell clones were tested against HLA-negative K562 cell line or EBV-LCLs negative for the recognized allo-HLA molecules, which were transduced with the particular allo-HLA molecule. The allo-HLA reactivity of 7 representative T-cell clones is shown in Figure 2A-G. The EBV-EBNA3A/HLA-A3–specific CD8 T-cell clone exhibited alloreactivity against all HLA-A*3101–expressing EBV-LCLs within the panel. The reactivity directed against allo–HLA-A*3101 was confirmed by transfection of K562 with HLA-A*3101 and subsequent recognition by this T-cell clone (Figure 2A). The EBV-EBNA3A/HLA-A3 clone also recognized the HLA-A*3101–negative EBV-LCL RSW, which however expressed HLA-A*3001. HLA-A*3101 and HLA-A*3001 are very similar in sequence and therefore we hypothesized that the molecules exhibit strong similarity in structure and peptide presentation, explaining recognition by this T-cell clone. To analyze whether the EBV-EBNA3A/HLA-A3 clone recognized HLA-A*3001, the clone was tested against 3 HLA-A*3001$^+$ EBV-LCLs, of which 1 is shown in Figure 2A, and 1 HLA-A*3101$^+$ EBV-LCL with or without blocking mAbs directed against HLA class I, HLA-A30/A31, and HLA-A2. All HLA-A*3001$^+$ EBV-LCLs and the HLA-A*3101$^+$ EBV-LCLs were

recognized, and this recognition was blocked by anti–HLA class I and anti–HLA-A30/A31 mAbs and not by anti–HLA-A2 mAb, indicating that the clone indeed also recognized HLA-A*3001. The CMV-pp50/HLA-A1–specific T-cell clone exhibited alloreactivity against all HLA-A*1101–expressing EBV-LCLs (Figure 2B). The allo–HLA-A*1101 reactivity could be confirmed by specific recognition of K562 transduced with HLA-A*1101 by this T-cell clone. In addition to reactivity against HLA-A*1101, the T-cell clone also exhibited low IFNγ production upon stimulation with a few EBV-LCLs negative for HLA-A*1101. Because these less recognized EBV-LCLs did not share one HLA molecule, we did not determine whether this alloreactivity was also based on allo-HLA cross-reactivity. The EBV-BRLF1–specific HLA-A3–restricted clone, shown in Figure 2C, exerted alloreactivity against all HLA-A*0201$^+$ EBV-LCLs. The CD8 T-cell clone did not recognize K562 transduced with HLA-A*0201, however it showed recognition of EBV-LCLs transduced with HLA-A*0201, suggesting that the peptide recognized by the clone in the context of HLA-A*0201 is not presented by K562 cells. The clone also showed recognition of HLA-A*0201$^+$ PHA-stimulated T-cell blasts (Figure 2C right panel), excluding the possibility that the clone recognized an EBV-derived peptide presented in HLA-A*0201. Next to allo–HLA-A*0201 reactivity, this T-cell clone also exhibited low cross-reactivity against EBV-LCLs negative for HLA-A*0201. Because these less recognized EBV-LCLs did not share 1 HLA molecule, we did not determine the alloreactivity in detail. The CMV-pp65/HLA-B7–specific CD8 T-cell clone exhibited alloreactivity against 2 HLA-DRB1*0801– and 1 HLA-DRB1*0806–expressing EBV-LCL present in the panel. The cross-reactivity exerted by this CD8 T-cell clone could be blocked with antibodies directed against HLA class II and HLA-DR and not by HLA class I, HLA-DQ, or HLA-DP antibodies, confirming that the cross-reactivity of this virus-specific CD8 T-cell clone was directed against HLA-DR8. The ability of CD8 T cells to cross-react against allo-HLA class II molecules was also demonstrated by the CMV-pp65–specific HLA-B35–restricted CD8 T-cell clone that was shown to be cross-reactive against HLA-DRB1*0401 (Table 3). As previously described, we observed that the EBV-EBNA3A/HLA-B8–specific T-cell clone exhibited alloreactivity against all EBV-LCLs expressing HLA-B*4402 (22). Furthermore, we observed alloreactivity against EBV-LCLs expressing HLA-B*5501 as was recently described by us (33). Allo-HLA reactivity against HLA-B*4402 and HLA-B*5501 was confirmed by recognition of K562 transfected with either HLA-B*4402 or HLA-B*5501 by this T-cell clone (Figure 2E).

In addition to CD8$^+$ T-cell lines and clones, we also analyzed the cross-reactive potential of CD4$^+$ T-cell clones to allo-HLA molecules. The alloreactivity of a Flu-HA/HLA-DR4–specific T-cell clone demonstrated to be directed against HLA-DRB1*1301. As shown in Figure 2F, both HLA-DRB1*1301–positive EBV-LCLs present in the panel were efficiently recognized by this T-cell clone. Allo–HLA-DRB1*1301 reactivity of the T-cell clone could be confirmed because the T cells recognized EBV-LCLs transduced with HLA-DRB1*1301, whereas nontransduced EBV-LCLs were not recognized. In addition, allo-HLA reactivity was demonstrated for 2 other CD4$^+$ T-cell clones of the 5 CD4$^+$ T-cell clones tested, indicating that virus-specific CD4$^+$ T cells also exert allo-HLA reactivity (Table 3).

For 5 of the 18 allo-HLA–reactive virus-specific T-cell clones, the recognized HLA molecules could not be determined because the recognized EBV-LCLs did not share 1 particular allo-HLA molecule. The alloreactivity of 1 of these clones, CMV-pp65/HLA-A2–specific clone

HRN 3, is shown in Figure 2G. We hypothesize that this recognition is mediated by the recognition of several HLA molecules because the reactivity could be blocked by mAb specific for HLA class I.

The results of the allo-HLA reactivity exerted by the virus-specific T-cell clones are summarized in Table 3, and demonstrate that approximately 45% of the virus-specific memory CD4 and CD8 T-cell clones exhibit cross-reactivity to allo-HLA molecules. The cross-reactivity of the CD8 and CD4 T-cell clones was directed primarily against HLA class I and II, respectively. However, cross-reactivity of CD8 T cells directed against HLA class II was also observed.

Figure 2. Allo-HLA reactivity of virus-specific T-cell clones.
Forty-one virus-specific T-cell clones, of which 7 are shown in this figure, were stimulated with a panel of EBV-LCLs for 18 hours, and IFNγ production was measured by ELISA. (A) The EBV-EBNA3A/HLA-A3–specific T-cell clone 1 exhibited alloreactivity against all EBV-LCLs expressing HLA-A*3101 and 1 EBV-LCL expressing HLA-A*3001. To confirm allo–HLA-A*3101 reactivity, clone 1 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transfected with HLA-A*3101 (K562+A31), and HLA-A*3101–negative (A31neg LCL) and HLA-A*3101–positive (A31+LCL) EBV-LCLs. To confirm the reactivity against HLA-A*3001, the clone was tested against 3 HLA-A*3001$^+$ EBV-LCLs, of which 1 is shown, and 1 HLA-A*3101$^+$ EBV-LCLs with or without blocking mAbs directed against HLA class I, HLA-A30/A31, and HLA-A2. (B) The CMV-pp50/HLA-A1–specific T-cell clone 24 exhibited alloreactivity against all HLA-A*1101–expressing EBV-LCLs. To confirm allo–HLA-A*1101 reactivity, clone 24 was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-A*1101 (K562+A11), and HLA-A*1101–positive EBV-LCLs (A11+LCL). (C) The EBV-BRLF1/HLA-A3–specific clone 19 exerted alloreactivity against all HLA-A0201$^+$ EBV-LCLs. This T-cell clone did not recognize K562 transduced with HLA-A*0201 (K562+A2). To confirm allo–HLA-A*0201 reactivity, clone 19 was tested against untransduced HLA-A*0201–negative EBV-LCLs (HLA-A2neg LCL) or transduced with HLA-A*0201 (A2 trans), and HLA-A*0201–positive EBV-LCLs (A2+LCL). To investigate whether this clone recognized an EBV-derived peptide in the context of HLA-A*0201, the clone was tested against HLA-A*0201$^+$ PHA blasts and HLA-A*0201–positive and –negative EBV-LCLs as controls. (D) The CMV-pp65/HLA-B7–specific T-cell clone 11 exhibited reactivity against all 3 HLA-DRB1*0801$^+$ EBV-LCLs. To confirm allo–HLA-DRB1*0801 reactivity, clone 11 was tested against the 3 HLA-DRB1*0801$^+$ EBV-LCLs, of which 1 is shown, in the presence of either no blocking mAbs (no block) or anti–HLA class I (class I), anti–HLA class II (class II), anti–HLA-DR (DR), anti–HLA-DR (DQ), and anti–HLA-DR (DP) blocking mAbs. (E) The EBV-EBNA3A/HLA-B8–specific T-cell clone 9 exhibited alloreactivity against all EBV-LCLs expressing either HLA-B*4402 or HLA-B*5501. To confirm HLA-B*4402 and HLA-B*5501 cross-reactivity, clone 9 was tested against K562 cells (K562), or K562 cells transfected with HLA-B*4402 (K562+B4402) or HLA-B*5501 (K562+B5501). As controls, clone 9 was tested against HLA-B*4402– and HLA-B*5501–negative EBV-LCLs (neg LCL), HLA-B*4402$^+$ EBV-LCLs (B4402+LCL), HLA-B*5501$^+$ EBV-LCLs (B5501+LCL), or HLA-B*0801$^+$ K562 loaded with viral peptide (K562+B8+pept). (F) The Flu-HA/HLA-DR4–specific clone 5 recognized all HLA-DRB1*1301$^+$ EBV-LCLs. To confirm allo–HLA-DRB1*1301 reactivity, clone 5 was tested against HLA-DRB1*1301$^+$ EBV-LCLs (FAQ DR13+ and IZA DR13+) as well as HLA-DR13–negative EBV-LCLs nontransduced (DR13 neg) or transduced with HLA-DRB1*1301 (DR13 trans). (G) The CMV-pp65/HLA-A2–specific clone HRN 3 recognized EBV-LCLs that did not share 1 particular allo-HLA molecule. To investigate whether this reactivity was based on allo-HLA recognition, the clone was tested against 4 of the recognized EBV-LCLs with and without blocking mAb directed against HLA class I. Experiments were performed in duplicate, mean values are shown ± SD.

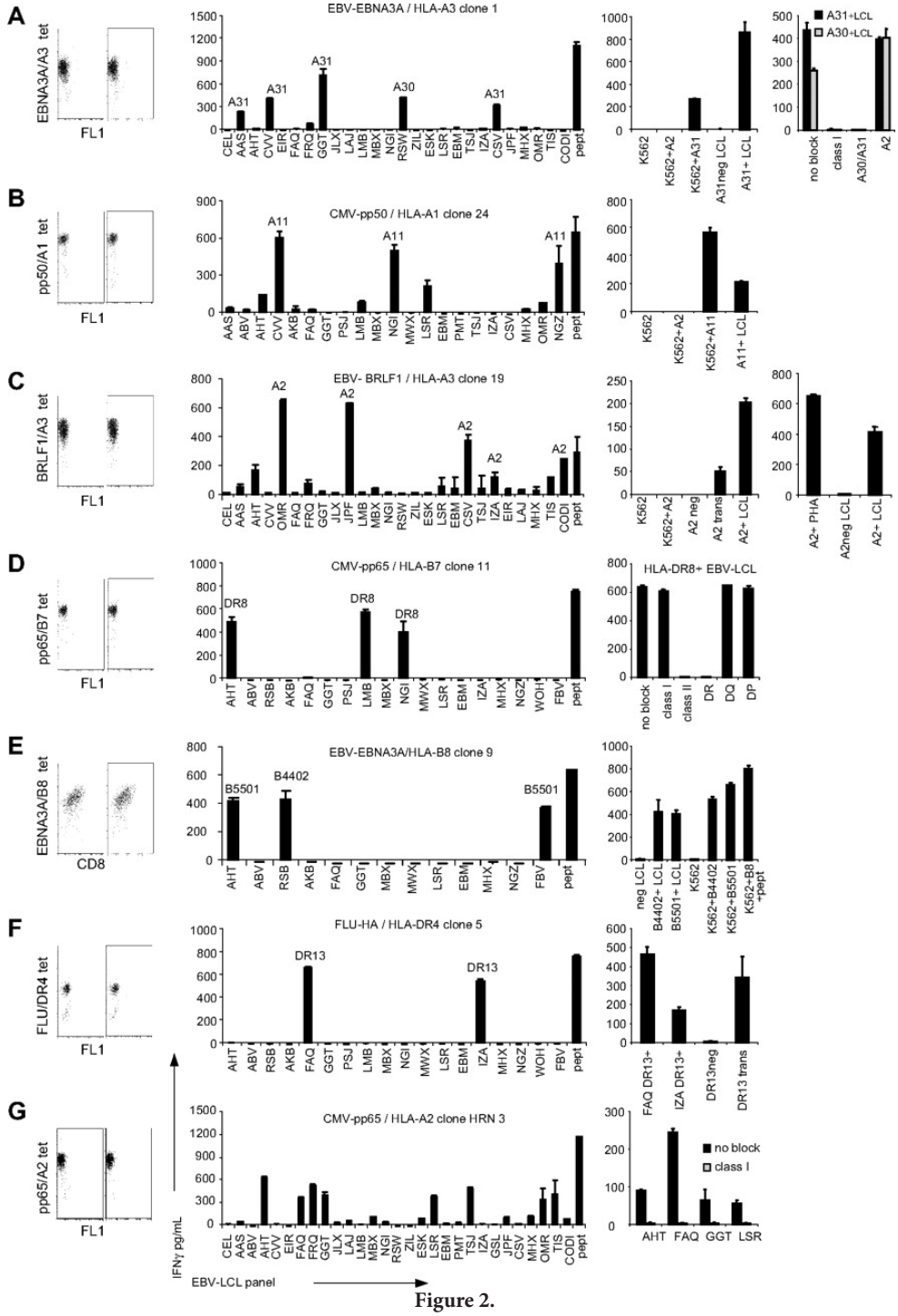
A
EBNA3A/A3 tet
FL1
EBV-EBNA3A / HLA-A3 clone 1
A31
A31
A31
A30
A31
CEL AAS AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM TSJ IZA CSV JPF MHX OMR TIS CODI pept
K562
K562+A2
K562+A31
A31neg LCL
A31+ LCL
A31+LCL
A30+LCL
no block
class I
A30/A31
A2
B
pp50/A1 tet
FL1
CMV-pp50 / HLA-A1 clone 24
A11
A11
A11
AAS ABV AHT CVV AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM PMT TSJ IZA CSV MHX OMR NGZ pept
K562
K562+A2
K562+A11
A11+ LCL
C
BRLF1/A3 tet
FL1
EBV- BRLF1 / HLA-A3 clone 19
A2
A2
A2
A2
A2
CEL AAS AHT CVV OMR FAQ FRQ GGT JLX JPF LMB MBX NGI RSW ZIL ESK LSR EBM CSV TSJ IZA EIR LAJ MHX TIS CODI pept
K562
K562+A2
A2 neg
A2 trans
A2+ LCL
A2+ PHA
A2neg LCL
A2+ LCL
D
pp65/B7 tet
FL1
CMV-pp65 / HLA-B7 clone 11
DR8
DR8
DR8
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
HLA-DR8+ EBV-LCL
no block
class I
class II
DR
DQ
DP
E
EBNA3A/B8 tet
CD8
EBV-EBNA3A/HLA-B8 clone 9
B5501
B4402
B5501
AHT ABV RSB AKB FAQ GGT MBX MWX LSR EBM MHX NGZ FBV pept
neg LCL
B4402+ LCL
B5501+ LCL
K562
K562+B4402
K562+B5501
K562+B8 +pept
F
FLU/DR4 tet
FL1
FLU-HA / HLA-DR4 clone 5
DR13
DR13
AHT ABV RSB AKB FAQ GGT PSJ LMB MBX NGI MWX LSR EBM IZA MHX NGZ WOH FBV pept
FAQ DR13+
IZA DR13+
DR13neg
DR13 trans
G
pp65/A2 tet
FL1
CMV-pp65 / HLA-A2 clone HRN 3
IFNγ pg/mL
EBV-LCL panel
CEL AAS ABV AHT CVV EIR FAQ FRQ GGT JLX LAJ LMB MBX NGI RSW ZIL ESK LSR EBM PMT TSJ IZA GSL JPF CSV MHX OMR TIS CODI pept
no block
class I
AHT FAQ GGT LSR

Figure 2.

Table 3. Allo-HLA reactivity of virus-specific T-cell clones

Specificity	Donor	TCR Vβ	EBV panel	HLA trans/block	Figure
pp50/A1	MBX	*	UD		
pp50/A1	MBX	1			
pp50/A1	MBX	5.1			
pp50/A1	MBX	3	A*1101	A*1101	2B
pp65/A2	MRJ	2			
pp65/A2	MRJ	13			
pp65/A2	HRN	8	UD		2G
pp65/A2	HRN	2			
pp65/A2	AMJ	3			
BMLF1/A2	GFS	*			
LMP2/A2	JVW	*			
FLU/A2	FKR	17	B*6401		
FLU/A2	FKR	17			
VZV/A2	PKN	14	B*5501	B*5501	3A
VZV/A2	PKN	*	B*5701	B*5701	3B
VZV/A2	PKN	21,3	A*0205	A*0205	3C
EBNA3A/A3	HRN	*	A*3101	A*3101	2A
BRLF1/A3	AKO	7.1			
BRLF1/A3	AKO	14			
BRLF1/A3	AKO	17			
BRLF1/A3	AKO	1			
BRLF1/A3	DVO	7.1	UD		
BRLF1/A3	DVO	8			
BRLF1/A3	DVO	14	UD		
BRLF1/A3	DVO	17	UD		
BRLF1/A3	DVO	*			
BRLF1/A3	DVO	7.2	A*0201	A*0201	2C
pp65/B7	BDV	7.2			
pp65/B7	BDV	7.2	DRB1*0801	DRB1*0801	2D
EBNA3A/B8	LDO	*	B*4402, B*5501	B*4402, B*5501	2E
BZLF/B8	AVK	7.1			
BZLF/B8	AVK	*			
BZLF/B8	AVK	5.1			
pp65/B35	MED	3			
pp65/B35	MED	5.1	DRB1*0401	DRB1*0401	
pp65/B35	MED	*			
pp65/DR1	CBH	2			
pp65/DR1	CBH	*			
pp65/DR1	MSV	8	DRB1*0901		
pp65/DR1	MSV	*	DRB3*0101	DRB3*0101	
FLU/DR4	VKY	3	DRB1*1301	DRB1*1301	2F

TCR indicates T-cell receptor; and UD, undetermined allo-HLA reactivity, which could not be characterized because the recognized EBV-LCLs did not share one particular allo-HLA molecule.
* The TCR Vβ of the clone could not be determined with the TCR Vβ kit.

Different allo-HLA recognition by T-cell clones with the same specificity but different TCR usage
Burrows et al showed that EBV-EBNA3A–specific HLA-B8–restricted T-cell clones, derived from different HLA-B44–negative persons, were all alloreactive against HLA-B44 (22,23). It could therefore be suggested that allo-HLA reactivity of virus-specific T cells can be predicted. However, the EBV-EBNA3A response in HLA-B8$^+$, HLA-B44$^-$ persons is a very homogeneous response in which all T cells express an almost identical public TCR (34). This is in contrast to most virus responses, which are usually oligoclonal and different among persons (16,35) (Table 3). To assess whether virus-specific T cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, we tested the alloreactivity of 3 T-cell clones derived from the same person, all specific for a peptide of the IE62 protein of VZV presented in HLA-A*0201 but with different TCR usage. As demonstrated in Figure 3A-C, all 3 VZV-specific T-cell clones recognized different allo-HLA molecules. Clone 5 showed

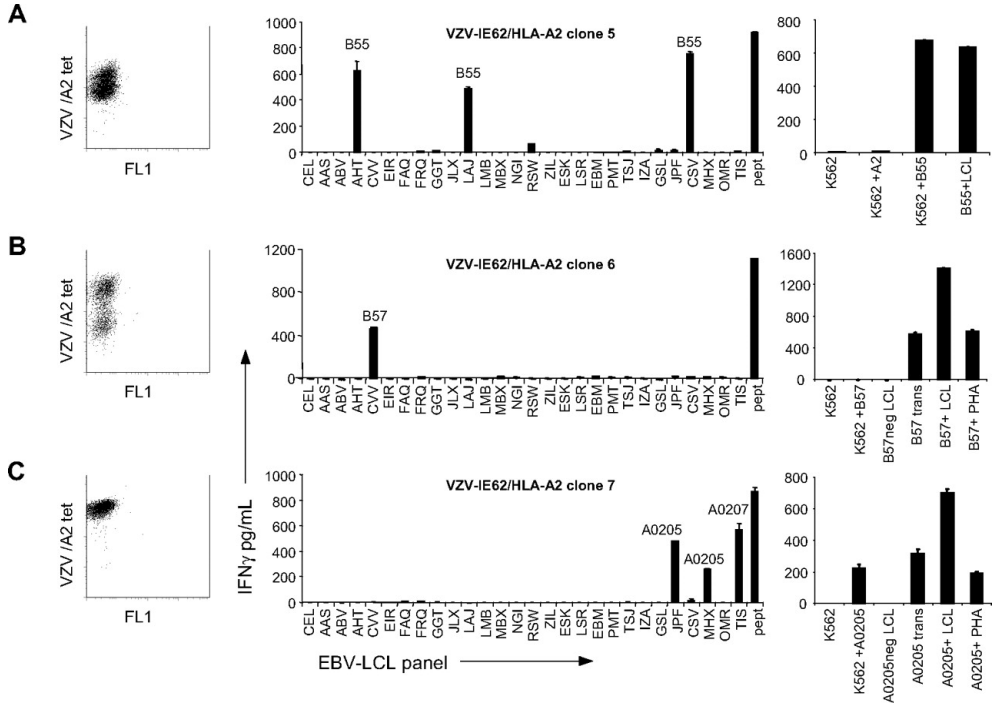

Figure 3. Variable allo-HLA recognition by T-cell clones with the same specificity but different TCR Vβ usage. To investigate whether virus-specific T cells sharing the same antigen specificity but expressing different TCRs exert the same allo-HLA reactivity, 3 VZV-IE62/HLA-A2–specific T-cell clones expressing different TCRs were stimulated for 18 hours with a panel of EBV-LCLs and IFNγ production was measured by ELISA. (A) VZV clone 5 showed alloreactivity against all HLA-B*5501$^+$ EBV-LCLs. To confirm allo–HLA-B*5501 reactivity, the clone was tested against K562 cells (K562), K562 cells transduced with HLA-A*0201 (K562+A2), K562 cells transduced with HLA-B*5501 (K562+B55), and HLA-B55$^+$ EBV-LCLs (B55+LCL). (B) VZV clone 6 was alloreactive against the HLA-B*5701$^+$ EBV-LCLs. Clone 6 did not show reactivity against K562 cells transduced with HLA-B*5701 (K562+B57). To confirm allo–HLA-B*5701 reactivity, clone 6 was tested against HLA-B*5701–negative EBV-LCLs (HLA-B57neg LCL) or transduced with HLA-B*5701 (B57 trans), HLA-B*5701$^+$ EBV-LCLs (B57+LCL), and HLA-B*5701$^+$ PHA blasts (B57+PHA). (C) VZV clone 7 exhibited cross-reactivity against all HLA-A*0205$^+$ and HLA-A*0207$^+$ EBV-LCLs. Allo–HLA-A*0205 reactivity was confirmed by testing the clone against K562 cells (K562), K562 transduced with HLA-HLA-A*0205 (K562+HLA-A0205), HLA-A*0205–negative EBV-LCLs (HLA-A0205neg LCL), or these EBV-LCLs transduced with HLA-A*0205 (A0205 trans), HLA-A*0205$^+$ EBV-LCLs (A0205+LCL), and HLA-A*0205$^+$ PHA blasts (A0205+PHA). The results demonstrate that virus-specific T cells with the same antigen specificity, but with different TCR usage, exert alloreactivity against different HLA molecules. Experiments were performed in duplicate, mean values are shown ± SD.

alloreactivity against HLA-B*5501, clone 6 was alloreactive against HLA-B*5701, and clone 7 exhibited allo–HLA-A*0205 and allo–HLA-A*0207 reactivity. The recognition of the 3 clones together was comparable with the recognition exerted by the VZV-IE62–specific line derived from the same person. This confirms that alloreactivity exerted by the virus-specific lines shown in Figure 1 is the sum of the alloreactivity of the various clonal populations present within the lines. The allo-HLA reactivities of the T-cell clones were confirmed by transduction of the allo-HLA molecules in K562 or in nonrecognized EBV-LCLs. Clones 5 and 7 recognized K562 transduced with HLA-B*5501 and HLA-*0205, respectively. Clone 6 was unable to recognize K562 transduced with HLA-B*5701, whereas EBV-LCLs transduced with HLA-B*5701 were recognized. Because this clone also recognized HLA-B*5701–expressing PHA-stimulated T-cell blasts, specificity against EBV-derived peptide in the context of allo–HLA-B*5701 is excluded. These results demonstrate that virus-specific T cells with the same antigen specificity, but with different TCR complexes, can exert alloreactivity against different HLA molecules. Because T-cell responses against viruses are usually oligoclonal and different among people, these results indicate that allo-HLA reactivity cannot be predicted.

The cytotoxic potential and affinity of the alloreactivity exerted by virus-specific T cells
Because cytotoxicity may be a relevant measure to predict the potency of the virus-specific T cells to induce GVHD or graft rejection in vivo, we investigated the allo-HLA–reactive cytotoxic capacity of the virus-specific T cells. Six virus-specific T-cell clones were tested against a panel of EBV-LCLs positive and negative for the recognized allo-HLA molecules. As shown in Figure 4A, all 6 T-cell clones tested showed cytotoxicity against the allo-HLA–expressing EBV-LCLs.

To investigate whether the affinity of the allo-HLA–reactive response was comparable with the affinity of the virus-specific response, the kinetics of recognition and antigen threshold were tested for both specificities. For this purpose, the allo–HLA-A*3101/A*3001 reactive EBV-EBNA3A/HLA-A3–specific clone 19 was tested against HLA-A*0301$^+$ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against 2 HLA-A*3101$^+$ EBV-LCLs, GGT and DSP at different effector-stimulator ratios. As shown in Figure 4B, the T-cell clone produced comparable amounts of IFNγ against the virus antigen–expressing EBV-LCLs as against the allo-HLA expressing–EBV-LCLs in the different effector-stimulator ratios, indicating that the kinetics of recognition and antigen threshold of the alloreactive response and the virus-specific T-cell response are comparable.

Normal cell subsets are recognized by virus-specific T cells
To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus-specific T-cell clones against allo-HLA–expressing B cells, CD40L-activated B cells, T cells, PHA blasts, monocytes, monocyte-derived DCs, and fibroblasts with and without IFNγ pretreatment. The results shown in Figure 4C demonstrate the reactivity of 3 virus-specific T-cell clones directed against the different cell subsets. pp65/HLA-B*0702–specific clone 11 showed high recognition of HLA-DRB1*0801–positive CD40L-activated B cells and low recognition of B cells and PHA blasts. VZV-IE62/HLA-

A*0201–specific clone 5 showed high recognition of HLA-B*5501–positive CD40L-activated B cells, DCs, and PHA blasts and low recognition of monocytes and T cells. This clone could unfortunately not be tested against fibroblasts because HLA-B*5501–positive fibroblasts were not available. VZV-IE62/HLA-A*0201–specific clone 6 highly recognized HLA-B*5701–positive DCs and showed low reactivity against CD40L-activated B cells, T cells, PHA blasts, and IFNγ-stimulated fibroblasts. These results indicate that virus-specific T cells can also be reactive against in vivo relevant normal cell subsets.

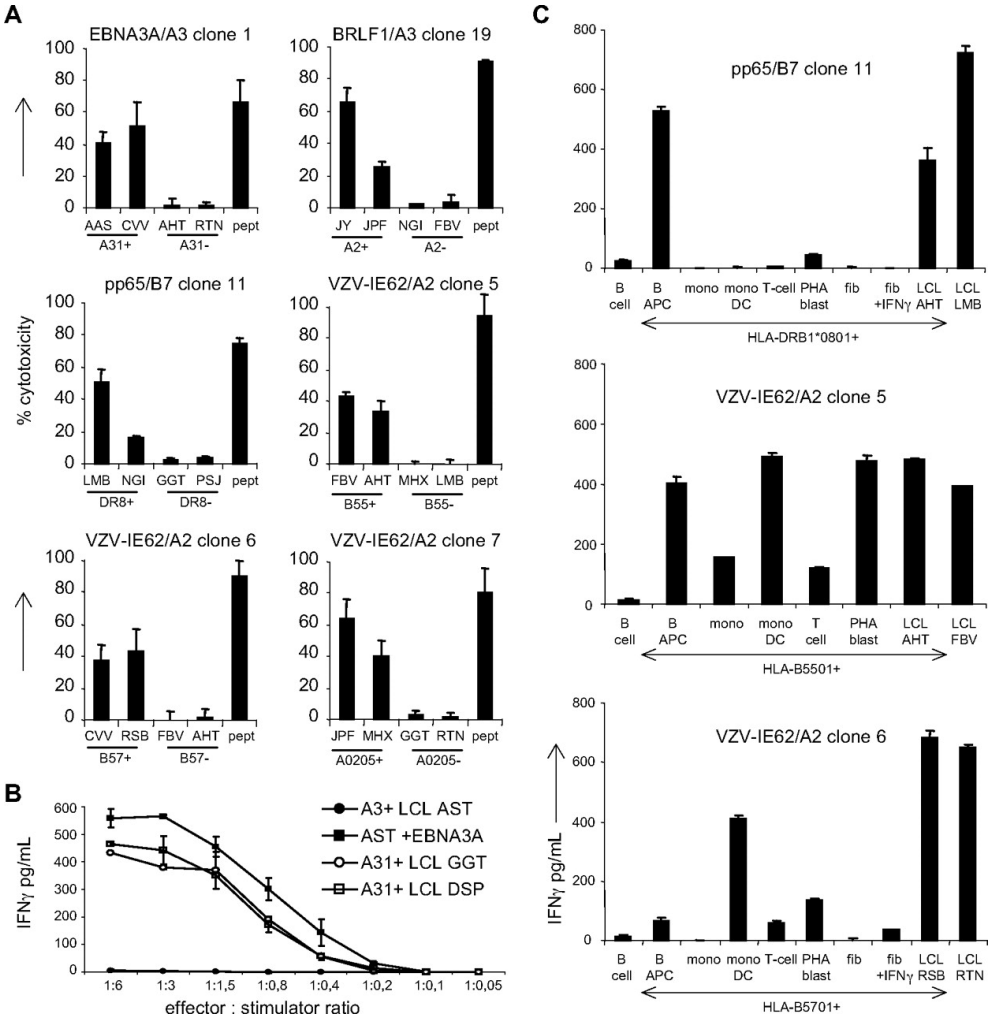

Figure 4.

Figure 4. The potency of the alloreactivity exerted by virus-specific T cells.
(A) To investigate the allo-HLA–reactive cytotoxic capacity of virus-specific T cells, 6 virus-specific cell clones were tested in cytotoxicity assays against 2 EBV-LCLs expressing the recognized allo-HLA molecules and 2 EBV-LCLs negative for the allo-HLA molecules. EBV-LCLs expressing the virus-specific restriction molecule were loaded with the viral peptide and used as positive control for cytotoxicity. (B) To compare the affinities of the allo-HLA–reactive response and the virus-specific response, the allo–HLA-A30/A31–reactive EBV-EBNA3A/HLA-A3–specific clone 19 was tested against the HLA-A*0301$^+$ EBV-LCL AST transduced with a retrovirus encoding for EBNA3A and against 2 HLA-A*3101$^+$ EBV-LCLs GGT and DSP. To compare the kinetics of the 2 responses, the clone was tested against the EBV-LCLs in different effector-stimulator ratios. (C) To extrapolate the results obtained with the EBV-LCLs and K562 cells to the recognition of normal cell subsets in vivo, we tested virus-specific T-cell clones against allo-HLA–expressing B cells, CD40 ligand–activated B cells (B APC), T cells, PHA blasts, monocytes, monocyte-derived DCs, and fibroblasts with and without IFNγ pretreatment. Experiments were performed in duplicate, mean values are shown ± SD.

One TCR complex mediates both virus specificity and allo-HLA reactivity
Because alloreactivity mediated by T cells may be explained by T cells expressing 2 TCR complexes at the cell surface (36,37) we wanted to exclude that the allo-HLA reactivity was mediated via another TCR than the virus-specific TCR. For this purpose, we determined the TCR usage of 2 representative allo-HLA–reactive virus-specific clones, the allo–HLA-A*0201–reactive BRLF1/HLA-A*0301–specific clone 19 (Figure 2C) and the allo–HLA-A*0205–reactive VZV-IE62/HLA-A*0201–specific clone 7 (Figure 3C). By reverse transcriptase–polymerase chain reaction, we established that the BRLF1/HLA-A*0301–specific clone 19 expressed 1 TCRβ gene transcript, BV7S2, and 2 TCRα transcripts, AV12S1 and AV18S1. However, 1 of the TCRα chains, AV12S1, contained a stop codon in the CDR3 region, indicating that this TCR was not expressed. Flow cytometric analysis confirmed that 100% of the T cells expressed TCR BV7S2 at the cell surface (data not shown). No antibodies were available for analysis of the specific TCRα chain expression at the cell surface. To investigate whether the BV7S2 and the AV18S1 mediated the dual recognition, IE1/HLA-A1–specific T cells were transduced with retroviral vectors encoding for these TCRα and TCRβ chains. The results shown in Figure 5A demonstrate that the BRLF1-TCR–transduced T cells exerted reactivity against HLA-A*0201–expressing target cells as well as against EBV-BRFL1 peptide–loaded HLA-A*0301$^+$ target cells. No reactivity directed against peptide-loaded or HLA-A*0201–expressing target cells was observed with mock-transduced T cells.

The VZV-IE62/HLA-A*0201 clone 7 expressed 1 TCRα transcript, AV6S1, and 1 TCRβ transcript, BV21S3. pp50/A1-specific T cells were transduced with retroviral vectors encoding for the VZV TCR chains. The results shown in Figure 5B demonstrate that the VZV-TCR–transduced T cells exerted reactivity against HLA-A*0205–expressing target cells as well as against VZV-IE62 peptide–loaded HLA-A*0201$^+$ target cells. No reactivity directed against peptide-loaded or HLA-A*0205–expressing target cells was observed with mock-transduced T cells. These results demonstrate that the virus specificity and the allo-HLA reactivity exerted by these virus-specific T cells were mediated via 1 TCR complex.

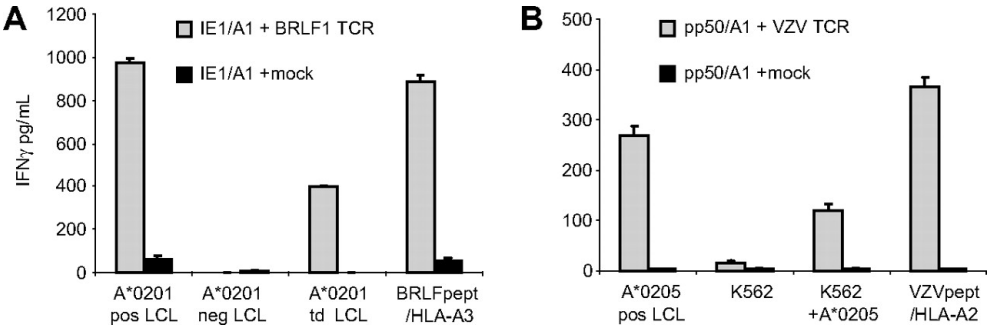

Figure 5. One TCR complex mediates both virus specificity and allo-HLA reactivity.
To exclude that allo-HLA reactivity was mediated via another TCR than the virus-specific TCR, the TCR of 2 representative clones was transferred to T cells with a different specificity. (A) IE1/A1-specific T cells transduced with viral vectors encoding for the TCR of BRLF1/A3-specific clone 19 (IE1/A1+BRLF1 TCR) and IE1/A1-specific T cells transduced with a mock viral vector (IE1/A1+mock) were tested for allo–HLA-A*0201 reactivity against HLA-A*0201–positive EBV-LCLs (A*0201 pos LCLs), HLA-A*0201–negative EBV-LCLs (A*0201 neg LCLs), and HLA-A*0201–negative EBV-LCLs transduced with HLA-A*0201 (A*0201 td LCLs) and for BRLF1 specificity against BRLF1 peptide–loaded HLA-A*0301–positive EBV-LCLs (BRLF1pept /HLA-A3). (B) Pp50/A1-specific T cells transduced with viral vectors encoding for the TCR of VZV clone 7 (pp50/A1+VZV TCR) and pp50-specific T cells transduced with a mock viral vector (pp50/A1+mock) were tested for allo–HLA-A*0205 reactivity against HLA-A*0205–positive EBV-LCLs (A*0205 pos LCLs), K562 cells (K562), and K562 cells transduced with HLA-A*0205 (K562+A*0205) and for VZV specificity against VZV-IE62 peptide–loaded HLA-A*0201–positive EBV-LCLs (VZVpept/HLA-A2). The results demonstrate that virus specificity and allo-HLA reactivity exerted by virus-specific T cells were mediated by 1 TCR complex. Experiments are shown in duplicate, mean values are shown ± SD.

DISCUSSION

In this study, we demonstrated that a high percentage of virus-specific memory T cells exhibits cross-reactivity against allogeneic HLA molecules. CD8 as well as CD4 virus-specific memory T cells were demonstrated to have allo-HLA–reactive potential. In addition, we determined that the alloreactivity exerted by CD8 T cells was directed against either HLA class I or HLA class II molecules, and that the alloreactivity of the T cells was mediated by cytotoxicity and cytokine production. Furthermore, we demonstrate that virus-specific T cells can exert allo-HLA reactivity against normal cell subsets, indicating the potential clinical relevance of the response. By TCR transfer, we confirmed that the allo-HLA reactivity and virus specificity were mediated via the same TCR.

Most virus-specific T-cell lines and 45% of virus-specific T-cell clones directed against EBV, CMV, VZV, and Flu exerted alloreactivity when tested against a panel of EBV-LCLs covering almost all common HLA molecules. The cross-reactivity exerted by the virus-specific T cells was confirmed to be based on allo-HLA recognition by testing the T-cell clones against

K562 cells and EBV-LCLs transduced with single HLA molecules. Some of the alloreactive virus-specific T-cell clones did not recognize K562 cells transduced with the specific allo-HLA molecules, but showed reactivity against EBV-LCLs transduced with the allo-HLA molecules. These data support the previous findings that allo-HLA reactivity is dependent on endogenous peptide (38). Allo-HLA cross-reactivity was directed not only against EBV-LCLs but also against PHA-stimulated T cells (Figure 3B), indicating that the peptides responsible for allo-HLA reactivity were not EBV derived. Differential recognition of HLA-transduced K562 cells and EBV-LCLs may indicate recognition of tissue-specific peptides in allo-HLA molecules. Therefore, it may be possible that we even underestimated the allo-HLA–reactive repertoire of T cells by initially screening only against an EBV-LCL panel.

Burrows et al showed that EBV-EBNA3A–specific HLA-B8–restricted T cells derived from different HLA-B44–negative persons all exert cross-reactivity against allo–HLA-B44 (22,23). Based on these findings, it could be suggested that the allo-HLA reactivity of virus-specific T cells can be predicted. The EBV-EBNA3A–specific T cells, however, express an almost identical public TCR in all HLA-B8$^+$ HLA-B44$^-$ persons (34), whereas most other virus responses are oligoclonal and the TCR usage of T cells directed against the same viral epitope is variable between persons (16,35) (Table 3). We have demonstrated that virus-specific T cells with the same antigen specificity, but expressing different TCRs, exhibit cross-reactivity against different HLA molecules (Figure 3). In addition, we have shown that 3 specific T-cell lines with the same specificity for CMV-pp50, but derived from different persons, exerted a very variable pattern of allo-HLA reactivity, ranging from no allo-HLA reactivity to very broad alloreactivity (Figure 1). These results together illustrate that the cross-reactive potential of antigen-specific T cells against allo-HLA molecules is difficult to predict.

The alloreactivity exerted by the virus-specific memory CD8 and CD4 T cells was directed primarily against allo-HLA class I and II molecules, respectively, suggesting that the coreceptors expressed by the T cells contributed to the affinity of the allo-HLA reactivity. However, we also demonstrated allo-HLA class II recognition by a small proportion of antigen-specific CD8 T cells, as was also shown recently by Rist et al (39). HLA class II allorecognition by CD8$^+$ T cells could indicate an HLA class II–TCR interaction that is independent of CD8 coreceptor binding. It is, however, also possible that CD8 coreceptors bind to HLA class I molecules expressed on the target cell and thereby strengthen the TCR–HLA class II interaction, as was previously shown (27). Although we did not observe HLA class I cross-reactive CD4 T cells, only a limited number of CD4 T-cell clones were tested, and therefore we cannot exclude that CD4 T cells may also cross-react with HLA class I complexes.

The results of our study illustrate that approximately 45% of all T cells exert allo-HLA cross-reactivity. However, because T cells were analyzed only for allo-HLA cross-reactivity against an EBV-LCL panel expressing most common HLA molecules, missing all infrequent HLA molecules as well as all tissue-specific peptides presented in allo-HLA molecules, we speculate that virtually all T cells may be allo-HLA reactive. Based on this assumption, and the fact that the TCR repertoire of humans is highly diverse, after HLA-mismatched transplantations sufficient allo-mismatched HLA cross-reactive T cells are likely to be present to induce acute GVHD or graft rejection. However, HLA-mismatched stem cell transplantation or solid organ

transplantations do not always lead to acute GVHD or graft rejection, indicating that other factors must be involved in these transplantation-related complications. A range of acute viral infections have been linked to initiating GVHD and graft rejection after transplantation (26), suggesting that virus-specific T cells may be mediators of GVHD and graft rejection. Because virus-specific T-cell responses usually have a restricted TCR usage, high numbers of T cells expressing an identical TCR can be found. In addition, herpes virus–specific T-cell populations can remain present at high percentages for long periods of time in healthy persons as well as in patients (18–21), and viral infections, leading to expansion of virus-specific T cells, are very common after HLA-mismatched SCT or solid organ transplantation (40–43). Therefore, given the high proportion of virus-specific T cells and the less stringent requirements for activation of memory T cells (12,13), it is tempting to speculate that if the HLA type of patient or the transplanted organ matches the cross-reactivity of the virus-specific T cells, these allo-HLA–reactive virus-specific memory T cells may easily induce GVHD or graft rejection.

The ability of virus-specific T cells to exert allo-HLA reactivity may also have implications for the clinical applicability of virus-specific T-cell lines. Because immune deficiency for viruses is a common complication after SCT, broad administration of virus-specific T cells lines over HLA barriers to SCT patients has been proposed (44,45). The results of this study demonstrate that administration of virus-specific T cells over HLA barriers may increase risk of GVHD, and indicate that virus-specific lines should be tested for alloreactivity against the patient before administration.

Based on our results, we postulate that virtually all antigen-specific T cells will be cross-reactive against allo-HLA class I or II molecules. The high alloreactive potential of particularly virus-specific memory T cells may have important clinical implications in transplantation settings.

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AUTHORSHIP

Contribution: A.L.A. performed research and wrote the paper; L.J.A.D. performed research; D.L.R. contributed to research design; M.M.L. helped in generation of EBV-LCL panel; R.S.H. generated TCR gene constructs; R.B. helped in generation of EBV-LCL panel and transduced the TCR gene constructs; M.A.W.G.H. generated virus-specific clones; M.G.D.K. generated tetramers; I.I.N.D. contributed to research design; J.H.F.F. supervised writing; F.H.J.C. supervised research; and M.H.M.H. supervised research and writing.

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Chapter 4

Tissue specificity of cross-reactive allogeneic responses by EBV EBNA3A specific memory T-cells

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ABSTRACT

Background: The cross-reactivity of Epstein-Barr virus (EBV EBNA3A) specific CD8 T-cells against allogeneic HLA-B*44:02 has been shown to be dependent on presentation of self-peptide EEYLQAFTY by the target antigen. Here we report that allogeneic HLA-B*44:02$^+$ proximal tubular epithelial cells (PTECs) and human umbilical vein endothelial cells (HUVECs) are poor targets for EBV EBNA3A specific T cells.
Methods: The EEY peptide was exogenously loaded onto HLA-B*44:02 and HLA-B*44:03 expressing PTECs and HUVECs. EEY peptide loaded, and unloaded, PTECs and HUVECs were then incubated with serial dilutions of our EBNA3A T-cell clone, in a cytotoxicity assay.
Results: While HLA-B*44:02 expressing PTECs were specifically lysed in proportion to the effector/target ratio by the EBNA3A T-cell clone, without peptide loading, lysis was greatly increased by exogenous EEY peptide loading (15% vs. 75%; $p<0.0001$). HLA-B*44:02 expressing HUVECs were only lysed when loaded with exogenous EEY peptide (0% vs. 64%; $p<0.0001$). Lack of HLA expression and lack of ABCD3 gene expression were excluded as a cause for these results. PTECs and HUVECs were specifically targeted by another alloreactive T-cell clone without exogenous peptide loading, suggesting that the lack of recognition of HLA-B*44:02$^+$ epithelial and endothelial cells by the EBV EBNA3A T-cell clone was due to lack of EEYLQAFTY peptide presentation.
Conclusions: Tissue specific (peptide dependent) alloreactivity may have important implications for transplantation monitoring and rejection.

INTRODUCTION

Viral infection is associated with solid organ transplant rejection and is a potent barrier to transplantation tolerance (1-10). It has recently been shown that allo-HLA crossreactivity from viral specific memory T-cells is far more common than predicted; using EBV transformed B-cells (EBV LCLs) and HLA-transfected target cells (11). This allo-HLA crossreactivity from viral specific memory T-cells has been shown to be dependent on endogenous self-peptide presentation by the donor cell (11-13), and therefore alloreactivity could be tissue cell type specific if the recognized peptide is differentially expressed by target tissues.

Early work suggested that the explanation for the presence of alloreactive memory T-cells in non-sensitized individuals could be crossreactivity from viral specific memory T-cells against allo-HLA molecules (14-15). Burrows and colleagues demonstrated the dual specificity of EBV EBNA3A specific T-cell clones for the immunodominant EBV peptide FLRGRAYGL presented on HLA-B*08:01 and the alloantigen HLA-B*44:02, to which the individual had never been exposed (15). The clinical relevance of this finding is reinforced by the fact that EBV EBNA3A specific CD8 T-cells are capable of specifically lysing HLA-B*44:02$^+$ target cells in cytotoxicity assays (15-16), and that HLA-B44 is an immunogenic mismatch in HLA-B8 kidney recipients (17).

This EBV EBNA3A T-cell allo-HLA-B*44:02 crossreactivity is dependent on presentation of the EEYLQAFTY self-peptide derived from the ABCD3 gene, via molecular mimicry (12). Despite extensive polymorphism between HLA-B*08:01 and HLA-B*44:02, and the disparate sequences of their bound viral and self-peptides respectively, the HLA-B8/FLR restricted TCR engages these different peptide/HLA complexes identically.

In our laboratory, various cell lines have been generated for in-vitro testing in kidney transplantation research. Proximal tubular epithelial cells (PTECs) are derived from proximal tubule cells taken from kidney transplant biopsy specimens (18-20), and are a useful model to examine alloreactivity from graft infiltrating lymphocytes (21-22). Human umbilical vein endothelial cells (HUVECs) are derived from healthy human post-partum umbilical tissue (23-25), but are also useful as a model of kidney vascular endothelial cell transplantation (26). Here we investigate tissue specificity of the crossreactive alloresponse by EBV EBNA3A specific memory T-cells, using PTECs and HUVECs as a model system for human kidney transplantation.

We report that in contrast to other allogeneic HLA-B*44:02$^+$ cell lines, allogeneic HLA-B*44:02$^+$ PTECs are poor targets for EBV EBNA3A specific CD8 T-cells, and HLA-B*44:02 HUVECs are not targeted at all. We hypothesized that this differential lysis of HLA-B*44:02 expressing PTEC and HUVEC cell lines could be explained by differential tissue presentation of the EEYLQAFTY self-peptide. Our work with the EBV EBNA3A T-cell clone, presented here, confirms that (peptide-dependent) tissue specificity of allo-HLA responses from viral specific memory T-cells may indeed be relevant in the kidney transplantation setting. Essentially, alloreactivity can be tissue specific.

RESULTS

*HLA-B*44:02 expressing epithelial and endothelial cell lines are poor targets for EBV EBNA3A specific CD8 memory T-cells*

We and others have previously shown that EBV EBNA3A specific T-cell clones exert cytolytic activity against allogeneic HLA-B*44:02$^+$ EBV LCLs, PHA Blasts and HLA-B*44:02 transfected K562 cells (SALs) (11, 15-16). We therefore performed cytolytic assays using HLA-B*44:02$^+$ PTEC and HUVEC targets to determine if allo-HLA crossreactivity from viral specific memory T-cells, as determined by hematological target cells types, corresponds with solid organ alloreactivity (Figure 1). In contrast to other allogeneic HLA-B*44:02$^+$ cell lines, allogeneic HLA-B*44:02$^+$ PTECs are poor targets for EBV EBNA3A specific CD8 T cells (specific lysis 12%). HLA-B*44:02$^+$ expressing HUVECs are not targeted by an EBV EBNA3A specific T-cell clone (specific lysis 0%).

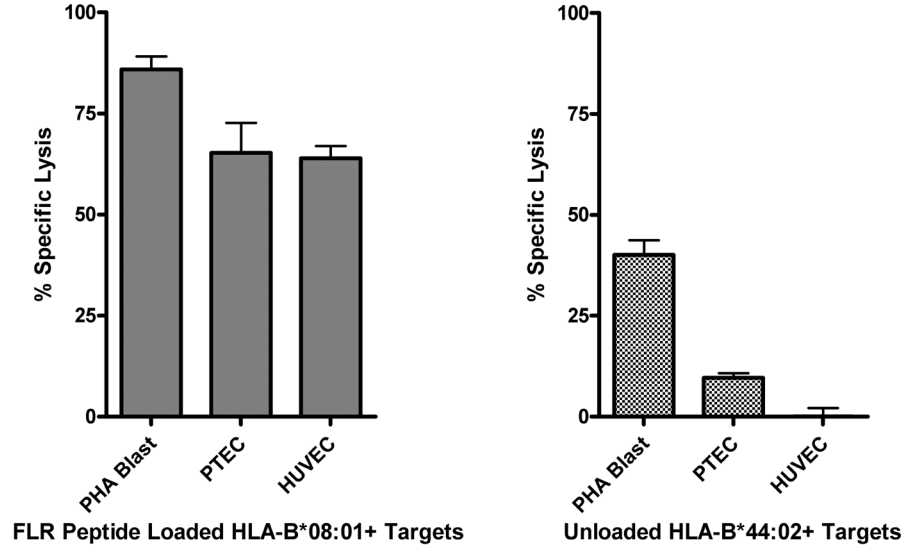

Figure 1. HLA-B*44:02 expressing PTECs and HUVECs are poor targets for EBV EBNA3A specific T-cells. In contrast to allogeneic HLA-B*44:02$^+$ PHA Blasts, allogeneic HLA-B*44:02$^+$ PTECs are poor targets for EBV EBNA3A specific CD8 T cells in a four hour cytotoxicity assay (specific lysis 12%). HLA-B*44:02$^+$ expressing HUVECs are not targeted by an EBV EBNA3A T-cell clone (specific lysis 0%). Experiments performed in triplicate, mean values shown with SD. E:T ratio 30:1, targets=5000.

*The lack of recognition of epithelial and endothelial cell lines is not due to lack of HLA-B*44:02 expression*

To exclude lack of HLA expression as the cause for these results we performed cytotoxicity assays before and after IFNγ stimulation of the PTEC and HUVEC targets. PTECs demonstrated higher baseline HLA-B*44:02 expression as compared to HUVECs (data not shown). IFNγ pre-stimulation was associated with increased HLA-B*44:02 surface expression in both PTECs and HUVECs (data not shown). Regardless, the increased HLA-B*44:02 expression following IFNγ stimulation did not result in increased lysis of the PTEC or HUVEC target cells (data not shown).

*The lack of recognition of HLA-B*44:02 epithelial and endothelial cells is not due to lack of ABCD3 gene expression*

Alloreactivity of the EBV EBNA3A T-cell clone against HLA-B*44:02^{+} cell lines is dependent on presentation of the EEYLQAFTY peptide derived from the ABCD3 gene (12). To exclude lack of ABCD3 gene expression in the epithelial and endothelial target cells we performed qPCR for ABCD3 gene specific mRNA. ABCD3 mRNA was detectable in both PTEC and HUVEC target cell types (Figure 2). Furthermore, an anti-ABCD3 monoclonal antibody demonstrated cytoplasmic presence of the protein product (data not shown).

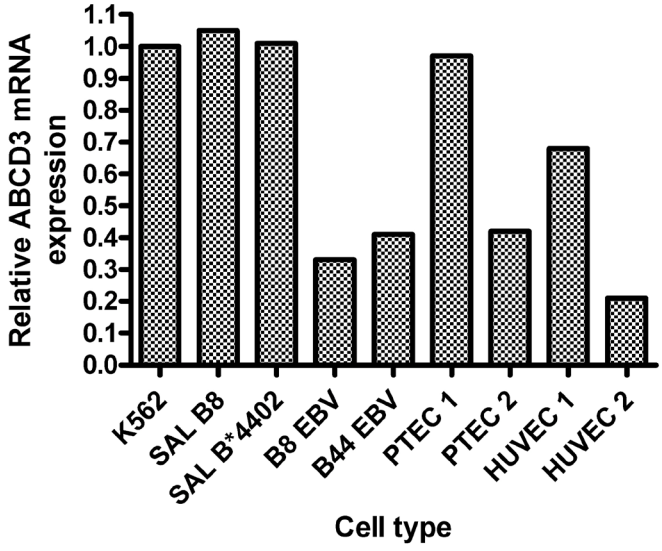

Figure 2. ABCD3 mRNA expression in PTEC and HUVEC cell lines.

ABCD3 mRNA expression, relative to household gene expression, was measured in HLA-transfected and non-transfected K562 cells, EBV LCLs, PTECs and HUVECs. ABCD3 mRNA expression in PTECs and HUVECs was comparable to EBV LCLs. Indicating that the lack of allorecognition of PTECs and HUVECs by the EBV EBNA3A T-cell clone was not due to absence of expression of the ABCD3 protein, from which the EEYLQAFTY self-peptide is derived. SAL= Single HLA transfected K562 cell, EBV=Epstein-Barr virus transformed B-cell.

*The lack of recognition of HLA-B*44:02 expressing epithelial and endothelial cells is likely due to quantitative lack of EEYLQAFTY peptide presentation on the cell surface*

To confirm that the lack of allorecognition of the HLA-B*44:02$^+$ epithelial and endothelial cell lines was due to lack of peptide presentation we performed cytolytic assays using EEYLQAFTY peptide loaded and unloaded PTEC and HUVEC targets. HLA-B*44:02 expressing PTECs were poorly targeted by an EBV EBNA3A T-cell clone without peptide loading (specific lysis 15%; P=0.004 vs. HLA-B*44:03 PTEC), and then only at high effector/target ratio (Figure 3a). The specific lysis of HLA-B*44:02 expressing PTECs was greatly increased by exogenous EEY peptide loading (15% vs. 75%; P<0.0001) (Figure 3a). HLA-B*44:02 expressing HUVECs were only targeted by an EBV EBNA3A specific clone when loaded with exogenous EEY peptide (0% vs. 64%; P<0.0001) (Figure 3b). Furthermore, stimulation of the EBV EBNA3A specific T-cell clone with HLA-B*44:02$^+$ HUVECs did not elicit cytokine production in a 24 hour luminex assay (data not shown). The EBV EBNA3A specific T-cell clone was able to target EEY peptide loaded HLA-B*44:02 PTECs and HUVECs even without IFNγ pre-stimulation, suggesting that the baseline HLA-B*44:02 expression in these cell lines is sufficient for CTL targeting if sufficient allopeptide is presented. HLA-B*44:03 PTECs and HUVECs were not targeted irrespective of peptide loading, as predicted (12). Thus organ (kidney) specificity of the allo-HLA crossreactivity from the EBV EBNA3A specific T-cell is dependent on endogenous self-peptide (EEY) processing and presentation.

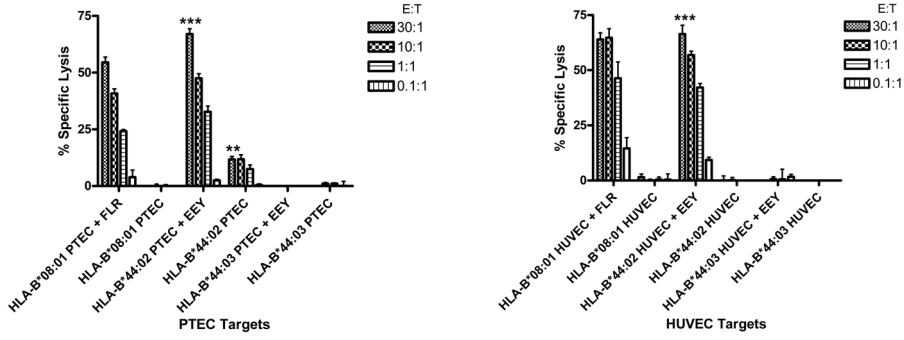

Figure 3. The lack of recognition of HLA-B*44:02$^+$ PTECs and HUVECs is likely due to lack of EEYLQAFTY peptide presentation on the cell surface. (a) HLA-B*44:02$^+$ PTECs were poorly targeted by the EBV EBNA3A specific T-cell clone (specific lysis 15%; **P=0.004 vs. HLA-B*44:03 PTEC). The specific lysis of HLA-B*44:02$^+$ PTECs was greatly increased by exogenous EEY peptide loading (specific lysis 15% vs. 75%; ***P<0.0001). (b) HLA-B*44:02$^+$ HUVECs were only targeted by an EBV EBNA3A specific T-cell clone when loaded with exogenous EEY peptide (specific lysis 0% vs. 64%; ***P<0.0001). The EBV EBNA3A T-cell clone did not recognize HLA-B*44:03$^+$ PTECs or HUVECs irrespective of peptide loading. Experiments shown here were performed without IFNγ pre-stimulation further demonstrating that the baseline HLA-B*44:02 expression is sufficient to elicit cytotoxicity if the EEY peptide is present. Experiments performed in triplicate, mean values shown with SD. Targets=5000.

Cognate antigen recognition and allorecognition increase in proportion to the concentration of exogenously added viral or allo-peptide

To determine the concentration of specific peptide required to elicit cytolytic effector function by the EBV EBNA3A specific T-cells, FLR or EEY peptide were loaded onto HLA-B*08:01$^+$ or HLA-B*44:02$^+$ target cells respectively, in a peptide dose-response experiment (Figure 4). Cognate viral antigen recognition and allorecognition increase in proportion to the concentration of exogenously added cognate or allo-peptide (Figure 4). Equivalent concentrations of the FLR cognate peptide on HLA-B*08:01$^+$ target cells and EEY allopeptide on HLA-B*44:02$^+$ target cells was required to elicit cytolytic effector function by the EBV EBNA3A specific T-cells. For both cognate and allo-peptides 50% of the maximum specific lysis occurred between 10μg/ml and 50μg/ml of exogenously added peptide.

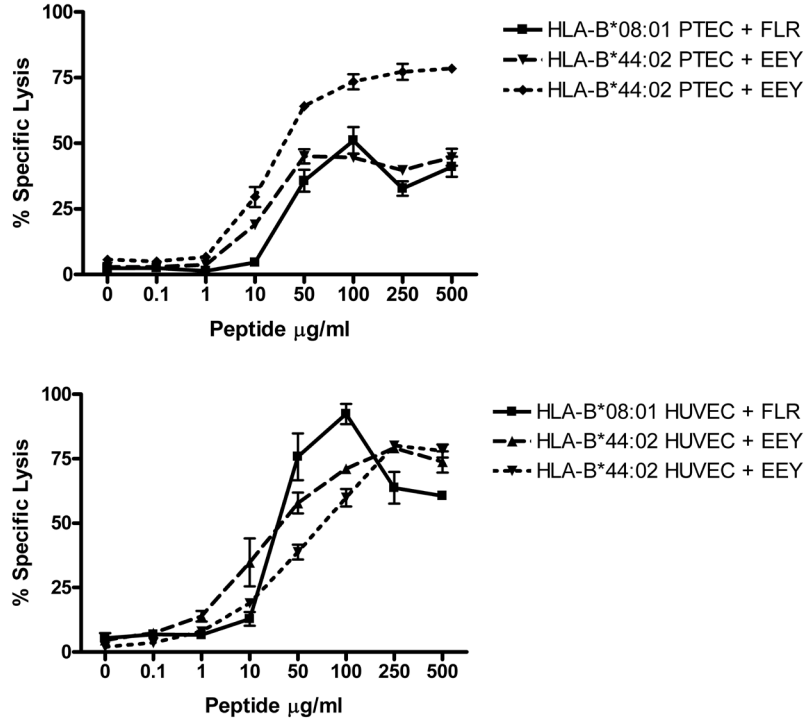

Figure 4. Cognate antigen recognition and allorecognition increase in proportion to the concentration of exogenously added viral or allo-peptide. To determine the concentration of specific peptide required to elicit cytolytic effector function by the EBV EBNA3A specific T-cells, FLR or EEY peptide was loaded onto HLA-B*08:01$^+$ or HLA-B*44:02$^+$ target cells respectively, in a peptide dose-response experiment. Cytolytic effector function of the EBV EBNA3A specific T-cells increases in proportion to exogenously added peptide concentration for both the cognate viral peptide and the allopeptide. Equivalent concentrations of the FLR cognate peptide on HLA-B*08:01$^+$ target cells and EEY allopeptide on HLA-B*44:02$^+$ target cells is required to elicit cytolytic effector function by the EBV EBNA3A specific T-cells. Assays performed with a HLA-B*08:01$^+$ and two different HLA-B*44:02$^+$ PTEC and HUVEC target cells. Experiments performed in triplicate, mean values shown with SD. Effector:target ratio 20:1, targets=5000.

Epithelial and endothelial cells are not resistant to lysis by CTL clones

Finally, to exclude the possibility that the EEYLQAFTY peptide is presented on the target HLA molecule but the cells are not targeted due to lytic resistance or CTL suppression we performed cytolytic assays using the EBV EBNA3A clone and a HLA-A2 alloreactive T-cell clone (JS132) in parallel. The JS132 clone specifically lysed HLA-A2^{+} B*44:02^{+} heterozygote PTECs and HUVECs, without any exogenous peptide addition (Figure 5). The EBV EBNA3A CD8 T-cell clone was unable to efficiently target the identical epithelial or endothelial cell lines without exogenous addition of EEYLQAFTY peptide (Figure 5). Thus, the epithelial and endothelial cells can be suitable targets for CTL clones without addition of exogenous peptide.

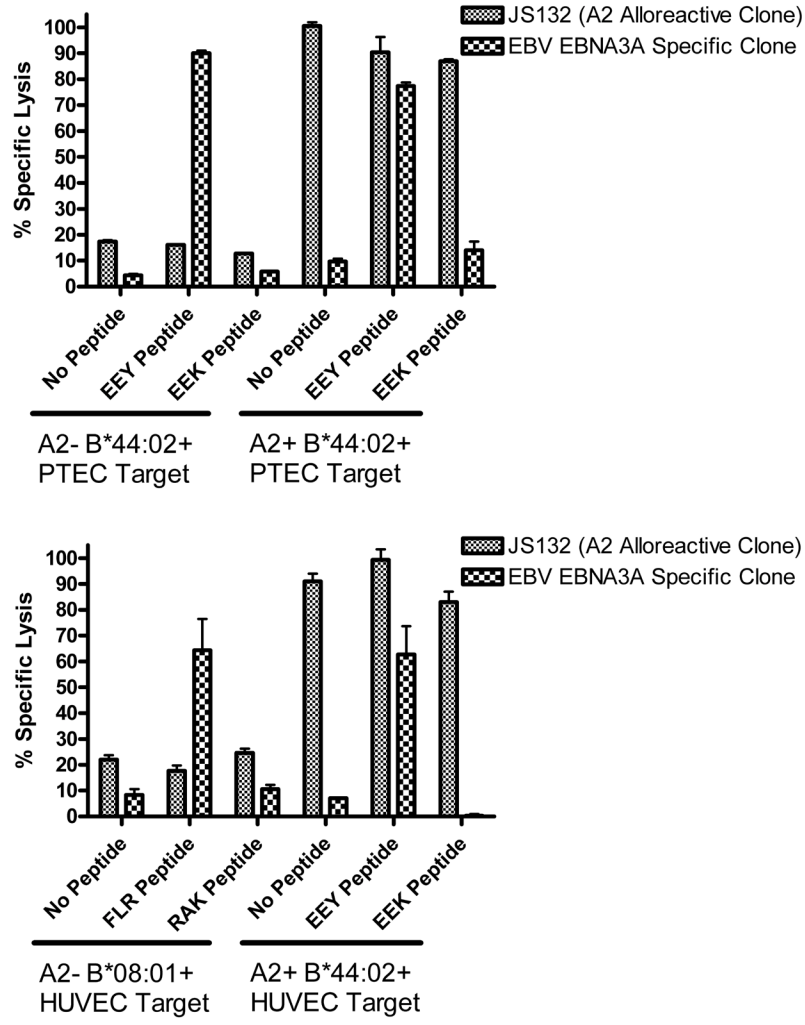

Figure 5.

Figure 5. PTECs and HUVECs are suitable targets for CTL mediated killing without exogenous peptide addition. The JS132 HLA-A2 alloreactive T-cell clone specifically lysed HLA-A*02$^+$ B*44:02$^+$ heterozygous PTECs and HUVECs irrespective of exogenous peptide loading (specific lysis >85%). The EBV EBNA3A CD8 T-cell clone was unable to efficiently target the identical epithelial or endothelial cell lines without exogenous addition of EEYLQAFTY peptide. Furthermore, the EBV EBNA3A clone lysed both HLA-B*08:01$^+$ and HLA-B*44:02$^+$ epithelial and endothelial cell lines when loaded with FLR peptide or EEY peptide respectively, but not RAK (HLA-B*08:01 control) nor EEK (HLA-B*44:02 control) peptides; confirming that the viral specificity and alloreactivity are peptide dependent and mediated by the same T-cell. Thus, it is highly likely that the lack of recognition of HLA-B*44:02$^+$ epithelial and endothelial cells is due to a quantitative lack of EEY peptide presentation. Experiments performed in triplicate, mean values shown with SD. Effector:target ratio 30:1, targets=5000.

DISCUSSION

In this report we demonstrate that allo-HLA crossreactivity by viral specific memory T-cells can be tissue cell-type specific because of differential tissue specific self-peptide presentation. We have confirmed that not only is the HLA-B*44:02 alloreactivity from the EBV EBNA3A specific T-cell clone self-peptide dependent but that normal allogeneic kidney cells may not be targeted unless sufficient EEY self-peptide is processed and presented. Alloreactivity is mediated by cytotoxicity, when the peptide is presented, indicating the potential clinical relevance of cross-reactive alloresponses against cell types present in kidney transplant tissue.

Our results do not suggest that allo-HLA crossreactivity from the EBV EBNA3A T-cell is irrelevant to kidney transplantation. The EBV EBNA3A specific T-cell does have cytolytic activity against HLA-B*44:02$^+$ kidney epithelial cells in a 4 hour assay. Memory T-cells persist and therefore could perform effector functions over a prolonged period, or at times when immunosuppression is tapered (2). Furthermore T-cells mediate effector functions through a variety of mechanisms, including cytokine production, not just cytotoxicity (27). The EBV EBNA3A specific immune response is a public TCR response present in all HLA-B8$^+$ B44$^-$ individuals (28) and HLA-B44 mismatching has been identified as high risk in HLA-B8 kidney recipients (17).

However results presented here suggest it is unlikely that EBV EBNA3A specific T-cells exhibit effector functions against HLA-B*44:02$^+$ endothelial cells present in solid organ tissue. Conversely, a viral specific T-cell that targets a kidney cell specific peptide presented on an allogeneic HLA molecule may not recognize PBMCs or spleen cells from the same allogeneic donor.

In light of our findings it is worth considering some of the possible mechanisms by which organ specific alloreactivity could occur. Quantitative differences in HLA expression could explain organ specific alloreactivity, but has been excluded in the present study. Differences in co-stimulation and accessory molecule expression are also feasible but there is little evidence for this as memory CD8 T-cells have reduced requirements for co-stimulation and do not require CD4 T-cell help (29-30). Furthermore, the EBV EBNA3A clone used here is clearly

capable of targeting HLA-B*44:02 transfected K562 cells which have absent co-stimulatory molecules (16).

Tissue specific expression of a protein that is the source of the self-peptide recognized on the allo-HLA molecule would be extremely likely to result in organ specific alloreactivity. For example, a peptide derived from a renal specific ion transporter will only be presented on renal tubular cells. Furthermore, alloreactivity might only be induced when the gene expression is up regulated.

Results presented here are of particular interest because we have demonstrated expression of the ABCD3 protein product in the target epithelial and endothelial cells. The HLA-B*44:02$^+$ PTECs were targeted albeit to a lower level of lysis (15%), therefore there must be naturally some EEY peptide presented on the cell surface but not enough to trigger a high percentage of specific lysis. The HLA-B*44:02$^+$ HUVECs are not targeted and therefore it is likely that insufficient EEY peptide is presented on the surface.

Differences in antigen processing and presentation could account for tissue specific alloreactivity, even if similar levels of the ABCD3 gene product are expressed within the epithelial and endothelial cells. For example, EBV LCLs, PHA Blasts and K562 cells constitutively express the immunoproteosome which may generate novel antigenic peptides. Furthermore, the study of Macdonald defines the EEYLQAFTY peptide as an antigenic target of the EBV EBNA3A T-cell presented via allogeneic HLA-B*44:02 (12), however this study does not exclude the possibility that several different peptides presented on HLA-B*44:02 are capable of activating the EBV EBNA3A specific T-cell. Theoretically, these additional peptides may not be presented by epithelial or endothelial cell types.

Alternatively, differences in expression of a protein that contains a peptide capable of competing with an antigenic peptide for the peptide-binding groove of the allogeneic molecule could also cause organ specific alloreactivity, as also suggested by others (31). A tissue specific competing peptide may reduce the amount of the target self-peptide/allo-HLA complex available for recognition by the alloreactive CTL.

HLA-B*44:02 is a highly tapasin-dependent HLA molecule (32-33) and therefore limited tapasin expression in PTECs and/or HUVECs could decrease EEY peptide presentation in these cell lines. However tapasin mRNA is strongly induced in endothelial cells following IFNγ treatment (34), and IFNγ treatment did not increase the targeting of HUVECs in our assays despite inducing elevated HLA-B44 expression. The HLA-B*44:05 molecule is also a target of the EBV EBNA3A T-cell (12) and can load peptides independently of tapasin, unfortunately no HLA-B*44:05 expressing PTECs or HUVECs are available.

Our assays using the JS132 clone exclude the possibility that the EEY peptide is presented but that epithelial and/or endothelial cells are resistant to lysis or are tolerogenic. The HLA-A2 alloreactive JS132 clone was generated by stimulating PBMCs with HLA-A2 mismatched irradiated EBV LCLs in-vitro. The JS132 allo-A2 reactivity is likely peptide dependent and therefore we conclude that the antigenic peptide recognized in the context of HLA-A2 is con-

stitutively presented by the epithelial and endothelial cell lines.

The ultimate proof that our results are attributable to lower/absent EEYLQAFTY peptide presentation could be provided by peptide elution studies. However elution of peptides from HLA-B*44:02^{+} PTECs and HUVECs is not feasible due to the large number of cells required for peptide elution and mass spectrometry analysis. Nonetheless, we favour the conclusion that the differential allorecognition of HLA-B*44:02^{+} PTECs and HUVECs by the EBV EBNA3A specific T-cell clone is the result of differential quantitative presentation of the EEYLQAFTY self-peptide by the target cells.

The finding of organ specific allorecognition is extensively described in mice (31, 35-38). For example, priming of mice with normal allogeneic spleen cells generated peptide-dependent Kb-specific alloreactive CTL clones that exhibited cell-type specific allorecognition (31). Human tissue specific alloreactivity has been suggested by studies using graft-infiltrating lymphocytes obtained from renal allografts undergoing rejection (21, 39-43). Graft infiltrating lymphocytes were shown to exhibit T cell functional activity against PTEC grown from the corresponding biopsy, but not donor derived splenocytes nor PTEC from biopsies obtained from other patients. For example, van der Woude and colleagues found that thirteen out of forty (33%) of graft infiltrating cell lines reacted in a donor-specific fashion to PTEC but not to donor splenocytes (41).

Results presented here may have important clinical implications for renal transplantation monitoring, rejection and tolerance. Monitoring of alloreactive T-cells may allow individualization of immunosuppression (44), but such assays routinely use donor PBMCS or spleen cells as stimulator. Allo-HLA crossreactivity by viral specific memory T-cells as defined against hematological target cell types will not correspond with solid organ alloreactivity unless the targeted self-peptide is ubiquitously and equally presented. If alloreactive CTL recognize allo-HLA presenting a specific peptide then it is possible that competitive peptides could be designed to inhibit allorecognition, as has also been suggested by others (31, 45). We have confirmed that the absence of a single tissue specific self-peptide is enough to abrogate alloreactivity. Also, long term immunosuppressive free graft survival is the ultimate aim of much transplantation research, but our work suggests induction of tolerance by using pre-transplant blood transfusion may not delete organ specific CTLs.

Finally, we acknowledge that this study uses umbilical vein endothelial cells as a model for kidney vascular endothelial cell transplantation, however, gene expression and/or functional differences have not been reported between kidney and umbilical endothelial cells. Others have also found that donor-derived gonadal vein endothelial cells can be specifically targeted by graft infiltrating alloreactive T-cells (39).

In conclusion, we show that the EBV EBNA3A T-cell exhibits tissue cell type specific alloreactivity because of quantitative differences in presentation of the recognized self-peptide. Tissue specific allorecognition may have important clinical consequences, especially for monitoring, rejection and tolerance induction of solid organ grafts. Future work should determine if tissue specific allorecognition is a common characteristic of human alloreactive CTL.

MATERIALS AND METHODS

Generation of EBV EBNA3A viral specific CD8 memory T-cell clone
The generation and allo-HLA-B*44:02 crossreactivity of the EBV EBNA3A CD8 memory T-cell clone used here has been previously described (16). Briefly, EBV EBNA3A specific CD8 T-cell clones (HLA-B8/FLRGRAYGL restricted) were derived from a healthy donor with HLA typing HLA-A*01:01,02:01; B*08:01,-; DRB1*03:01,-; using single cell sorting based on viral peptide/tetramer complex staining. Clonality of the T-cell clone was confirmed using RT-PCR to determine TCR AV and BV usage (16).

Generation of JS132 clone
The generation and the allo-HLA-A2 alloreactivity of the JS132 CD8 T-cell clone have been previously described (46-47). Briefly, PBMCs from healthy donor JS (HLA-A3,3; B7,7; DR2,2; DQ1,1) were stimulated with irradiated EBV transformed B-cell line JY (HLA-A2,2; B7,7; DR4,6). Following several rounds of stimulation and enrichment the HLA-A2 alloreactive population was cloned by limiting dilution at 0.5 cell/well.

Generation and culture of PTECs and HUVECs
Generation of PTECs (18-19) and HUVECs (23-24) has been previously described. PTECs were cultured from cortical tissue of human kidneys not suitable for transplantation because of anatomical reasons or from pretransplant biopsies, and HUVECs from umbilical vein of human umbilical cord. Morphologic appearance and immunofluorescence staining confirmed specific outgrowth of PTECs and HUVECs.

HLA typing and FACS staining for HLA expression of epithelial and endothelial cells
Molecular typing for class I and class II was performed in the tissue typing laboratory Leiden University Medical Centre, the Netherlands. The relative amount of HLA surface expression was determined using human monoclonal antibodies specific for the HLA molecule expressed. Epithelial and endothelial cell lines were treated with trypsin, harvested and then washed two times. Cells were incubated with the human HLA specific monoclonal antibody for 30 minutes and then washed twice. Cells were then labeled with a rabbit-anti-human-FITC secondary detection antibody for a further 30 minutes and then washed three times. HLA expression was determined before and after IFNγ treatment, 500 units/ml for 24 hours.

ABCD3 gene expression in PTEC and HUVEC cell lines
For detection of ABCD3 mRNA expression cells were harvested and preserved in RNAlater solution (Qiagen, Chatsworth, CA, USA). RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturers instructions. RNA was treated with DNase (Qiagen) on the spin columns. RNA quantity was assessed with a spectrophotometer (Nanodrop technologies, Wilmington, DE, USA) and all samples showed A260/A280 ratios between 1.9 and 2.1. Quantitative PCR was performed as per standard protocols. The forward and reverse primer

sequences used in the quantitative PCR for ABCD3 mRNA were CCTGGTGCTGGAGAAATCAT and CCCCAGATCGAACTTCAAAA respectively, giving an amplicon of 118 bp. The PCR was performed using an iCycler MyiQ (Bio-Rad). The PCR program was finalized with a melting curve analysis. The signal of the stably expressed household genes β-actin and GAPDH served as normalization factors.

Cytotoxicity Assays

The EBV EBNA-3A specific T-cell clone and/or the JS132 CD8 T-cell clone were evaluated for cytotoxicity by incubating 5000 PTEC or HUVEC target cells with serial dilutions of the T-cell clone(s) for 4 hours in ^{51}Cr release assays. HLA-B*44:02^{+} target cells were loaded with either the EEYLQAFTY allopeptide or EEKLIVVLF control peptide, or no peptide. HLA-B*08:01^{+} target cells were loaded with either FLRGRAYGL cognate peptide or RAKFKQLL control peptide, or no peptide. In the peptide dose-response assays HLA-B*08:01^{+} target cells or HLA-B*44:02^{+} target cells were incubated with different concentrations of the FLRGRAYGL cognate peptide or the EEYLQAFTY allopeptide respectively, for one hour and then washed twice. The peptide-dose response assays were performed with an effector:target ratio of 20:1 only. Target cells were incubated with chromium for 60 minutes. Supernatants were harvested for gamma counting: *percent specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%*. Results are expressed as the mean of triplicate samples, with standard deviation.

Statistics

Values for specific lysis are presented as the mean of triplicate wells, with standard deviation. Comparative analyses are non-parametric (unpaired) t-tests, $p<0.05$ is considered to be significant. Statistics are derived using Graph Pad Prism 4 for Windows (Version 4.02, 2004).

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Chapter 5

Vaccine induced allo-HLA reactive memory T-cells in a kidney transplantation candidate

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Submitted for publication

ABSTRACT

Background: Allo-HLA reactivity by naturally acquired viral specific memory T-cells is common. However, the effect of successful vaccination on the alloreactive memory T-cell repertoire is unclear. We hypothesized that vaccination could specifically induce allo-HLA reactive memory T-cells.
Methods: A varicella zoster virus (VZV) IE62 specific CD8 memory T-cell clone was single cell sorted from a VZV seronegative renal transplant candidate, following response to live attenuated varicella vaccination. To analyze the allo-HLA reactivity, the VZV IE62 specific T-cell clone was tested against HLA typed target cells and target cells transfected with HLA molecules, in both cytokine production and cytotoxicity assays.
Results: The varicella vaccine induced VZV IE62 specific T-cell clone specifically produced IFNγ when stimulated with HLA-B*55:01 expressing Epstein-Barr virus (EBV) transformed B-cells and HLA-B*55:01 transfected K562 cells (SALs) only. The clone also demonstrated specific cytolytic effector function against HLA-B*55:01 SALs and PHA Blasts. Cytotoxicity assays using proximal tubular epithelial cell (PTEC) and human umbilical cord endothelial cell (HUVEC) targets confirmed the kidney tissue specificity of the allo-HLA-B*55:01 reactivity, and the relevance of the crossreactivity to clinical kidney transplantation. The results also suggest that molecular mimicry, and not bystander proliferation, is the mechanism underlying vaccine induced alloreactivity.
Conclusions: Varicella vaccination generated a *de novo* alloreactive kidney cell specific cytolytic effector memory T-cell in a patient awaiting renal transplantation. Vaccination induced alloreactivity may have important clinical implications, especially for vaccine timing and recipient monitoring.

INTRODUCTION

Allo-HLA reactivity by naturally acquired viral specific memory T-cells is far more common than anticipated, and the allo-HLA reactivity and virus specificity are mediated via the same T-cell receptor (TCR) (1). 45% of virus specific CD4 and CD8 memory T-cell clones have been shown to be cross-reactive against allo-HLA molecules. Allo-HLA crossreactivity has been shown for Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Influenza virus and Varicella-Zoster Virus (VZV) specific T-cells (1). Alloreactive memory T-cells are a major barrier to successful transplantation because they resist immunosuppression and can rapidly engage effector functions (2-10).

Vaccine preventable infections remain a major source of morbidity and mortality in transplant recipients (11-12). Guidelines for recommended vaccinations to be given before and after transplantation are provided by many centres (11) and whenever possible the full complement of vaccines should be administered prior to transplantation (12). Adaptive immunity and the development of pathogen specific memory T-cells underlie successful vaccination. However the effect of vaccination on the allo-HLA reactive T-cell pool is largely unknown.

Danziger-Isakov and colleagues recently demonstrated that influenza vaccination can have a significant impact on the potency of the alloreactive T-cell repertoire, as determined by IFNγ production in a mixed lymphocyte culture (13). However this study did not determine the origin of the alloreactive memory T-cells or if the alloreactivity was elicited by non-specific reactivation, bystander proliferation or molecular mimicry.

VZV seronegative transplant recipients who contract varicella may suffer lethal consequences (14). Therefore, guidelines for vaccination of solid organ transplant candidates recommend that live attenuated varicella-zoster virus (VZV) vaccination be administered prior to transplantation, amongst others (11). Consequently at the Erasmus Medical Centre all kidney transplantation candidates with negative varicella serology are given the recommended live attenuated varicella vaccine (15). We hypothesized that this varicella vaccination could specifically induce allo-HLA reactive memory T-cells in patients awaiting renal transplantation.

We therefore sorted a VZV specific T-cell clone from a seronegative renal transplant candidate with a demonstrable VZV specific T-cell response following varicella vaccination, using VZV specific peptide/tetramer complex staining. Successful vaccination generated *de novo* HLA-specific alloreactive memory T-cells.

RESULTS

Confirmation of monoclonality and TCR repertoire analyses of the vaccine induced VZV IE62 specific CD8 T-cell clone

Post-vaccine VZV seroconversion was confirmed (Figure 1a). VZV IE62 specific T-cells were not detectable in the blood prior to vaccination. HLA-A2/ALW tetramer positive CD8 T-cells were detectable in the blood only following live attenuated varicella vaccination (Figure 1b), and were single cell sorted based on tetramer staining. VZV IE62 specific memory T-cell clones were confirmed to bind viral peptide/HLA-A2 tetramer complexes (Figure 1c). RT-PCR and sequencing were performed to confirm monoclonality and determine the TCR usage of the sorted VZV IE62 specific CD8 memory T-cell clones. All clones isolated expressed an identical Va2s1 Vb14s1 TCR, and therefore only a single clone for testing was generated. Sequence analysis of the TCR CDR3 region is shown in table 1.

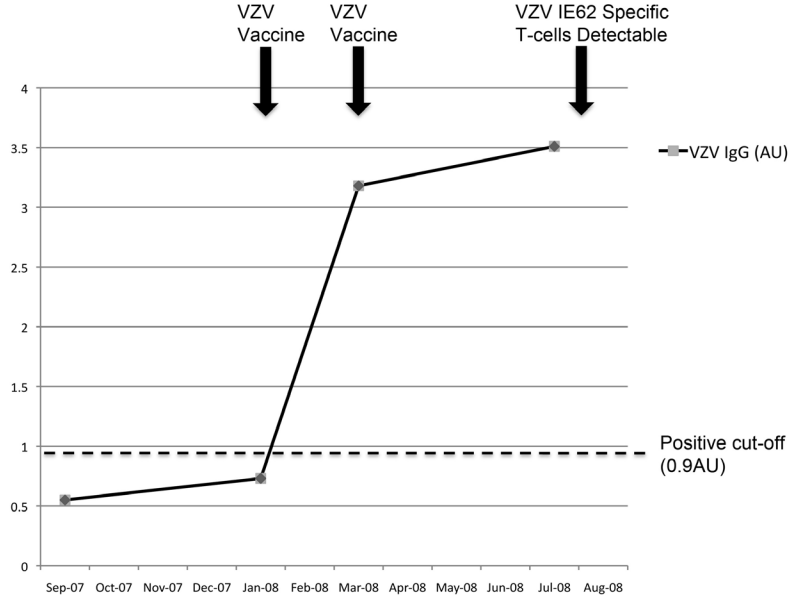

Figure 1a.

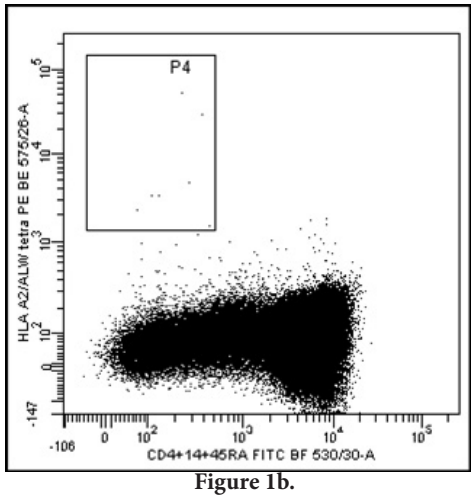

Figure 1b.

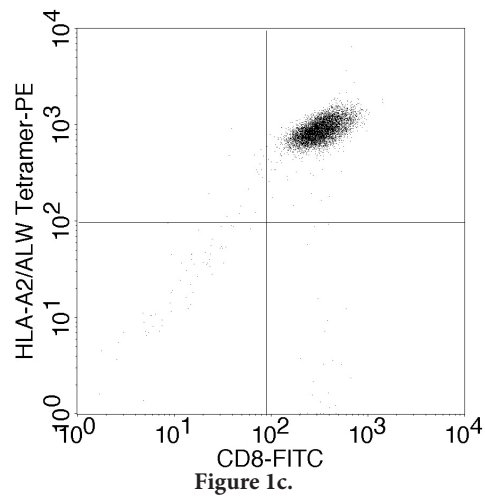

Figure 1c.

Figure 1. Varicella vaccine induced VZV IE62 specific CD8 memory T-cells.
Generation of the T-cell clone was performed by single cell sorting based on HLA-A*0201/ALWALPHAA specific tetramer staining. (a) A VZV seronegative renal transplantation candidate was given varicella vaccination, with seroconversion confirmed after 6 weeks. A booster vaccination was then given. Vaccine was administered immediately after collection of serum samples. Following vaccination VZV IE62 specific T-cells became detectable in the peripheral blood, and were then single cell sorted based on HLA-A2/ALW tetramer staining. VZV IE62 specific T-cells were not detectable in the blood prior to vaccination. AU=Arbitrary Units. Seropositivity cut-off value=0.9AU (b): HLA-A2/ALW specific T-cells were sorted 5 months following varicella vaccination of a VZV seronegative transplantation candidate. P4=Sort gate. Total events=200000. Sorted events=16. (c) T-cell clone is >99% HLA-A2/ALW tetramer binding and clonality was confirmed with TCR PCR. All sorted clones possessed an identical Va2s1 Vb14s1 TCR. HLA typing of renal transplantation candidate from whom VZV IE62 specific clone was sorted - HLA-A*02:01,-; B*13,40; Cw3,Cw6; DRB1*07,-.

Allo-HLA crossreactivity of the vaccine induced VZV IE62 specific T-cell clone
To screen for the ability of the varicella vaccine induced T-cell clone to exert alloreactivity, the clone was tested against a panel of EBV-LCLs selected to cover almost all frequently occurring HLA class I and II molecules. The T-cell clone produced IFNγ when stimulated with EBV-LCLs 5 and 19 only (Figure 2). These two EBV LCLs shared expression of the HLA-B*55:01 molecule, which was not expressed on any other tested EBV LCL. The T-cell clone recognized HLA-A*02:01 expressing EBV LCLs only when exogenously loaded with the ALW peptide (positive control), and did not produce IFNγ when cultured in IL-2 containing medium alone (negative control). Therefore the screening results were highly suggestive that varicella vaccination had induced a de novo HLA-B*55:01 alloreactive memory T-cell.

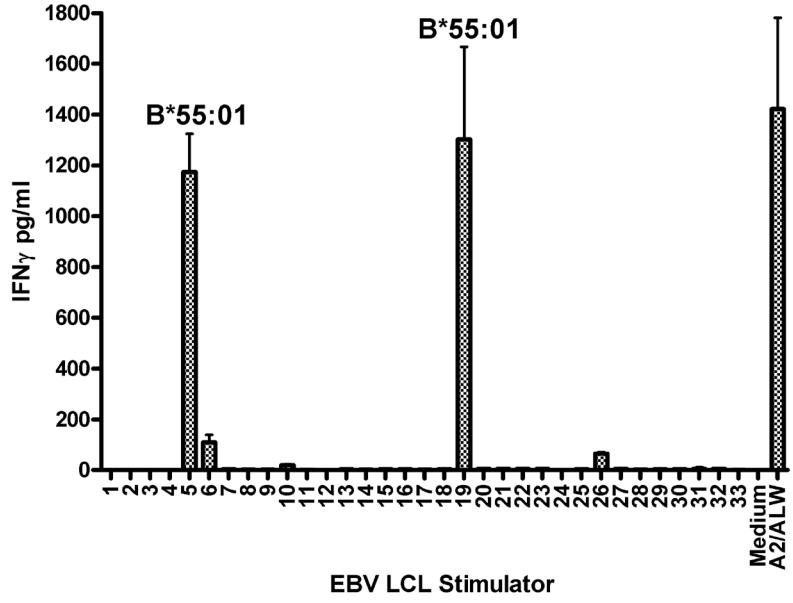

Figure 2. Allo-HLA-B*55:01 crossreactivity by the varicella vaccine induced VZV IE62 specific T-cell clone. The VZV IE62 specific T-cell clone was stimulated with a panel of EBV LCLs, selected to cover all common HLA class I and II molecules, in a 24 hour IFNγ ELISA. The T-cell clone produced IFNγ when stimulated with EBV-LCLs 5 and 19 only. These two EBV LCLs shared expression of the HLA-B*55:01 molecule, which was not expressed on any other tested EBV LCL. The T-cell clone recognized a HLA-A*02:01 EBV LCL only when loaded with the ALW peptide (Positive control– A2/ALW). Responder=10000 cells, Stimulator=50000 cells. Experiments performed in duplicate, mean values shown with SD. The screening results strongly suggest that varicella vaccination had induced a de novo HLA-B*55:01 alloreactive memory T-cell.

Table 1. Sequence analysis of the VZV TCR CDR3 region

	AV2S1	N	Ja40	BV14S1	NDN	Jb2.7
VZV	C A V	F	T S G T Y K Y I F	C A S S S	D R G	Y E Q Y F
	tgt gcc gtg	tt	t acc tca gga acc tac aaa tac atc ttt	tgt gcc agc agt t	cc gac cgg ggt	tac gag cag tac ttc

TCR sequences were analysed using IMGT/V-QUEST version 3.2.16 (Brochet et al. Nucl. Acids Res. (2008) 36 (suppl 2): w503-508). The junction analysis of the TCRα -and TCRβ chains are shown left to right. The one-letter amino acid code is shown above the first nucleotide of the codon. Variable gene segments are depicted according to Arden nomenclature (Arden et al. Immunogenet 1995).

*Confirmation of allo-HLA-B*55:01 crossreactivity by the vaccine induced VZV IE62 specific T-cell clone*

To confirm the allo-HLA crossreactivity of the VZV IE62 specific T-cell clone against the allogeneic HLA-B*55:01 molecule, the T-cell clone was tested for IFNγ production using HLA transfected K562 cells (SALs) as stimulators. Strong IFNγ production was only elicited by the HLA-B*55:01 transfected SAL (Figure 3) (***p<0.0001). IFNγ production was also elicited by HLA-A*02:01 SAL loaded with ALW peptide (positive control). No IFNγ production was elicited by culture with medium alone, non transfected K562 cells or HLA-A*0201 SALs (negative controls).

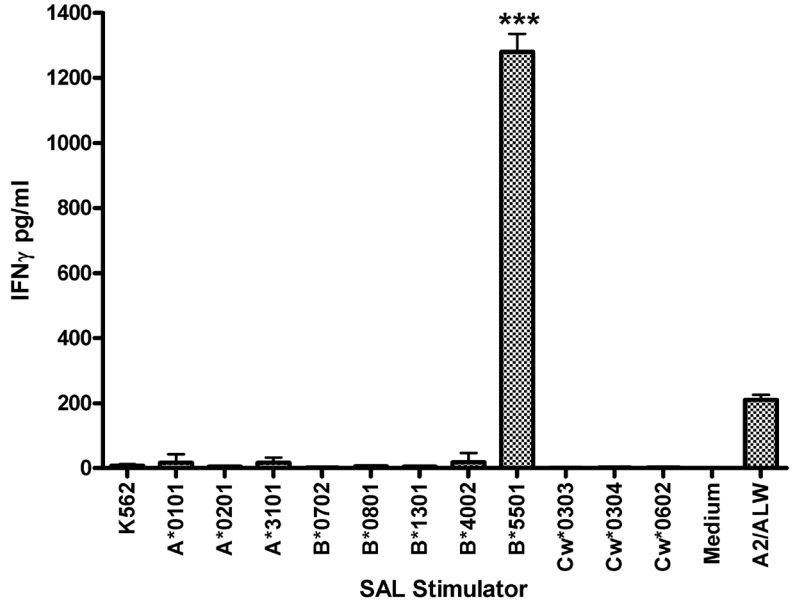

Figure 3. Confirmation of allo-HLA-B*55:01 reactivity by the varicella vaccine induced VZV IE62 specific T-cell clone. The VZV IE62 specific T-cell clone recognized only K562 cells transfected with allogeneic HLA-B*55:01, in a 24 hour IFNγ ELISA (***p<0.0001; comparison to K562 cell). SAL A2 was recognized only when exogenously loaded with ALW peptide (Positive control – A2/ALW). Non-transfected K562 cells, SAL A*02:01 (HLA restriction of clone), HLA matched and allo-HLA transfected SALs (other than HLA-B*55:01) were not recognized. Responder=10000 cells, Stimulator=50000 cells. Experiments performed in duplicate, mean values shown with SD.

Cytotoxicity of the vaccine induced VZV IE62 specific T-cell clone

To confirm that the VZV IE62 specific CD8 T-cell clone was also cytolytic against allogeneic cells expressing the HLA-B*55:01 molecule, we performed a cytotoxicity assay using SALs and PHA blasts as target cells (Figures 4a and 4b). The VZV IE62 specific T-cell clone specifically lysed HLA-B*55:01 expressing SALs and PHA blasts in proportion to the E/T ratio (Figure 4a and 4b) (**p=0.0007 and **p=0.0002 respectively), whereas HLA-A*02:01 expressing PHA blasts and SALs were not lysed unless exogenously loaded with the ALW peptide. Cytotoxicity was low, as compared to the HLA-A2$^+$ viral peptide loaded positive controls after 4 hours, but increased after overnight cytotoxicity assay (Figure 4b) (***p<0.0001).

Tissue Specificity of the vaccine induced VZV IE62 specific T-cell clone

To investigate tissue (kidney) specificity of the crossreactive alloresponse by the VZV IE62 specific memory T-cells, we used PTECs and HUVECs as a model system for human kidney transplantation. The VZV IE62 specific T-cell clone demonstrated specific cytolytic effector function against a HLA-B*55:01 expressing PTEC, in a 4 hour cytotoxicity assay (Figure 5a). This confirms that the vaccine induced memory T-cells in this kidney transplantation candidate can recognize normal kidney tissue cell types present in a transplanted kidney. However a HLA-B*55:01 expressing HUVEC was not targeted by the VZV IE62 specific T-cell clone, in a 4 hour cytotoxicity assay, even with IFNγ pre-stimulation to increase the amount of HLA-B*55:01 expression (Figure 5b).

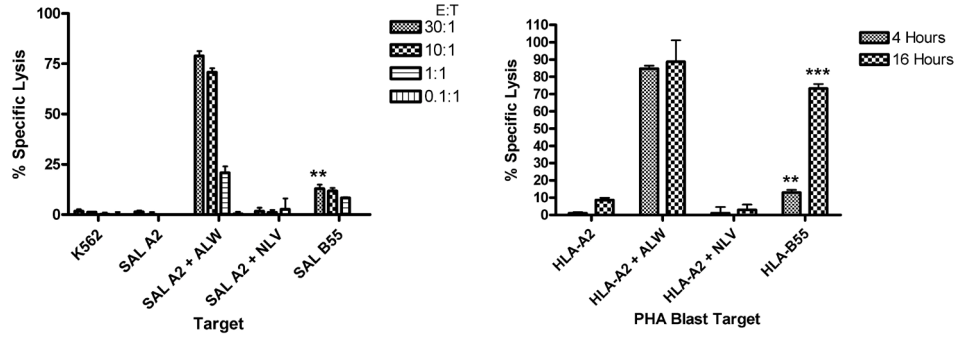

Figure 4. Varicella vaccine induced VZV IE62 specific memory T-cell clone is cytolytic against allogeneic HLA-B*55:01 expressing target cells. (A) Cytotoxicity assay using effector T-cell clone demonstrates cytotoxic effector function against HLA-B*55:01 SAL (**p=0.0007; comparison to K562 cell ratio 30:1), in proportion to effector:target ratio. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD. (B) VZV IE62 allogeneic cell cytotoxicity capacity increases with time. Cytotoxicity assay using effector T-cell clone demonstrates cytotoxic effector function against HLA-B*55:01 expressing PHA blasts (** p=0.0002; comparison to HLA-A2 PHA blast at 4 hours), in a 4 hour cytotoxicity assay. Cytotoxicity capacity of the VZV IE62 specific T-cell clone against HLA-B*55:01 PHA blast increased with longer co-culture time (***p<0.0001; comparison between HLA-B*55:01 PHA blast target cell at 4 and 16 hours). E:T ratio=30:1, Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD.

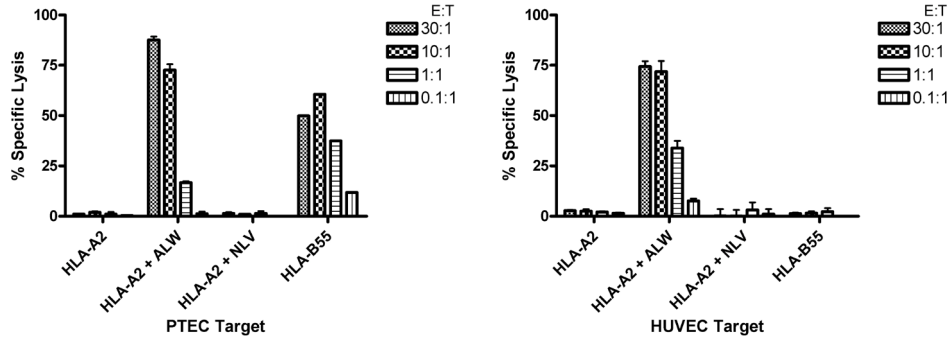

Figure 5. Tissue specificity of the allo-HLA reactivity by the VZV IE62 specific T-cell clone.
(A) The VZV IE62 specific T-cell clone demonstrates specific cytolytic effector function against a HLA-B*55:01 expressing PTEC, in a 4 hour cytotoxicity assay. Thus confirming that the vaccine induced memory T-cells can recognize normal renal tissue cell types present in a transplanted kidney. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD. Assay with HLA-B*55:01 PTEC target performed in single due to low target cell numbers. (B) A HLA-B*55:01 expressing HUVEC was not targeted by the VZV IE62 specific T-cell clone, in a 4 hour cytotoxicity assay, even following IFNγ pre-stimulation to increase the HLA-B*55:01 expression. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD.

DISCUSSION

Transplant candidates are at increased risk of infectious complications and every effort should be made to assure that they complete the full complement of recommended vaccinations prior to transplantation. We addressed whether successful vaccination may also induce alloreactive memory T-cells. In this study we show that live attenuated varicella vaccine can induce *de novo* human cytolytic allo-HLA reactive memory T-cells, in a kidney transplantation candidate. It also suggests that successful vaccination induces alloimmunity against specific allo-HLA molecules via peptide dependent molecular mimicry.

The VZV IE62 specific memory T-cells specifically recognized the allogeneic HLA-B*55:01 molecule. This allorecognition resulted in both cytokine production *and* cytotoxicity. We also demonstrated that this VZV specific T-cell from a kidney transplantation candidate can exert allo-HLA reactivity against normal kidney cell types present in transplantation tissue, thus demonstrating the clinical relevance of the vaccine induced response to kidney transplantation.

Lower cytotoxicity against allo-HLA-B*55:01 expressing PHA blasts and SALs in a four hour cytotoxicity assay, as compared to the positive controls (ALW peptide loaded HLA-A2$^+$ target

cells), is not unexpected. The HLA-A2 expressing PHA blasts and SALs were exogenously loaded with excess amount of viral peptide, while the cross-reactive alloresponses are dependent on presentation of endogenous self-peptide. Furthermore the lower percentage of specific lysis against PHA blasts, increased to levels comparable to the positive controls after an overnight cytotoxicity assay. Alloreactive memory T-cells are able to persist and may be capable of causing long-term damage, especially at times when immunosuppression is tapered (3).

The lack of recognition of HLA-B*55:01$^+$ HUVECs supports the conclusion that alloreactivity is tissue specific, and that the vaccine induced alloreactivity is HLA and endogenous peptide dependent. These results support molecular mimicry, and not bystander proliferation, as the mechanism for the vaccine induced alloreactivity. Similarly a HLA-DR3 restricted human tetanus toxoid-specific T-cell clone was previously found to give HLA-DR4 specific alloreactivity (16).

The presently described VZV IE62 specific T-cell expresses a Vβ14 TCR and recognizes the allogeneic HLA-B*55:01 molecule. Another T-cell clone with the same specificity and the same Vβ usage sorted from an individual with naturally acquired VZV infection was previously also reported to give allogeneic HLA-B*55:01 crossreactivity (1). VZV IE62 specific T-cell clones have also been reported to give allogeneic HLA-A*02:05 and HLA-B*57:01 reactivity, however these T-cell clones did not express Vβ14 TCRs (1).

The crossreactive potential of antigen specific T-cells is difficult to predict. However data presented here supports the notion that memory T-cells with the same antigen specificity and the same Vβ usage will exert alloreactivity against the same allo-HLA molecule.

VZV infects about 95% of the population (15,17-18). The immediate early (IE62) protein is required for the initiation of VZV replication, and VZV IE62 specific T-cells are correlated with immunity after VZV infection (19-20). VZV IE62 specific T-cells were found in 12/19 (63%) of stem cell transplantation patients with VZV reactivation, and 3/18 (17%) serologically positive healthy donors, indicating that this HLA-A2 restricted epitope is commonly used in HLA-A2 positive individuals (21). Here we show that live attenuated varicella vaccination also induces memory T-cells with identical specificity. Therefore HLA-B*55:01 mismatching may be an unacceptable mismatch in HLA-A2$^+$ transplantation recipients, but further database studies are warranted.

Live attenuated vaccines are very potent at eliciting protective T-cell immunity because they possess many antigenic targets, provide natural co-stimulation and can usually replicate to a limited extent. Recently activated effector memory T-cells may have differential or even no requirements for co-stimulatory signals, as compared to resting peripheral blood memory T-cells (22-23), and the CD8 memory T-cell state of readiness is antigen dependent and actively maintained but reversible (24-25). Therefore live attenuated vaccination may enable quantitatively and qualitatively stronger allo-HLA reactivity from the newly generated crossreactive memory T-cells. Similarly alloreactive memory T-cells in an already seropositive transplant candidate or recipient could be activated by booster vaccination, thereby enabling alloreactive

effector function in the absence of co-stimulation.

Alloreactive memory T-cells are central mediators of the immune-mediated injury to the allograft and therefore results presented here may have important clinical implications. We suggest vaccination could be given at least three months prior to expected transplantation to avoid the peak of specific cellular alloreactivity, as also suggested by others (13). Closer monitoring could be applicable to transplanted patients following recent vaccination. Live attenuated vaccination may be particularly potent at activating alloreactive memory T-cells. Further clinical studies on the effects of vaccination on the alloreactive T-cell repertoire are required.

Finally, Danziger-Isakov and colleagues demonstrated an effect of Influenza vaccination on cellular alloreactivity in humans (13), and favoured the conclusion that the observed increase in T-cell alloreactivity may be due to non-specific reactivation of a variety of T-cells clones. However their work lacked mechanistic studies to help understand the immunological process behind the reported observations. Furthermore they did not study HLA specificities of the cellular alloreactivity. Nonetheless, the absence of self responses in those transplant recipients who also showed evidence of anti-influenza reactivity suggests that specific allo-HLA reactivity is likely. Our previous work (1,26) combined with results presented here strongly suggests that molecular mimicry underlies the effect of vaccination on cellular alloreactivity.

Transplant candidates are at increased risk of infectious complications mandating a full complement of recommended vaccinations prior to transplantation. We provide evidence that live attenuated vaccination is associated with the generation of de novo HLA specific alloreactive memory T-cells, likely via molecular mimicry. Vaccination induced alloreactivity may have important clinical implications, especially for vaccine timing and recipient monitoring. Future work should determine if induction of HLA specific alloreactivity is a common characteristic of human vaccination.

MATERIALS AND METHODS

Renal transplantation candidate

HLA-A*02:01^{+}, varicella seronegative renal transplantation candidates were considered for participation in this study. The patient from whom the VZV specific T-cell clone was sorted is a 52 year old male with end stage renal disease due to diabetes mellitus type 2, receiving haemodialysis therapy, with HLA typing HLA-A*02:01,-; B*13,40; Cw3,Cw6; DRB1*07,-. The candidate had never previously received an organ transplant. History taking confirmed no previous varicella infection or vaccination. Two separate serum samples taken before varicella vaccination were both VZV seronegative (Figure 1a). A commercially available ELISA kit was used (Vidas Varicella Zoster IgG, BioMerieux, Marcy l'Etoile, France). The patient was vaccinated with a live attenuated varicella vaccine (Varilix, GlaxoSmithKline) on two occasions, separated by six weeks.

Generation of VZV IE62 specific CD8 memory T-cell clone

We used the newly validated ALWALPHAA/HLA-A*02:01 restricted epitope from the immediate early 62 (IE62) protein of varicella zoster virus to detect de-novo virus specific memory T-cells generated following varicella vaccination (21). VZV IE62 specific T cells were isolated from the peripheral blood as previously described (1,26). Briefly, PBMCs were harvested and labeled with HLA-A2/ALW tetrameric complexes for 30 minutes at 4 ^{0}C in RPMI without phenol red, supplemented with 2% FCS, washed three times and single cell sorted at 4 ^{0}C using the FACS vantageTM (Becton Dickinson). Tetramer positive CD8 T-cells were non-specifically stimulated every 2 weeks with feeder cell mixture containing irradiated allogeneic PBMCs (3500 Rad), 800ng/ml phytohaemagglutinin (PHA), 100 IU/ml IL-2 in IMDM medium supplemented with glutamine, human serum (5%) and fetal calf serum (5%).

Confirmation of T-cell clonality

TCRα and TCRβ rearrangements were analyzed on the VZV IE62 specific T-cell clone.
Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Oligo dT primed first-strand cDNA was synthesized from 1 µg RNA template using Superscript III reverse transcriptase (Invitrogen corporation, Carlsbad, CA, USA). First RT-PCR was performed to determine the TCR AV and BV usage, using primers that cover the complete TCR repertoire. Sequencing templates were obtained performing high fidelity PCR using Pfx50 DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Each reaction contained forward primers targeting the Va2S1 or Vb14S1 variable region and reverse primers specific for the alpha and beta chain constant region. Amplicons spanning the variable, CDR3 and joining regions were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) according to manufacturer's protocol. Thermo sequenase primer cycle sequencing (GE healthcare) reactions were performed using a CY5 labeled M13 sequencing primer (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol. Sequencing reactions were run on an ALFexpress DNA sequencer (GE Healthcare), and analyzed with sequence analyser 2.10 software (GE Healthcare).

Cytokine assays
Screening for allo-HLA crossreactivity of the VZV IE62 specific T-cell clone was done using a panel of EBV transformed B-cells (EBV LCLs) selected to cover almost all frequently occurring HLA class I and II molecules, using IFNγ production as readout. IFNγ production was also used to confirm the allo-HLA crossreactivity by the VZV IE62 specific T-cell clone, using single HLA transfected K562 cells (SALs) as stimulator (27). 10000 T-cells were co-cultured with 50000 irradiated stimulator cells in a final volume of 150µL IMDM culture medium supplemented with 10% fetal calf serum and 100IU/mL IL-2. After 18 hours of incubation, supernatants were harvested and IFNγ production was measured using standard enzyme-linked immunosorbent assay (ELISA, U-Cytech, Netherlands).

Cytotoxicity assays
The VZV IE62 specific T-cell clone was evaluated for cytotoxicity by incubating 5000 target cells with serial dilutions of the T-cell clone for 4 hours in a standard ^{51}Cr-release cytotoxicity assay. Target cells used in the standard cytotoxicity assays include PHA Blasts, single HLA transfected K562 cells (SALs) (26-27), proximal tubular epithelial cells (PTECs) (28-31) and human umbilical vein endothelial cells (HUVECs) (32-34). Cytotoxicity assays involving PTEC and HUVEC target cells were performed before and after IFNγ treatment, 500 units/ml for 24 hours. PHA blasts were also incubated with the T-cell clone in an overnight cytotoxicity assay (16 hours), however, SALs were not suitable targets in an overnight assay due to ^{51}Cr leakage. Target cells were incubated with ^{51}Cr for 60 minutes. Supernatants were harvested for gamma counting: *percent specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.* Results are expressed as the mean of triplicate samples.

Statistics
Values for specific lysis are presented as the mean of triplicate wells, with standard deviation (SD). Values for IFNγ production are presented as the mean of duplicate wells, with standard deviation. Comparative analyses are non-parametric (unpaired) t-tests, $p<0.05$ is considered to be significant. Statistics are derived using Graph Pad Prism 4 for Windows (Version 4.02, 2004).

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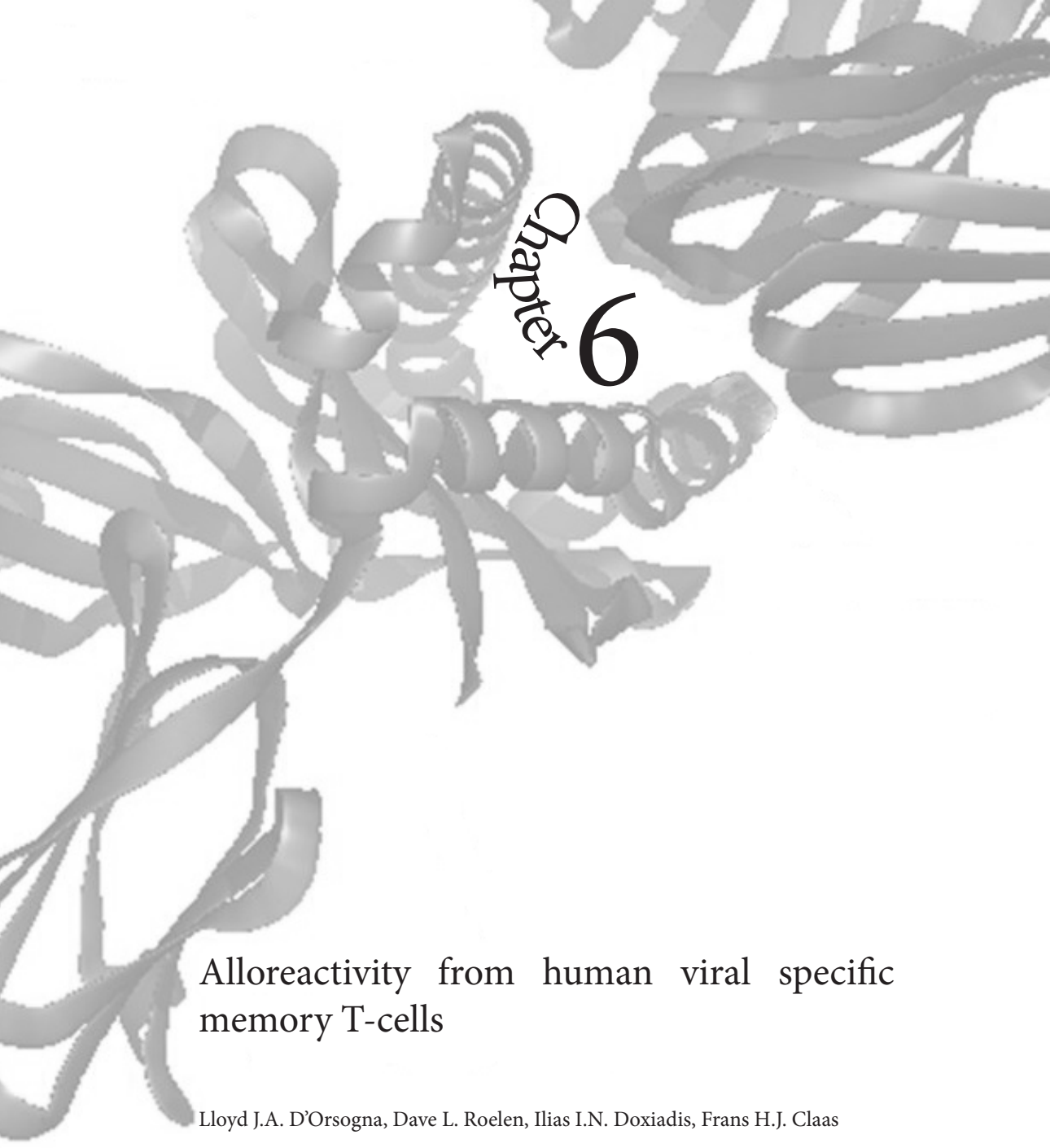

Chapter 6

Alloreactivity from human viral specific memory T-cells

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ABSTRACT

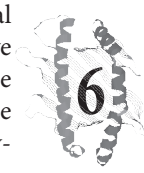
The mechanisms by which alloreactive memory T-cells are generated in non-sensitized individuals have begun to be elucidated. It is generally accepted that a very high level of crossreactivity is an essential feature of the T-cell receptor. Indeed it has recently been shown that alloreactivity from viral specific memory T-cells is far more common than predicted, 45% of viral specific T-cell clones were found to be allo-HLA crossreactive. In this overview the evidence for crossreactive alloresponses from human viral specific memory T-cells is discussed with special emphasis on the unexpected high frequency of these crossreactive responses, the peptide and tissue specificity of the responses, and the mechanistic insights gleaned from the elucidation of the crystal structure of an allo-HLA crossreactive viral specific TCR. The possible implications for clinical solid organ and bone marrow transplantation and tolerance induction will be discussed.

1. NON-SENSITIZED TRANSPLANTATION RECIPIENTS HAVE STRONG "MEMORY" RESPONSES FOR ALLO-HLA

Transplantation recipients can be sensitized against alloantigen by pregnancy, blood transfusion or previous transplantation. B-cell sensitization is revealed by the presence of HLA specific antibodies, which are not detectable in non-sensitized individuals. However, even in non-sensitized individuals a substantial portion of the pre-existing memory T-cell repertoire is already alloreactive (1-4), which is far greater than the proportion of T-cells that respond to any individual pathogen. The origin of these high-frequency pre-existing alloreactive memory T-cells in non-sensitized individuals was previously unclear, but has been hypothesized to relate to crossreactive allo-HLA responses from viral specific memory T-cells (5-7).

In humans, acute rejection has been associated with varying viral infections, and CMV prophylaxis with oral ganciclovir is associated with improved long-term renal graft survival (8). Mismatched donor HLA antigens have differential impact on graft survival depending on the HLA phenotype of the recipient (9), and one possible explanation for the occurrence of these harmful HLA combinations may be that patients have had previous immunological contact with pathogens that elicit T-cell responses which crossreact against the HLA mismatches (6-7,9). The fact that cord blood T-cells are less able to mediate graft vs. host disease (GvHD) than marrow derived T-cells because of their naïve status supports this theory (10-11).

In-vivo, the presence of virally induced alloreactive T-cell memory is a potent barrier to transplantation tolerance in mice (12-17). Many strategies have been used to successfully induce tolerance to transplanted tissue in mice, most of which primarily block the CD80/CD86/CD28 and/or CD40/CD154 co-stimulatory pathways. For example, donor specific transfusion and anti-CD154 antibody readily induce tolerance to solid organ grafts in pathogen free mice; however, all these protocols fail in pathogen exposed mice as viral infections induce alloreactivity and abrogate the induction of transplant tolerance (18-22). Furthermore, Adams clearly demonstrated a viral dose effect whereby mice previously exposed to multiple viral infections were refractory to tolerance induction and rejected their allografts, whereas naïve mice or single pathogen exposed mice were susceptible to tolerance induction (15). Evidence for virally induced alloreactive T-cell memory in mice is already extensively reviewed in the literature (12-15), therefore this review will focus on the evidence for allo-HLA crossreactivity by human T-cell clones.



Once generated, viral specific memory T-cells persist in high frequency and have lower activation requirements with novel co-stimulatory pathways that may be constitutively expressed (12, 23). Upon activation, memory T-cells produce a wide variety of cytokines including IL-2, IL-4, IFNγ, TNFα and are capable of rapid up-regulation of cytolytic effector function without the need for CD4 T-cell help (24). Taken together these factors provide strong support for the ability of viral specific memory T-cells to directly elicit acute rejection, and for viral memory having a negative influence on graft survival and/or tolerance induction.

2. EBV SPECIFIC CLONES ARE CROSSREACTIVE AGAINST ALLO HLA-B*4402 VIA MOLECULAR MIMICRY

Early work suggested that the explanation for the presence of alloreactive memory T-cells in non-sensitized individuals could be crossreactivity from viral specific memory T-cells against allo-HLA molecules (5-6). Burrows and colleagues demonstrated the dual specificity of EBV EBNA3A specific T-cell clones for the immunodominant peptide FLRGRAYGL presented on HLA-B*0801 and the alloantigen HLA-B*4402, to which the individual had never been exposed (6). This data also showed that the T-cell alloresponse can be dominated by a cross-reactive CTL induced by a single viral epitope.

In fact the HLA-B8/FLR restricted response in a HLA-$B8^+$ $B44^-$ individual gives rise to a public BV6S2 TCR which always cross-reacts against allogeneic HLA-B*4402 (25). This finding has been reproducibly found in different individuals from different genetic backgrounds using different techniques (2, 26-27). For example, we confirmed the alloreactivity of the EBV EBNA3A specific T-cell against HLA-B*4402 using single antigen expressing cell lines (single HLA transfected K562 cells) (26). In theory, viral infections that give rise to public TCR responses could therefore be used to determine unacceptable mismatches based solely on immunological history. Indeed HLA-B44 mismatching has been identified as higher risk among HLA-$B8^+$ renal transplant recipients (28).

The EBV EBNA3A T-cell allo-HLA-B*4402 crossreactivity is dependent on presentation of the EEYLQAFTY self-peptide derived from the ABCD3 gene (29). Molecular mimicry, as revealed by crystallography studies, is the mechanism for this human T-cell alloreactivity from a viral specific memory T-cell (e.g. see figure 1). Despite extensive amino acid differences between HLA-B*0801 and HLA-B*4402, and the disparate sequences of their bound viral and self peptides respectively, the HLA-B8/FLR restricted TCR engages these peptide-HLA complexes identically. The viral and allopeptides adopted similar conformations after TCR ligation, revealing that molecular mimicry is associated with TCR specificity. This paper highlights the exquisite specificity of the TCR and the self peptide dependence of the T-cell alloreactivity.

It is also suggested that molecular mimicry operates in other alloreactions (30-33). Nonetheless, more definitive data on the mechanisms of T-cell allo-HLA crossreactivity from other clonotypes are still required.

3. ALLOREACTIVITY FROM VIRAL SPECIFIC MEMORY T-CELLS IS COMMON

Recently we reported that allo-HLA responses from viral specific memory T-cells are in fact far more common than anticipated (27). To analyze allo-HLA crossreactivity from viral specific T-cells, T-cell clones were tested against a panel of HLA typed target cells, and target cells transduced with single HLA molecules. These studies showed that 80% of virus specific T-cell lines and 45% of virus specific T-cell clones crossreact against certain allo-HLA mol-

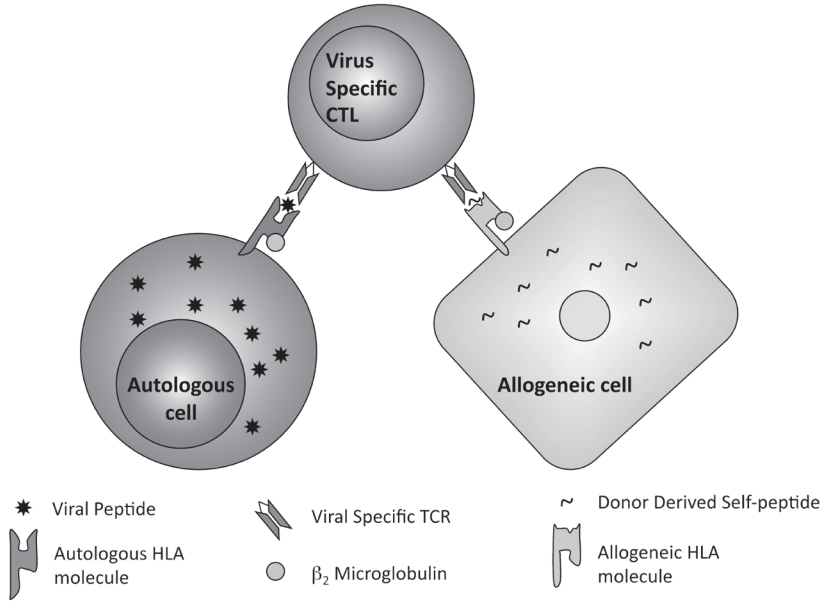


Figure 1. Allo-HLA crossreactivity by viral specific memory T-cells.
Viral specific memory T-cells target virus infected autologous cells presenting viral peptides in a self-HLA restricted fashion. The same viral specific TCR may crossreact against an allogeneic HLA molecule presenting a self-peptide. CTL=Cytotoxic T Lymphocyte.

ecules. Allo-HLA crossreactivity was shown from EBV, CMV, VZV and influenza specific T-cell clones (27). Multiple viral specific CD8 T-cell clones were shown to be alloreactive against allogeneic class I molecules, and likewise several viral specific CD4 T-cell clones were shown to crossreact against allogeneic class II molecules. Surprisingly, two separate CMV specific, class I restricted T-cell clones recognized allogeneic class II molecules (27), as has also been reported by others (34).

Additionally others have demonstrated allo-HLA crossreactivity by viral specific T-cell clones, although the target HLA molecule was not always clearly defined. HLA-A*0201 restricted HSV-2 specific T-cell clones have been shown to crossreact against the HLA-B44 family (35), and CMV specific CD8 T-cells have been shown to crossreact against undefined class I alloantigens by another group (36). EBV and tetanus toxoid specific CD4 T-cell clones have also been shown to exhibit allo-HLA class II responses (37-38). Table 1 lists human viral specific memory T-cells reported to give allo-HLA crossreactivity while table 2 compares the methods used for detection of the allo-HLA crossreactivity.

The importance of these findings are reinforced by functional studies showing that the vari-

ous viral specific CD8 T-cell clones can lyse multiple different target cells expressing the target HLA molecule, in a 4 hour ^{51}Chromium release assay (6,26-27). The fact that the same TCR complex mediates both virus specificity and allo-HLA crossreactivity has been confirmed by TCR PCR, viral tetramer inhibition and TCR transfection assays (6,26-27).

The multiple mechanisms of T-cell receptor crossreactivity have been reviewed extensively by others (39-42). Despite peptide/HLA diversity and TCR plasticity, these T-cell responses always exhibit exquisite HLA and peptide specificity.

3.1 Peptide dependence of allo-HLA crossreactivity from viral specific T-cells

It is now generally accepted that alloreactive T-cells recognize allo-HLA molecules presenting self-peptides (7,27,29,43-44). Macdonald and colleagues have provided clear structural evidence that self-peptide dependent molecular mimicry underpins the alloreactivity of the EBV EBNA3A specific T-cell against allogeneic HLA-B*4402 (29).

Furthermore, the peptide dependence of the allo-HLA crossreactivity from other viral specific memory T-cells is reinforced by differing potency of the alloreactivity exerted by virus specific T-cells against different cell targets. For example, a VZV specific HLA-A2 restricted T-cell clone recognizes allogeneic HLA-B*5701 expressing EBV LCLs, PHA Blasts and monocyte derived DCs, but does not recognize HLA-B*5701 expressing B-cells, T-cells, monocytes nor fibroblasts (27). Therefore allo-HLA expression is not solely sufficient to elicit target killing. Presumably the cell types that are not recognized do not present the relevant self-peptide.

In contrast to allogeneic HLA-B*4402$^+$ EBV LCLs and SALs, allogeneic HLA-B*4402$^+$ human umbilical vein endothelial cells (HUVECs) and proximal tubular epithelial cells (PTECs) are poor targets for EBV EBNA3A specific CD8 T cells. HLA-B*4402 expressing PTECs are specifically lysed by an EBNA3A T-cell clone without peptide loading albeit at high effector/target ratio only. The specific lysis of HLA-B*4402 expressing PTECs is greatly increased by exogenous EEY peptide loading. HLA-B*4402 expressing HUVECs are only targeted by an EBV EBNA3A clone when loaded with exogenous EEY peptide. The lack of recognition of endothelial and epithelial cells was not due to the lack of HLA-B*4402 expression. Thus organ (kidney) specificity of the alloresponse from the EBNA3A specific T-cell is dependent on endogenous self-peptide (EEY) processing and presentation.

3.2 Viral specific T-cell responses may not give predictable allo-HLA crossreactivity

Unlike the public BV6S2 TCR response against FLR peptide presented on HLA-B8, immune responses against other common pathogens are not so immunodominant and memory CD8 T-cells generated following viral infections often demonstrate a wide diversity of Vβ usage and therefore allo-HLA crossreactivity (Table 1). For example, Burrows showed that EBV EBNA3A specific T-cells that do not use the Vb6S2 TCR are alloreactive against HLA-B14 and -B35, but not HLA-B*4402 (45). Several other examples of differing alloresponses from T-cell clones with the same viral peptide/HLA restriction are also reported by Amir (27) and summarized in Table 1.

Table 1. Reported allo-HLA crossreactivity by human viral specific memory T-cells. # = Not determined.

Reference	Virus	Viral antigen	HLA restriction	Viral peptide restriction	Vb	Allo-HLA crossreactivity	Target cell(s)
Viral specific CD8 T-cells recognizing allo-class I							
27	CMV	pp50	A*0101	VTEHDTLLY	#	#	EBV LCLs
27	CMV	pp50	A*0101	VTEHDTLLY	3	A*1101	EBV LCLs
27	CMV	pp65	A*0201	NLVPTMVATV	8	#	EBV LCLs
36	CMV	pp65	A*0201	NLVPTMVATV	#	#	Splenocytes, EBV LCLs
36	EBV	BMLF1	A*0201	GLCTLVAML	#	#	Splenocytes, EBV LCLs
27	H. Influenzae	IMP	A*0201	GILGFVFTL	17	B*6401	EBV LCLs
27	VZV	IE62	A*0201	ALWALPHAA	14	B*5501	EBV LCLs
27	VZV	IE62	A*0201	ALWALPHAA	#	B*5701	EBV LCLs
27	VZV	IE62	A*0201	ALWALPHAA	21.3	A*0205	EBV LCLs
35	HSV-2	VP13/14	A*0201	FLVDAIVRVA	#	B*4402, B*4403, B*4407	EBV LCLs
35	HSV-2	VP13/14	A*0201	GLADTVVAC	#	B*4404	EBV LCLs
27	EBV	EBNA3A	A*0301	RLRAEAQVK	#	A*3101	EBV LCLs
27	EBV	BRLF1	A*0301	RVRAYTYSK	7.1	#	EBV LCLs
27	EBV	BRLF1	A*0301	RVRAYTYSK	14	#	EBV LCLs
27	EBV	BRLF1	A*0301	RVRAYTYSK	17	#	EBV LCLs
27	EBV	BRLF1	A*0301	RVRAYTYSK	7.2	A*0201	EBV LCLs
5	EBV	#	B8	#	#	B35, B62, B12 (B44/B45)	EBV LCLs
5	EBV	#	B8/B14	#	#	A3, A11	EBV LCLs
6	EBV	EBNA3A	B*0801	FLRGRAYGL	BV6S2	B*4402	PHA Blasts
26	EBV	EBNA3A	B*0801	FLRGRAYGL	BV6S2	B*4402 & B*5501	PHA Blasts, SALs
27	EBV	EBNA3A	B*0801	FLRGRAYGL	BV6S2	B*4402 & B*5501	EBV LCLs
2	EBV	EBNA3A	B*0801	FLRGRAYGL	#	B*4402	PHA Blasts
45	EBV	EBNA3A	B*0801	FLRGRAYGL	BV7S1B	B14	PHA Blasts
45	EBV	EBNA3A	B*0801	FLRGRAYGL	BV7S5	B35	PHA Blasts
5	EBV	#	B62	#	#	B57	EBV LCLs
Viral specific CD4 T-cells recognizing allo-class II							
27	CMV	pp65	DRB1*0101	KYQEFFWDANDIYRI	8	DRB1*0901	EBV LCLs
27	CMV	pp65	DRB1*0101	KYQEFFWDANDIYRI	#	DRB3*0101	EBV LCLs
37	EBV	#	DRB1*0101	#	#	DRB1*0404	EBV LCLs
27	H. Influenzae	HA	DRB1*0401	PKYVKQNTLKLAT	3	DRB1*1301	EBV LCLs
37	EBV	LMP2	DRB1*1001	ICLTWRIEDPPFNSILFALL	#	DRB1*0406	EBV LCLs
37	EBV	EBNA3C	DRB1*16	PHDITYPYTARNIR	#	DRB1*0101	EBV LCLs
38	Tetanus Toxoid	#	DR3	#	#	DR4	EBV LCLs
37	EBV	EBNA2	DRB3*0202	PRSTVFYNIPPMPLPPSQL	#	DRB1*0101	EBV LCLs
37	EBV	EBNA1	DPB1*1001	NPKFENIAEGLRALLARSHV	#	DRB1*0102	EBV LCLs
37	EBV	LMP2	DQB1*0601	TYGPVFMSLGGLLTMVA	#	DRB1*0901	EBV LCLs
Viral specific CD8 T-cells recognizing allo-class II							
27	CMV	pp65	B*0702	RPHERNGFTVL	7.2	DRB1*0801	EBV LCLS
27	CMV	pp65	B*3501	IPSINVHHY	5.1	DRB1*0401	EBV LCLs
34	CMV	pp65	Cw*0602	TRATKMQVI	13	DRB1*0401	EBV LCLs, B-cells

Variable allo-HLA crossreactivity by T-cell clones sorted from the same individual with the same specificity, but different TCR Vβ usage, was also reported. Single cell sorting of VZV IE62 specific T-cells from an individual with VZV infection generated three different clones with usage of Vβ 21.3, Vβ 14 and an undetermined Vβ (27). These T-cell clones cross-reacted against allo HLA-A*0205, HLA-B*5501 and HLA-B*5701 respectively. Demonstrating how a single viral peptide/HLA restricted immune response can generate different clonotypes with differing allo-HLA crossreactivity within the same individual.

Furthermore, a single EBV EBNA3A specific memory T-cell clone was able to recognize both allogeneic HLA-B*4402 and B*5501, in addition to the viral peptide presented on HLA-B8 against which it was originally selected (26).

Table 2. Cellular Targets used for Detection of Allo-HLA Crossreactivity by Viral Specific Memory T-cells

Detection method	Advantages	Disadvantages
EBV LCLs	Cells easily generated and maintained	Does not allow conclusive definition of crossreactive HLA molecule
	Suitable for screening of alloreactivity against many common HLA molecules	Viral responder cells may recognize EBV peptides presented on HLA molecules
	High expression of HLA molecules	
Single HLA antigen cell lines	Allows definitive confirmation of crossreactive HLA molecule	Requires HLA molecule transfection for generation
	Once produced are relatively easily maintained and grown	Requires ML1 laboratory facilities
	K562 cell is an immortalized cell line	K562 cells possess many genetic variations which may give rise to recognition of tumour peptides
	Viral peptide free	Poor targets in 4 hour chromium release assay
PHA blasts	High expression of HLA molecules	Cannot be used in IFNγ based assays
	Easily generated	
	Viral peptide free[a]	
PBMC or splenocytes	Suitable for screening of alloreactivity against many common HLA molecules	Does not allow definitive definition of crossreactive HLA molecule without blocking by monoclonal antibodies
	Easily obtained	
	Viral peptide free[a]	
Tissue type specific cells e.g. PTEC/ HUVEC	Able to detect tissue specific alloresponses	Technically difficult and laborious to grow

* The authors have found that our EBV EBNA3A specific T-cell clone does not recognize HLA-B8^{+} EBV seropositive PBMCs or PHA Blasts without addition of exogenous FLR peptide

Pan HLA recognition is inherent in germline TCR sequences (46). T-cells can presumably exit the thymus due to their high crossreactivity, as they are "positively" selected by self-HLA molecules. These alloreactive T-cells are unable to discriminate between self and non-self peptides presented on allo-HLA molecules (29). Only T-cells with high affinity for self-peptides presented on self-HLA molecules are "negatively" selected from the pan HLA reactive T-cell repertoire. Indeed, in HLA-B8/B44 heterozygotes the public Vb6S2 TCR expressing EBV EBNA3A clonotype is deleted from the T-cell repertoire (47). CTLs from HLA-B8$^+$B44$^+$ individuals express different TCR gene combinations which maintain HLA-B8/FLR specificity, but do not possess HLA-B*4402 reactivity, thereby preventing auto-immunity. Self-tolerance shapes the TCR repertoire available to respond to any individual viral antigen (47-48), thereby also altering the allo-HLA crossreactivity of the viral specific TCR pool.

Therefore, alloreactive T-cells do escape thymic deletion and are subsequently activated by viral infection. However virus specific T-cells with the same antigen specificity, but with different TCR Vb usage, clearly exert alloreactivity against different HLA molecules. It is currently not known if viral specific T-cells from different individuals with the same specificity *and* the same Vβ usage will always demonstrate similar allo-HLA crossreactivity. This knowledge is essential in order to be able to predict (un)acceptable mismatches based on donor-recipient HLA mismatches and immunological history of the recipient.

4. PREVIOUS VIRAL (PATHOGEN) INFECTION IS CRITICAL TO INDUCTION OF THE ALLOREACTIVE T-CELLS

Memory T-cells demonstrate critical functional differences versus their naïve counterparts, such as immediate cytotoxicity without the need for co-stimulation nor CD4 T-cell help. For example, EBV EBNA3A specific memory T-cells demonstrate immediate cytolytic effector function against HLA-B*4402$^+$ PHA blasts in a 4 hour ^{51}chromium release assay (6,26). A CCR7$^+$ CD45Ra$^+$ naive T-cell with the same TCR (e.g. from a EBV seronegative individual), upon first contact with antigen, will secrete only IL2, is not cytolytic and requires CD4 T-cell and B-cell help within the germinal centre to initiate an immune response before expanding into the memory T-cell pool. Naïve T-cells recognizing an alloantigen without the appropriate co-stimulatory signals and T-cell help may gain regulatory function, be deleted or become anergic (49-50). This illustrates the critical importance of previous viral infection to the activation of alloreactive T-cells.

5. CLINICAL IMPLICATIONS OF STUDIES USING VIRAL SPECIFIC T-CELL CLONES

In humans, alloreactive memory T-cells are frequently generated by viral infection. This allo-HLA crossreactivity is likely peptide dependent but not predictable based on donor-recipient HLA mismatches alone. Allo-HLA crossreactivity from viral specific memory T-cells may have important clinical implications for the alloimmune response after transplantation

because memory T-cells have lower activation requirements, no need for CD4 T-cell help and can have immediate cytotoxic effector function as compared to their naïve counterparts. Therefore if truly alloreactive in-vivo, pre-existing memory T-cells may represent a common source of acute and/or chronic rejection and be a major obstacle to tolerance induction.

The frequency of memory T-cells are highest for the chronically persistent viruses such as human herpes viruses EBV and CMV. It remains to be determined if the alloreactive memory T-cell pool consists of many responding memory T-cells each of different specificities and each of low precursor frequency, or of a single (or few) viral specific memory T-cells that individually account for a large portion of the alloresponse. If alloreactive T-cells are driven by reactivation of viral infection then anti-viral therapy may decrease the proportion of these allo-HLA crossreactive alloresponses. Supportive evidence is provided by the finding that CMV prophylaxis with oral ganciclovir is associated with less acute rejection and improved long-term renal graft survival (8). Alternatively vaccination could induce alloreactivity, as suggested by others (38,51).

Given the longevity of viral specific memory T-cells, it is likely that allo-HLA crossreactive memory T-cells generated after infection are maintained and able to elicit acute rejection, particularly when immunosuppression is tapered (12). Ex-vivo staining for the presence of viral specific T-cells within rejecting kidney or GvHD biopsy samples may help confirm the clinical relevance of crossreactive allo-HLA responses.

Long term antigen specific tolerance to engrafted tissue is the ultimate goal in transplantation but despite numerous successful rodent models clinical human tolerance has remained an elusive goal. The presence of viral specific memory T-cells may be responsible for the failure to induce tolerance in clinical settings (12,15), although it is unclear what role primary infection vs. reactivation may play. For example, mice work reveals that viral infection abrogates the tolerance induced by donor specific transfusion and anti-CD154 blocking (13). Similar effects from memory T-cells following viral infection are also reported in many other studies (12,14-15,18). This relates to decreased dependence of memory T-cells on co-stimulatory pathways. Humans are not immunologically naïve and we propose that memory T-cells generated after environmental exposures may account for the difficulty in transferring tolerance studies from mice into the human setting. Therefore, we suggest caution when interpreting tolerance protocols studied in pathogen free animals.

The self-peptide dependency of alloreactivity from viral specific memory T-cells, as confirmed by Macdonald and colleagues (29), is of interest and may present several therapeutic opportunities. Allo-HLA recognition from viral specific T-cells may exhibit different tissue specificities depending on household gene expression and self-peptide presentation. For example, preliminary work shows that PTEC and HUVEC cell lines are poor targets for EBV EBNA3A specific T-cells, likely due to decreased EEY peptide presentation. Therefore a HLA-B*4402 mismatch in a HLA-B8^{+} B44^{-} kidney recipient may not be associated with high risk of rejection if the EEY peptide is not presented on the donor cell surface.

Conversely, a HLA-B8 mismatch in a HLA-B8^{-} B*4402^{+} bone marrow recipient could theoret-

ically be associated with graft vs. leukaemia (GvL) effect but low risk of GvHD. Interestingly, haploidentical bone marrow transplantation may be associated with increased GvL effect. Exploitation of the differential peptide and tissue specificity of alloreactivity from viral specific T-cells for therapeutic benefit should become a major research focus.

Peptide dependent alloreactivity also implies that immunomodulating techniques could be used to inhibit these harmful T-cell clonotypes, as suggested by Burrows (52). While the alloreactivity of the EBV EBNA3A specific T-cell has been confirmed to be dependent on peptide dependent molecular mimicry, and not degenerate recognition, further structural studies on the mechanisms of allorecognition are clearly warranted.

Given the abundant crossreactivity contained within the T-cell repertoire deletion of any individual virus specific clonotype might not be associated with viral reactivation. While the CD8 memory T-cell pool created after a viral infection has a distinct immunodominant hierarchy, many clonotypes are capable of recognizing the viral peptide/HLA complex. Nonetheless, it can not yet be excluded that successful tolerance induction may occur at the expense of a T-cell clone that has an important role in controlling a chronic viral infection, possibly leading to viral reactivation.

Monitoring of alloreactive T-cells is also critical as this may allow individualization of immunosuppression (53). Currently in-vitro assay for renal transplantation monitoring does not have adequate sensitivity or specificity to enter routine clinical practice. However such assays routinely use donor PBMCS or spleen cells as stimulator, and it is unclear if these target cells present a comparable peptide pool to that presented by the relevant donor (kidney) cells. Perhaps future studies of transplantation monitoring could use a pool of tissue specific self-peptides which are known to be presented by the donor organ. At the current point in time HLA matching remains the best predictor of long-term renal graft survival.

DR-matching has beneficial effects on transplantation survival. Allo-HLA class II crossreactivity from class I restricted viral specific T-cells was previously unreported (27,34). We suggest that DR matching may, in part, be associated with improved graft survival due to the inability of viral specific T-cells to crossreact against allogeneic HLA class II. Further examination of MHC class II restricted pathogen specific CD4 T-cells is required, as it is likely that this T-cell population plays a dominant role in allograft rejection (2,54-55).

Ultimately not only HLA phenotype, but also immunological history, may be used to determine donor-recipient suitability. However major studies on the public nature of anti-viral responses in individuals of different HLA background are still required. Early work suggests that, unlike the HLA-B8/FLR restricted immune response, most viral specific T-cell responses do not give rise to a public TCR nor predictable allo-HLA crossreactivity. Nonetheless studies of viral peptide/HLA restricted T-cell responses, TCR Vb usage and allo-HLA crossreactivity are ongoing.

Even if immunological history can not be utilized to avoid alloreactivity, selective therapies at the time of transplantation may allow inhibition of allo-HLA crossreactivity from pre-ex-

isting memory T-cells while still allowing de-novo naïve responses against viral antigens. For example, selective blockade of ICOSL and CD86 which represent two major co-stimulatory signals for the activation of resting peripheral blood memory T-cells (12,56) may still allow immune responses via the CD40/CD154 and/or CD70/CD27 co-stimulatory pathways which are important for naïve T-cell activation. While the effect of immunosuppressive drugs on allo-HLA crossreactivity from viral specific T-cells has not been studied, the calcineurin inhibitors are able to inhibit proliferation and cytokine production from effector CD4 memory T cells (54). Unfortunately leukocyte depleting therapies such as antithymocyte globulin and alemtuzumab are less able to diminish the memory T-cell pool (54).

Adoptive transfer of virus or fungal specific T-cells offers an effective option for the management of specific immune defects in an immune compromised host (57), particularly following allogeneic BMT. However given the high frequency of allo-HLA crossreactivity from viral specific T-cells, it is not surprising that adoptive transfer has already been associated with GvHD. For example, adoptive transfer of CMV specific T-cells to nine recipients after allogeneic BMT resulted in three cases of GvHD, including one patient who died (58). Similarly, TCR gene transfer to induce anti-leukaemia reactivity is associated with α and β chain rearrangements and therefore the formation of mixed dimer TCRs (59), which could also be alloreactive. Screening of adoptively transferred antigen or leukaemia specific T-cells for allo-HLA crossreactivity may help prevent GvHD.

Consistent with this theory cord blood T cells are less able to mediate GvHD than marrow derived T-cells because of their naïve status (49,60).

Finally, some groups have suggested that allo-HLA crossreactivity by viral specific T-cells does not play a significant role in transplantation. Nickel and colleagues found no association between CMV specific memory T cells and alloreactivity (61). However this study only measured CMV specific responses against viral peptides loaded on autologous cells and did not specifically document if these responses were crossreactive against mismatched donor HLA molecules. While 45% of virus specific T-cells have demonstrable allo-HLA crossreactivity against one HLA molecule (27), the target HLA molecule may not have been present on the donor cell. All kidney recipients received anti-IL2R mAb, calcineurin inhibitor, mycophenolate mofitil and steroids as induction therapy, possibly suppressing allo-HLA crossreactive responses until the immunosuppressive drugs were tapered. Furthermore, recipients received pre-emptive ganciclovir therapy guided by asymptomatic CMV viraemia. While we agree that CMV specific T-cell responses that are not allo-HLA crossreactive are likely to benefit a recipient, this study does not exclude the role of allo-HLA crossreactivity from viral specific T-cells in kidney rejection.

Therefore, crossreactivity by viral specific memory T-cells or "heterologous immunity" is common. While this crossreactivity by pathogen specific memory T-cells may help protect against subsequent unrelated infections, in the transplantation setting such crossreactivity may give rise to harmful alloresponses.

6. CONCLUSION

An essential feature of the T-cell response is the ability to recognize a diverse array of potentially unlimited antigens, necessitating that the TCR be inherently crossreactive. The memory T-cells that are specific to previously encountered pathogens accumulate following repeated infectious exposure and have low activation thresholds. Mice in-vivo, and human in-vitro, experiments reveal that these viral specific memory T-cells are commonly crossreactive with allo-HLA molecules in a self-peptide specific manner. Thus, getting a certain infection in an individual with a certain HLA type might have significant adverse consequences in the event of organ or marrow transplantation. Human ex-vivo studies are clearly warranted. We suggest that current research objectives should focus on the human in-vivo relevance of allo-HLA crossreactivity from viral specific memory T-cells, and specifically how self-peptide dependent allorecognition from viral specific T-cells alters tissue specificity. Allo-HLA crossreactivity could also have serious adverse effects in the setting of adoptive transfer and TCR transfection of viral specific T-cells. New understandings of the origin of alloreactivity may lead to an era whereby donor suitability is defined not only by HLA typing but also using immunological history, and hopefully toward successful antigen-specific transplantation tolerance.

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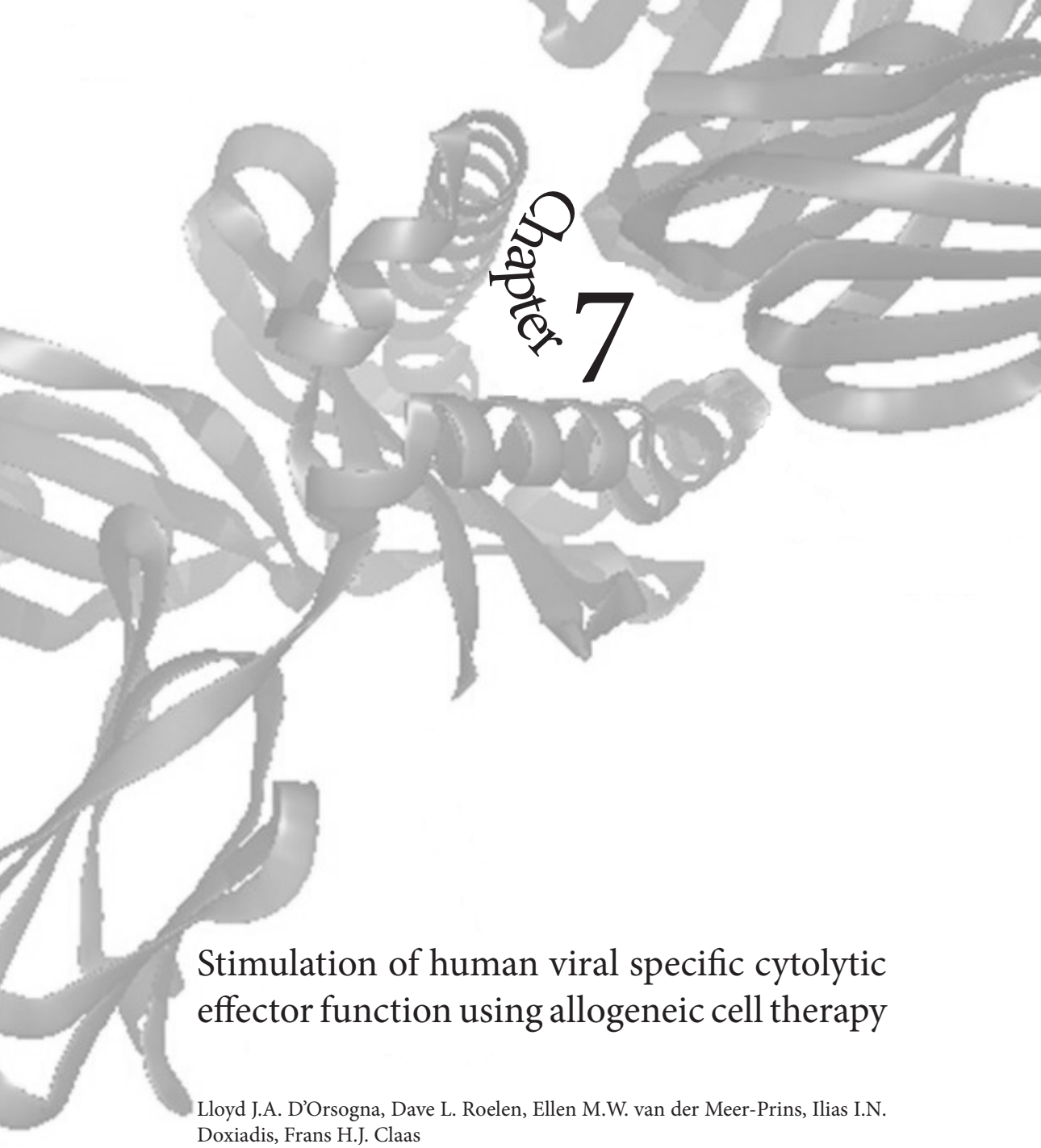

Chapter 7

Stimulation of human viral specific cytolytic effector function using allogeneic cell therapy

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Manuscript in preparation

ABSTRACT

Viral infection is a major cause of morbidity and mortality, and there are few therapeutic options available to augment a viral specific T-cell response. While allo-HLA crossreactivity from viral specific memory T-cells is common, it is unclear if priming with allogeneic cells could conversely elicit a viral peptide/self-HLA restricted T-cell response. Firstly we used the previously described allo-HLA-B*44:02 crossreactivity by EBV peptide/HLA-B*08:01 restricted T-cells, to determine if allogeneic HLA stimulation can elicit a cytolytic immune response against Epstein-Barr virus. HLA-B*08:01^{+} HLA-B*44^{-} EBV seropositive PBMCs were stimulated with either HLA-B*44:02^{+} or HLA-B*44:03^{+} mismatched irradiated PBMCs in a 7-10 day mixed lymphocyte reaction. The stimulated responder cells were then evaluated for cytotoxicity using EBV peptide loaded autologous target cells and unloaded HLA-B*08:01^{+} EBV LCL target cells. PBMCs from EBV seropositive donors gained EBV specific cytolytic effector function following specific allo-HLA stimulation. Finally, as a proof-of-principle, we also elicited cytolytic CMV specific responses using allogeneic cell stimulation, to confirm that this technique can be used to elicit viral peptide/self-HLA restricted responses against any virus or specificity. Allogeneic cell stimulation used as a cell therapy may be a potential tool to augment an anti-viral T-cell response in patients with viral infection.

INTRODUCTION

Control of viral replication depends primarily on viral specific memory T-lymphocyte activity (1,2). In the normal course of viral infections, anti-viral immunity and non-infectivity correlates with the development of virus specific effector memory T-cells. Absence of HIV-specific CD8 T-cells is associated with progression to AIDS in HIV infected individuals (3), and use of lymphocyte targeted biological therapies has recently been associated with viral reactivation which may not respond to anti-viral antibiotics (4). For example, while allogeneic marrow depleted of T-cells prevents acute and chronic forms of graft versus host disease (GvHD) posttransplant, the risk of infections, particularly with Epstein-Barr virus (EBV) and cytomegalovirus (CMV), are increased (5). Furthermore, viral infection can cause severe morbidity and mortality, even in individuals without defined immune deficiency.

Currently there are no in-vivo autologous therapies to increase the number or effector function of viral specific T-cells. Antiviral prophylaxis can be toxic and does not result in an increase in viral-specific T-cells nor achieve long-term eradication. Adoptive transfer of 3rd party cell lines is associated with GvHD or failure due to allogeneic rejection (6), and is technically difficult (7). While antigen specific T-cell responses are actively maintained, they are reversible and short lived in the absence of antigen (8-10).

We have recently confirmed that alloreactivity from viral specific T-cells is common, and that the allo-HLA reactivity and virus specificity is mediated via the same T-cell receptor (TCR) (11). 45% of virus specific CD4 and CD8 T-cell clones were shown to be cross-reactive against allo-HLA molecules. For example, EBV infection in a HLA-B*08:01^{+} individual always selects for a dominant "public" Vb6S2 TCR (12), which cross-reacts against allo-HLA-B*44:02(13). We confirmed the previously described alloreactivity of this EBV EBNA3A specific T-cell (HLA-B8/FLRGRAYGL restricted) against allogeneic HLA-B*44:02 (11,14). Allo-HLA cross-reactivity was also shown for cytomegalovirus (CMV), varicella-zoster virus (VZV) and influenza virus specific T-cells (Amir) which express non-public TCRs.

A very high level of cross-reactivity against allo-HLA molecules is therefore an essential feature of the virus specific memory TCR (11-21). This allo-HLA crossreactivity by viral specific T-cells can be reproducibly predicted in-vitro. However, currently it is unknown if stimulation with allogeneic-HLA molecules could conversely specifically augment a HLA-restricted viral specific T-cell response.

The purpose of this study was therefore to assess if allogeneic HLA challenge could be a useful tool to augment a HLA-restricted anti-viral CD8 T-cell response, as determined by cytolytic functional assays. We used viral specific tetramers to confirm that in-vitro allogeneic challenge of EBV and CMV seropositive individuals, resulted in proliferation of human virus specific CD8 T-cells. Furthermore, we confirmed that this proliferation was associated with increased cytolytic effector function from the allo-HLA primed cells against viral antigens. Our proof-of-principle results demonstrate that allo-HLA stimulation may be a potential tool to augment cytolytic anti-viral CD8 T-cell effector responses in patients with viral infection.

RESULTS

EBV specific CD8 T-cells proliferate following allogeneic cell stimulation

To determine whether an allogeneic HLA challenge could specifically stimulate a viral specific CD8 T-cell response within whole blood, a modification of the MLC assay was used. EBV EBNA3A specific T-cells proliferated only in response to stimulation with HLA-B*44:02^{+}, and not HLA-B*44:03^{+}, mismatched irradiated PBMCs implying specific stimulation of cross-reactive viral specific T-cells by allogeneic HLA molecules (Figure 1). EBV EBNA3A specific T-cells did not proliferate in response to stimulation with allogeneic HLA-B*0801^{+} HLA-B*44^{-} PBMCs, excluding the possibility that the cells could be responding to EBV peptides contained within the culture medium or presented via stimulator cells (Data not shown). Proliferation was associated with a specific increase in the proportion of EBV EBNA3A specific T-cells within the CD8 T-cell compartment (Figure 2 and table 1), and no proliferation of HLA-A2/GLC or HLA-B8/RAK restricted T-cells was detected (data not shown); thereby excluding bystander proliferation and confirming the allo-HLA dependency of the stimulation. The observed response was abrogated when heterozygote HLA-B*08:01^{+} HLA-B*44:02^{+} responder PBMCs were used, consistent with specific thymic editing of the T-cell repertoire (Data not shown). These results confirm that viral specific CTL can directly recognize and proliferate in response to allogeneic HLA to which they are crossreactive and have never been exposed.

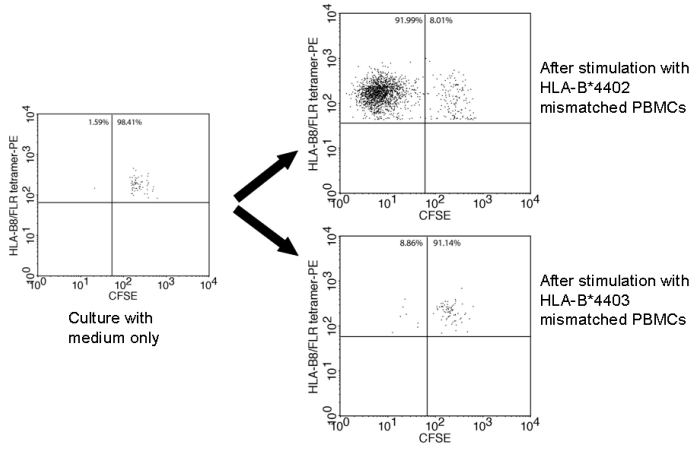

Figure 1. EBV specific CD8 memory T-cells specifically proliferate following allogeneic cell stimulation.

EBV EBNA3A specific T-cells are specifically stimulated to proliferate following 7-10 day in-vitro co-culture with heterozygous HLA-B*44:02^{+}, but not HLA-B*44:03^{+}, mismatched irradiated PBMCs. Bystander activation was excluded. FACS plots gated on total HLA-B8/FLR tetramer complex positive lymphocytes. Assay repeated 4 times with different responder-stimulator pairings, with similar results. A representative result is shown. Responder HLA-A*02,31; B*08:01,39; DRB1*03,16. HLA-B*44:02^{+} stimulator HLA-A*11,-; B*44:02,51; DRB1*12,15. HLA-B*44:03^{+} stimulator HLA-A*02,68; B*44:03,51; DRB1*08,13.

Viral specific T-cell proliferation may be greater following homozygote cell stimulation
To determine if homozygote allo-HLA is a greater stimulus for viral specific T-cells HLA-B*08:01^{+} B*44^{-} responder PBMCs were stimulated with either homozygous or heterozygous, HLA-B*44:02 or HLA-B*44:03, allogeneic cells. EBV EBNA3A specific T-cells accounted for 20.8% of the total CD8 T-cell population following homozygous HLA-B*44:02 allogeneic cell stimulation, but only 5.04% of the total CD8 T-cell population following heterozygous HLA-B*44:02 cell stimulation (Table 1 and Figure 2). The proportion of EBV EBNA3A specific CD8 T-cells was not significantly altered by homozygous or heterozygous allo-HLA-B*44:03 stimulation in the same assay (Table 1). The percentage of EBV EBNA3A specific CD8 T-cells prior to stimulation was 1.5%.

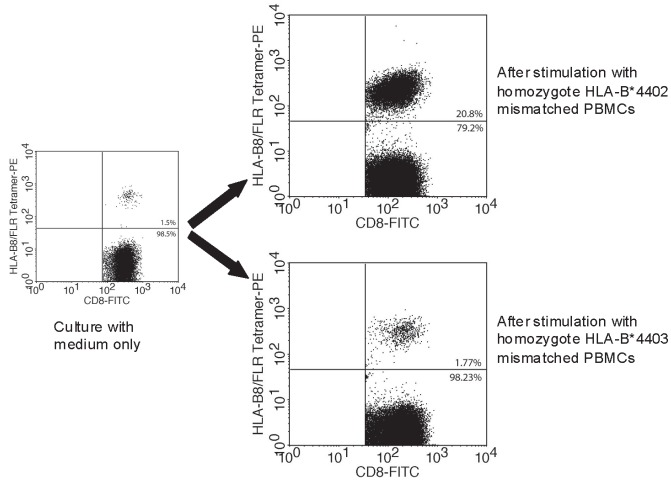

Figure 2. The proportion of EBV specific CD8 T-cells is specifically increased following allogeneic cell stimulation.
EBV EBNA3A specific CD8 T-cells accounted for 20.8% of total CD8 T-cells, following 8 day co-culture with homozygote HLA-B*44:02 mismatched irradiated PBMCs. The proportion of EBV EBNA3A specific CD8 T-cells was unaltered by co-culture with homozygote HLA-B*44:03^{+} PBMCs. FACS plots gated on total CD8 T-cell population. The primed responder cells shown here were then harvested and used as effector cells in the cytolytic assays shown in Figure 5. Responder HLA-A*01,02; B*08:01,-; DRB1*03,-. HLA-B*44:02^{+} stimulator HLA-A*02,68; B*44:02,-; DRB1*07,14. HLA-B*44:03^{+} stimulator HLA-A*02,32; B*44:03,-; DRB1*01,08.

Table 1. Viral specific T-cell proliferation may be greater following homozygote allogeneic cell stimulation.

	HLA-B*44:02	HLA-B*44:03
Heterozygous stimulation	5.04%	2.19%
Homozygous stimulation	20.80%	1.77%

HLA-B*08:01$^+$ responder PBMCs were stimulated with either homozygous or heterozygous, HLA-B*44:02 or HLA-B*44:03, allogeneic cells. The proportion of EBV EBNA3A specific CD8 T-cells was measured using viral specific tetrameric complexes and results are expressed as percentage of EBV EBNA3A specific CD8 T-cells within the total CD8 T-lymphocyte population. The percentage of EBV EBNA3A specific CD8 T-cells prior to stimulation was 1.5%. The responder PBMCs stimulated with HLA-B*44 homozygous allogeneic cells are shown in figure 2, and were harvested and used as the effector cells in the assays shown in figure 5.

Table 2. Screening for allo-HLA crossreactivity using pools of allogeneic cells.

	Medium	Pool 1 (S1-4)	Pool 2 (S5-8)	Pool 3 (S9-12)	Pool 4 (S13-16)
Responder 1 A2/NLV	-	-	+	-	+
Responder 2 A2/NLV	-	-	-	-	+
Responder 3 A2/NLV	-	-	-	-	-
Responder 4 B35/IPS	-	-	-	-	+

Pools of 4 different allogeneic cells were first used to screen for allo-HLA crossreactivity of CMV specific CD8 T-cells within whole blood, using CFSE staining of proliferating responder cells. The specific allogeneic cell giving the stimulation was then easily identified in a second assay. Specific allogeneic stimulation was associated with not only proliferation but also increased cytolytic activity against the original cognate viral antigen. Specific allogeneic cells stimulating a viral specific T-cell response were identifiable for most responders and specificities.
+ Specific proliferation detected.
– No proliferation detected.

CMV specific CD8 T-cells proliferate following allogeneic cell stimulation

To determine whether allo-HLA stimulation can elicit proliferation of T-cells specific for any viral peptide/self-HLA restriction of interest, we screened for responder CMV specific T-cell proliferation using pools of PBMC stimulator cells. Proliferation of CMV specific CD8 memory T-cells was detectable using pools of 4 different PBMC stimulators together (Table 2). The individual PBMC giving the specific stimulation was then easily determined in a second assay. For example, CMV pp65 specific T-cells (HLA-A2/NLV restricted) from a healthy donor (Responder 2) proliferated in response to a PBMC pool of 4 different PBMCs (Pool 4 - Figure 3a and table 2). The same responder was then tested individually against the stimulators present in the screening pool in order to identify the specific stimulator (Figure 3b). Proliferation was associated with a specific increase in the proportion of CMV pp65 specific T-cells within the CD8 T-cell compartment (Figure 4). Screening experiments were repeated multiple times with different responders and for different CMV CD8 T-cell specificities. Using this technique proliferation of HLA-A2/NLV and HLA-B35/IPS restricted CD8 T-cells from different responders was elicited (Table 2). Furthermore this stimulation is demonstrable without the need to generate viral specific T-cell clones from the responder, even when the viral specific T-cell of interest does not express a public TCR, thereby confirming that allogeneic cells stimulating viral-peptide/HLA restricted T-cells from any given responder are readily identifiable in the routine laboratory.

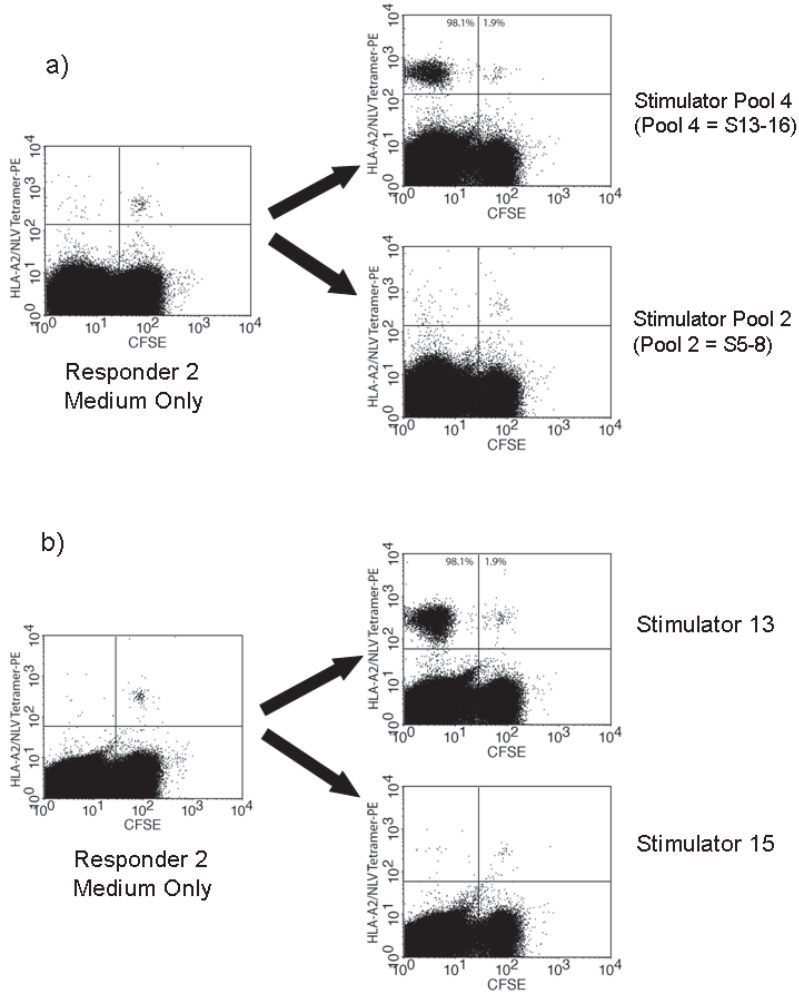

Figure 3. Screening for allo-HLA crossreactivity of viral specific memory T-cells.
(a) CMV pp65 specific CD8 Memory T-cells (A2/NLV restricted) from Responder 2 (R2) proliferate following stimulation with a pool of 4 PBMCs (Pool 4 containing stimulators 13-16), but not other pools of 4 different stimulator PBMCs (Pool 2 shown). (b) Responder 2 was then tested individually against all four stimulators present in pool 4 (S13-16). R2 proliferated only when stimulated with S13 and not when stimulated with the other 3 stimulators present in pool 4 (S15 shown). Thereby confirming the CMV pp65 specific T-cells from responder 2 were specifically stimulated by only S13 allogeneic cells. HLA typing of responder 2 HLA-A*02,11; B*35,40; DRB1*11,15. Stimulator 13 HLA-A*02:01,02:05; B*18,50; DRB1*11,13. Stimulator 15 HLA-A*23,29; B*15,53; DRB1*11,13.

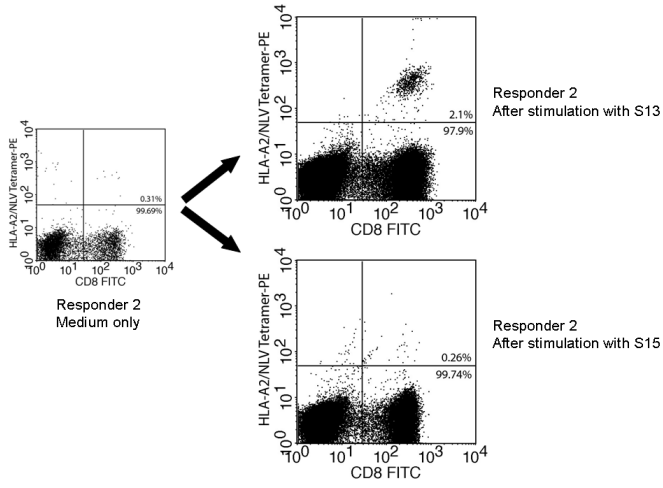

Figure 4. The proportion of CMV specific CD8 T-cells is specifically increased following allogeneic cell stimulation.
CMV pp65 specific CD8 T-cells accounted for 2.1% of total CD8 T-cells from responder 2 (R2), following 8 day co-culture with Stimulator 13 (S13). The proportion of CMV pp65 specific CD8 T-cells was unaltered by co-culture with Stimulator 15 (S15) or IL-2 containing medium alone. The primed responder cells shown here were then harvested and used as effector cells in the cytolytic assay shown in Figure 6. HLA typing of responder 2 HLA-A*02,11; B*35,40; DRB1*11,15. Stimulator 13 HLA-A*02:01,02:05; B*18,50; DRB1*11,13. Stimulator 15 HLA-A*23,29; B*15,53; DRB1*11,13.

EBV and CMV specific CD8 memory T-cells gain viral peptide/self-HLA restricted cytolytic effector function following specific allo-HLA stimulation

For viral protection it is essential that the proliferation of viral specific T-cells following allogeneic stimulation is associated with a gain of cytolytic effector function against the original viral peptide/self-HLA restricted target antigen. We therefore performed a cytolytic assay using responder HLA-B*08:01^{+} EBV seropositive healthy donor PBMCs following in-vitro stimulation with either homozygote HLA-B*44:02 or HLA-B*44:03 mismatched irradiated PBMCs, and with viral peptide loaded autologous cells and unloaded EBV transformed B-cells (EBV LCLs) as target cells. Following 7-10 day stimulation with HLA-B*44:02 homozygote mismatched irradiated PBMCs, primed responder cells from a HLA-B*08:01^{+} EBV seropositive healthy donor showed increased cytolytic effector function against both HLA-B*08:01^{+} EBV LCLs and FLR peptide loaded autologous target cells, but not HLA-B8^{-} EBV LCLs nor RAK peptide loaded autologous target cells (Figure 5); as compared to the same PBMCs co-cultured with either HLA-B*44:03 mismatched PBMCs or culture with IL-2 containing medium alone. This increased cytolytic effector function was associated with proliferation

and an increase in the proportion of EBV EBNA3A specific CD8 T-cells (Figure 2). Likewise, specific stimulation of CMV specific CD8 T-cells with allo-HLA resulted in increased cytolytic effector function against CMV peptide loaded autologous cells (Figure 6). Once again confirming that allogeneic HLA challenge can indeed increase the (in-vitro) cytolytic effector function of human viral specific T-cells against their original cognate viral antigen. We argue these proof-of-principle results may have important implications for treatment of viral infections, if confirmed in-vivo.

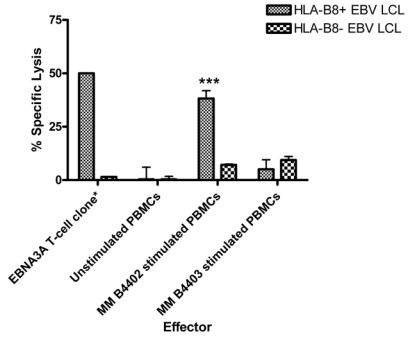

Figure 5a. EBV specific cytolytic effector function of allo-HLA primed cells using EBV LCL target cells. PBMCs from a HLA-B*08:01$^+$ EBV seropositive donor gain EBV specific cytolytic effector function following allogeneic HLA-B*44:02$^+$ cell stimulation, ***P<0.0001 versus HLA-B*08$^-$ EBV LCL. Unstimulated HLA-B*08:01$^+$ PBMCs and HLA-B*44:03 stimulated HLA-B*08:01$^+$ PBMCs do not demonstrate cytolytic effector function against HLA-B*08*01$^+$ EBV LCLs. Effector:target ratio 50:1, targets 2000. *Positive control EBNA3A T-cell clone is previously described (14), and responder PBMCs used in this assay are also obtained from the same donor. HLA typing of responder PBMCs and EBNA3A T-cell clone HLA-A*01,02; B*08:01,-; DRB1*03,-. HLA-B8$^+$ EBV LCL HLA-A*01,-; B*08:01,-; DRB1*03,-. HLA-B8$^-$ EBV LCL HLA-A*03,-; B*07,-; DRB1*15,-.

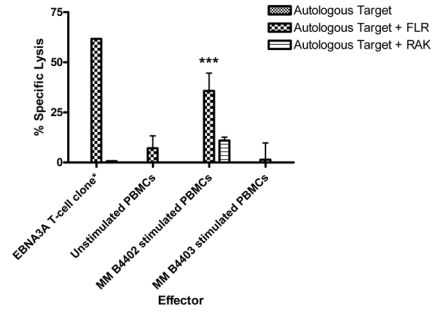

Figure 5b. EBV specific cytolytic effector function of allo-HLA primed cells using viral peptide loaded autologous target cells. PBMCs from a HLA-B*08:01$^+$ EBV seropositive donor gain HLA-B8/FLR restricted cytolytic effector function following allogeneic HLA-B*44:02 stimulation. ***P=0.0094 versus RAK peptide loaded autologous cells. Unstimulated HLA-B*08:01$^+$ PBMCs and HLA-B*44:03 stimulated HLA-B*08:01$^+$ PBMCs do not demonstrate cytolytic effector function against FLR peptide loaded autologous cells. Effector:target ratio 50:1, targets 2000. *Positive control EBNA3A T-cell clone is previously described (14), and responder PBMCs used in this assay are also obtained from the same donor. HLA typing of responder PBMCs, autologous target PBMCs and EBNA3A T-cell clone HLA-A*01,02; B*08:01,-; DRB1*03,-.

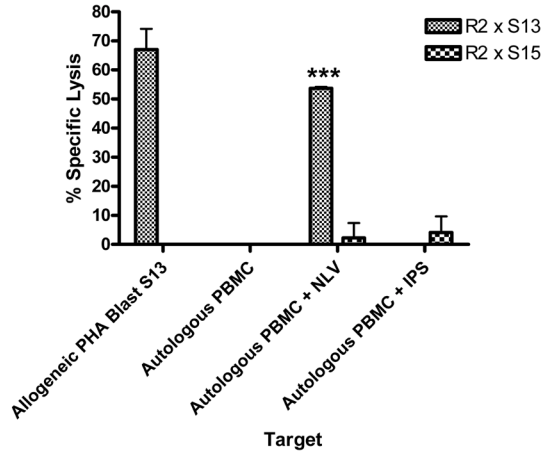

Figure 6. CMV specific cytolytic effector function of allo-HLA primed cells.
PBMCs from a CMV seropositive HLA-A*02:01^{+} donor (R2) gain HLA-A2/NLV restricted cytolytic effector function following heterozygote allogeneic cell stimulation with stimulator 13 cells (R2 x S13). ***P<0.0001 versus IPS loaded autologous cells. S15 stimulated PBMCs do not demonstrate cytolytic effector function against NLV peptide loaded autologous cells (R2 x S15). A strong secondary response against S13 is demonstrated from R2 responder cells primed with S13 (Positive Control), but not S15. Effector:target ratio 100:1, targets 2000. Responder 2 HLA-A*02,11; B*35,40; DRB1*11,15. Stimulator 13 HLA-A*02:01,02:05; B*18,50; DRB1*11,13. Stimulator 15 HLA-A*23,29; B*15,53; DRB1*11,13.

DISCUSSION

This study demonstrates that human viral specific memory T-cells gain cognate viral antigen specific cytolytic effector function following stimulation with allogeneic HLA molecules against which they are crossreactive. Stimulation of peripheral blood from a non-sensitized HLA-B*08:01^{+} EBV seropositive healthy donor with HLA-B*44:02 mismatched irradiated PBMCs increases (in-vitro) cytolytic effector function against EBV. Furthermore, we show this technique can be used to elicit cytolytic effector function against any potential viral antigen, as shown for CMV. These results provide proof-of-principle evidence that specific allogeneic cell therapy could be useful for treatment of viral infections.

The importance of our findings are reinforced by functional studies showing that the proliferation of EBV and CMV specific CD8 memory T-cells corresponded with a specific increase of cytolytic effector function against viral peptide loaded autologous cells, which was not detectable without specific allo-HLA stimulation. Cytolysis of the EBV LCLs by the HLA-B*44:02 primed effector cells suggests that virus infected cells can spontaneously process and present viral peptides via HLA class I molecules in the course of normal infection, and that the

amount of peptide present is sufficient to trigger killing from allo-HLA primed effector cells.

For the EBV specific cytotoxicity assays we used homozygote HLA-B*44:02^{+} PBMCs to stimulate the EBV EBNA3A specific T-cell response, as this should provide a larger antigenic stimulus. However our CMV specific cytotoxicity assay clearly demonstrates that heterozygote cell stimulation is sufficient to prime viral specific cytolytic effector functions. Nonetheless, further studies may be required to determine if homozygote allogeneic cell therapy truly provides a significantly better stimulation over heterozygote allogeneic cell therapy.

EBV infection in a HLA-B*08:01^{+} HLA-B*44^{-} individual selects for a public BV6S2 TCR which cross-reacts against allogeneic HLA-B*44:02 (12). While not all viral specific immune responses give rise to a public TCR, the allo-HLA crossreactivity of virus specific T-cells from a given individual can be easily detected in-vitro using techniques we have described here and elsewhere (11,14). Indeed, successful stimulation of cytolytic effector function against CMV antigen reveals that this technique can be reproducibly used to elicit T-cell cytolytic effector function against any virus or specificity. Furthermore, identification of the allogeneic cells that stimulated the anti-viral cytotoxicity did not require generation of virus specific T-cell clones. Techniques described here should therefore be reproducible in most routine laboratories.

We have confirmed that these effects are mediated by leukocytes present in the blood components and are related to the expression of HLA antigens. We used irradiated isolated PBMCs for stimulation of the viral specific memory T-cells thereby excluding any contributions by plasma, platelets and/or erythrocytes. Therefore we suggest allogeneic cell therapy should be investigated using only isolated leukocytes as stimulators.

Immunological memory is one of the hallmarks of the adaptive immune response. Functional viral specific memory T-cells are essential for proper host defense as in the periphery infected cells can now be targeted for immediate killing, both during the initial infection and on subsequent re-infection or viral reactivation.

Results presented here suggest that specific allogeneic cell therapy could prime and/or maintain viral specific memory. The proportion of EBV EBNA3A specific T-cells in the CD8 compartment increased from 1.5% to 20.8% following stimulation with homozygote HLA-B*44:02^{+} allogeneic cells and the proportion of CMV pp65 specific T-cells from 0.31% to 2.1% following heterozygote allogeneic cell stimulation. Data from preliminary clinical studies suggest that CMV specific CD8 T-cell levels greater than 1x107/L of peripheral blood may correlate with protection (22), therefore the total number of viral specific T-cells induced by proliferation following allogeneic cell stimulation may be important in isolation.

However, others have also shown that the memory T-cell state of readiness is actively maintained and reversible, requiring ongoing specific TCR signaling (8,10). Transfer of memory T-cells to naïve mice, in the presence or absence of priming antigen, reveals that maintenance of T-cell memory is short lived in the absence of TCR mediated signaling (8). Furthermore, recently activated memory T-cells can bypass the requirement for CD28/CD80/CD86 co-

stimulation, as compared to resting memory T-cells that are still dependent on CD28 triggering for their activation (23). Although at baseline in our EBV specific assays 1.5% of CD8 T-cells in the peripheral blood of the individual were EBV EBNA3A specific T-cells, prior to allo-HLA-B*44:02 stimulation no cytolysis of FLR peptide loaded autologous cells could be detected, suggesting allogeneic cell priming was important to induce the observed cytolysis. Therefore, the allogeneic stimulation used in our assays may also have increased cytolytic effector function of the viral specific T-cells via triggering TCR signaling and/or abrogating co-stimulation requirements, irrespective of the changes to the total number of cells.

To evade these cytolytic CD8 T-cell responses viruses have evolved many different strategies for immune evasion (24-26), most of which interfere with the various steps necessary for MHC class I restricted antigen presentation. For example, CMV evades MHC class I antigen presentation by reducing the stability of class I heavy chains (27) and also by dislocating MHC class I heavy chains from the endoplasmic reticulum (28). The co-ordinated function of murine CMV genes can completely inhibit CTL lysis (29). Amongst others, the EBV EBNA1 protein contains an element that interferes with its proteasomal proteolysis and the HSV ICP47 protein inhibits the TAP complex (30-31). Many other viral immune evasion strategies are also described (32-36).

Allogeneic cell therapy may be capable of bypassing all these viral strategies of immune evasion as the viral specific memory T-cells are directly stimulated via molecular mimicry (37). The allo-HLA molecule against which the virus specific T-cell is crossreactive is constitutively expressed and occupied by the stimulating self-peptide. Theoretically allogeneic cell therapy could even stimulate additional virus specific responses other than the specificity of interest. Steffens and colleagues demonstrated that pre-emptive CMV specific CD8 T-cell immunotherapy, guided by viral DNA load, prevented lethal disease and reduced the risk of virus recurrence (38). Similarly, allogeneic cell therapy may ensure a high proportion of pre-existing activated virus specific memory T-cells to prevent disease and accelerate the resolution of productive infection.

HIV specific effector memory CD8 T-cells are present in most HIV infected individuals and play a critical role in controlling viral replication and disease progression, however HIV is also highly efficient at evading immune responses (39). Recent data demonstrate that HIV escape mutations may impair dendritic cell function (3) and that the HIV-1 Vpu protein modulates MHC class II presentation (40), thereby possibly impairing later CD4 and/or CD8 T-cell responses to the same and other epitopes. High viraemia is also associated with in-vivo downregulation of MHC class I in rhesus macaques infected with SIV (41). The maintenance of early differentiated, highly avid HIV specific CD8 T-cells by allogeneic cell therapy could induce a non-progressive course of the disease. Further in-vitro studies are warranted using responder PBMCs from HIV infected individuals.

Poorly controlled viral infections are also associated with malignancy. Post-transplant lymphoproliferative disease (PTLD) is a well recognized complication of both solid organ and allogeneic bone marrow transplantation, and is associated with a deficient cellular response from the host to EBV infected B-cells. Most PTLD occurring in solid organ setting arise from

recipient cells, therefore, allogeneic HLA-B*44:02 cell therapy may elicit an anti-tumour response in a HLA-B*08:01^{+} recipient with PTLD. Results presented here strongly support this hypothesis.

Results presented here demonstrate the stimulation of cytolytic effector function from pre-existing memory T-cells. It is unclear if allogeneic cell therapy could also be used to stimulate a de-novo viral specific response from naïve T-cells. However it is likely that stimulation of de-novo viral specific T-cell responses using allogeneic cells, from naïve T-cells, would require additional co-stimulatory factors than those provided by irradiated allogeneic PBMCs alone (42-44).

Finally, we acknowledge that further work is required before allogeneic cell therapy can be used in the clinical setting to treat viral infections. In these experiments we have used healthy blood donors as responder PBMCs, not cells from immunosuppressed patients. While infusion of irradiated leukocytes should not be associated with chimerism or engraftment, this possibility should be considered in an extremely immunodeficient recipient. Repeated allogeneic cell therapy may cause sensitization of a recipient to future transplantations. Nonetheless results demonstrated here suggest cell therapy may have potential as an alternative to adoptive transfer or pharmacological therapy to treat viral infections.

The high frequency of allo-HLA crossreactivity by viral specific T-cells in the transplantation setting is increasingly being recognised. We provide (in-vitro) evidence that allogeneic cell therapy may be useful to conversely stimulate a beneficial anti-viral cytolytic effector response for treatment of viral infection. This proof-of-principle technique could provide important future options for the treatment of viral infections. This approach should be investigated further.

MATERIALS AND METHODS

Preparation of responder, stimulator and target cells

Responder and stimulator cells were both obtained using blood samples from healthy donors, after informed consent. PBMC were isolated from heparinized blood by standard density gradient centrifugation, and were subsequently cryopreserved until use. Epstein-Barr virus-transformed B-cell lines (EBV LCLs) were generated using standard procedures, and were cultured in Iscoves Modified Dulbeccos Medium (IMDM, Cambrex) with 10% fetal calf serum (FCS). The HLA type of all cells used in our experiments was determined molecularly by SSO and SSP genotyping at the Leiden University Medical Centre, Dept of Immunohematology and Blood Transfusion, the Netherlands.

Proliferation Assays for EBV EBNA3A specific T-cell responses

For the proliferation assays 1x10*6 Carboxyfluoresceinsuccinimidyl ester (CFSE)-labeled PBMC from a HLA-B*08:01^{+} HLA-B*44^{-} EBV seropositive healthy donor, were co-cultured with 1x10*6 HLA-B*44:02^{+} or HLA-B*44:03^{+} mismatched irradiated PBMCs (3000 Rad) also from healthy donors, in a 24 well flat bottom plate. Cells were incubated for 7-10 days in IMDM culture medium with 15% human serum and IL-2 (25IU/ml). Then, fluorescence activated cell sorter analysis was performed, after staining the cells with CD8-APC (Becton-Dickinson) and PE-labeled HLA-B8/FLR tetrameric complexes to detect cell division. In all experiments HLA-A2/GLC and HLA-B8/RAK tetrameric complex staining served as negative controls. The proportion of EBV EBNA3A specific T-cells within the total CD8 T-cell population of a single responder was also determined before and after homozygous vs. heterozygous allo-HLA-B*44 cell stimulation in a separate assay without CFSE labeling. The HLA typing of the selected responder-stimulator examples is given below the figures.

Proliferation Assays for CMV specific T-cell responses

To determine if allo-HLA stimulation could elicit an anti-viral response against any virus or specificity, we had to first determine a new method whereby specific allogeneic cells stimulating the proliferation of viral specific T-cells from any given individual could be identified. 1x10*6 Carboxyfluoresceinsuccinimidyl ester (CFSE)-labeled PBMC from CMV seropositive healthy donors, were first co-cultured with a pool of 1x10*6 total mismatched irradiated PBMCs (3000 Rad) from 4 different healthy donors (0.25x10*6 cells of each individual stimulator), in a 24 well flat bottom plate. Each responder was screened against 4 different pools of PBMCs. The 16 total different allogeneic stimulator cells were selected to cover the most common occurring HLA molecules. Cells were incubated for 7-10 days in RPMI culture medium with 15% human serum and IL-2 (25IU/ml). Fluorescence activated cell sorter analysis was performed after staining the cells with PE-labeled CMV specific tetrameric complexes to detect cell division. If proliferation of CMV specific cells was detected following stimulation with a screening pool of 4 different allogeneic PBMCs, then the same responder PBMCs were tested individually against the 4 stimulator PBMCs to determine which allogeneic cell(s) elicited proliferation of the CMV specific T-cells. The proportion of CMV specific tetramer positive T-cells within the total CD8 T-cell population were also determined before and after al-

logeneic cell stimulation using routine FACS analyses. The CMV seropositive responder cells were then stimulated with the individual relevant PBMCs (or control) in a new assay (without CFSE labeling), following which the allo-HLA primed responder cells were harvested and used as effector cells in the cytotoxicity assays (see methods below). The HLA typing of the selected responder-stimulator examples is given below the figures.

Cytotoxicity Assays

To confirm that allogeneic cell stimulation resulted in increased viral specific cytolytic effector function, not just proliferation, from the stimulated PBMCs we performed cytolytic assays using autologous cells loaded with the relevant viral peptide or unloaded EBV LCLs as target cells. Responder PBMCs from EBV or CMV seropositive healthy donors were first specifically stimulated in a 7-10 day mixed lymphocyte reaction with allo-HLA mismatched irradiated cells to stimulate a viral specific memory T-cell of interest (see methods above). The stimulated PBMCs were then evaluated for cytotoxicity by incubating serial dilutions with 2000 viral peptide loaded autologous target cells or EBV LCL target cells, in a 4 hour ^{51}Cr release assay. Cognate viral peptide or control viral peptide was directly added to the autologous target cells and incubated for 60 minutes, simultaneously with chromium incubation, and then washed three times. Supernatants were harvested for gamma counting: *per cent-specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.*

Statistical Analysis

Values for specific lysis are presented as the mean of triplicate wells with standard deviation. Comparative analyses are nonparametric (unpaired) t-tests, and $P<0.05$ is considered significant. Statistics are derived using Graph Pad Prism 4 for Windows (version 4.02, 2004).

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose.

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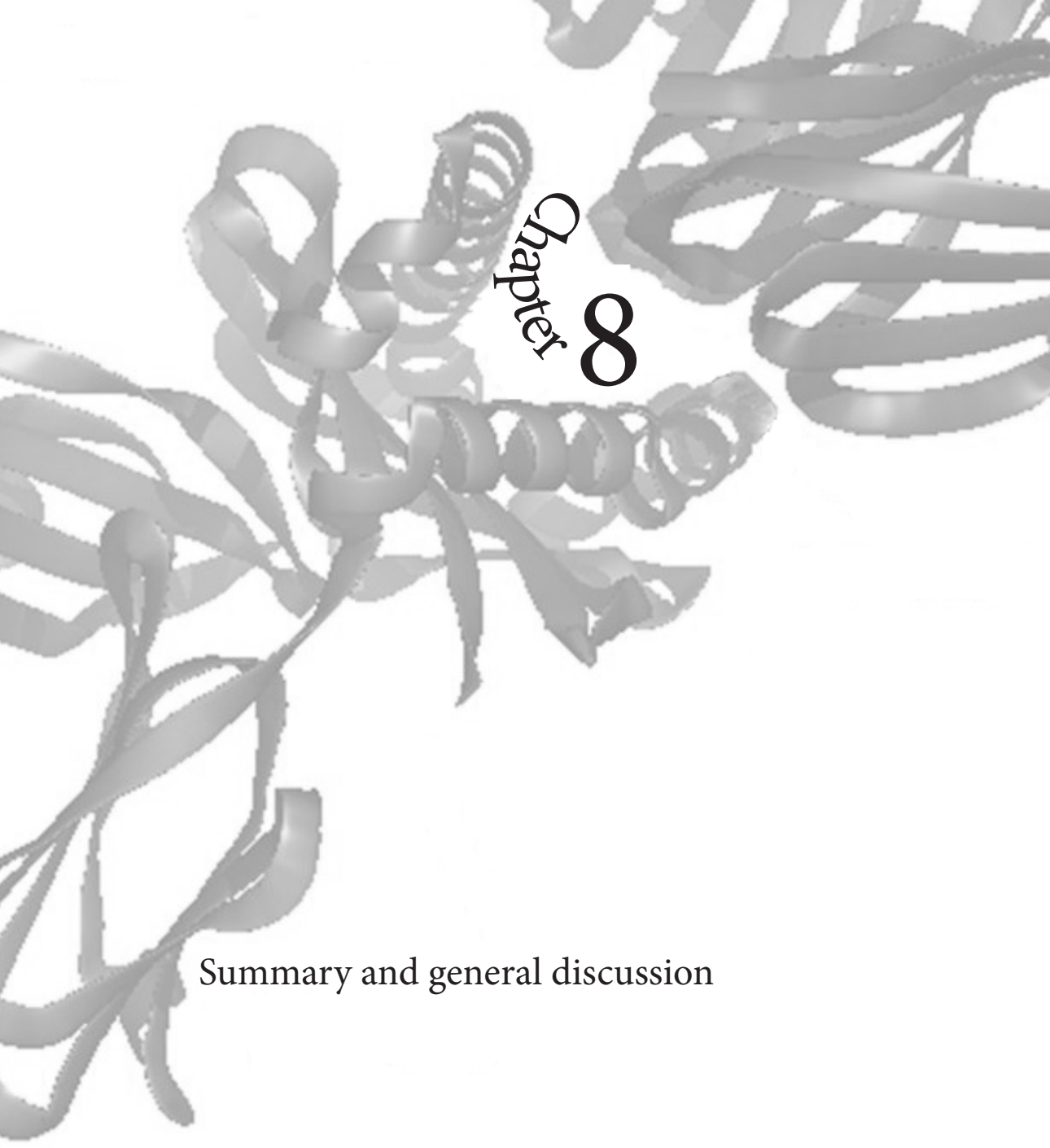

Chapter 8

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

T-cell memory is a hallmark of the adaptive immune response and is critical for protective immunity against pathogens. However, many studies reveal that pre-existing memory T-cells also pose a potent barrier to transplantation tolerance, even in non-sensitized individuals (1-12). It was previously unclear how these alloreactive memory T-cells arose in non-sensitized organ recipients.

A possible explanation is that alloreactive memory T-cells arise via exposure to environmental antigens (13-19). Recipients might have had immunological contact with pathogens that lead to crossreactive immune responses with the HLA mismatches. Limited evidence for this phenomenon exists in both mouse and human models, but was thought to be a rare occurrence. Allo-HLA crossreactivity from viral specific memory T-cells may have important clinical implications for the alloimmune response after transplantation because memory T-cells have lower activation requirements, no need for CD4 T-cell help and can have immediate cytotoxic effector function as compared to their naïve counterparts (20-24). Therefore if truly alloreactive in-vivo, pre-existing memory T-cells may represent a common source of acute and/or chronic rejection and be a major obstacle to tolerance induction.

The molecular mechanisms that might underlie such crossreactivity were also unexplained.

In order to study the effect of environmental exposure on the alloreactive T-cell repertoire viral specific T-cell clones were single cell sorted based on viral peptide/HLA tetrameric complex staining, from healthy (non-sensitized) individuals. This technique proved to be the basis of an effective system for detection of allo-HLA crossreactivity (heterologous immunity) by viral specific memory T-cells. Results presented in this thesis indicate that heterologous immunity is much more common than anticipated. Furthermore, it has been confirmed that the virus specificity and alloreactivity are mediated by the same TCR and that the allo-HLA crossreactivity can not be predicted based solely on immunological history and HLA mismatch alone. Future clinical studies are now clearly warranted.

Several novel and important discoveries for the field of transplantation have been made in this thesis and are summarized below:

Alloreactivity from viral specific memory T-cells is common
This thesis confirms that allo-HLA responses from viral specific memory T-cells are in fact far more common than anticipated (25) (Chapter 3). 80% of virus specific T-cell lines and 45% of virus specific T-cell clones crossreacted against individual allo-HLA molecules. Allo-HLA crossreactivity was shown from EBV, CMV, VZV and influenza specific T-cell clones. Multiple viral specific CD8 T-cell clones were shown to be alloreactive against allogeneic class I molecules, and likewise several viral specific CD4 T-cell clones were shown to crossreact against allogeneic class II molecules. Surprisingly, two separate CMV specific, class I restricted T-cell clones recognized allogeneic class II molecules (25).

The fact that the same TCR complex mediates both virus specificity and allo-HLA crossreactivity has been confirmed by TCR PCR, viral tetramer inhibition and TCR transfection assays (25-26) (Chapters 2 and 3). Vaccination with live attenuated virus can also induce alloreactive memory T-cells, as shown in chapter 5 of this thesis for varicella vaccination.

The importance of these findings are reinforced by functional studies showing that the various viral specific CD8 T-cell clones can lyse multiple different target cells expressing the target HLA molecule, in a 4 hour cytotoxicity assay (25-26) (Chapters 2, 3 and 5). Further examination of MHC class II restricted pathogen specific CD4 T-cells is required, as it is likely that this T-cell population also plays a dominant role in allograft rejection (10,27-28). Ex-vivo staining for the presence of viral specific T-cells within rejecting kidney or GvHD biopsy samples may help confirm the clinical relevance of these in-vitro crossreactive allo-HLA responses.

Human viral specific memory T-cells reported to give allo-HLA crossreactivity are summarized in table 1 of chapter 6 of this thesis (29).

HLA alloreactivity by viral specific memory T-cells is (self) peptide dependent
It is now generally accepted that alloreactive T-cells recognize allo-HLA molecules presenting self-peptides (25,30-33). Macdonald and colleagues have provided clear structural evidence that self-peptide dependent molecular mimicry underpins the alloreactivity of the EBV EBNA3A specific T-cell against allogeneic HLA-B*44:02 (31). The EBV EBNA3A specific T-cell crossreactivity against allogeneic HLA-B*44:02 is dependent on presentation of EEYLQAFTY peptide (derived from the ABCD3 protein) by the target tissue.

In this thesis, the peptide dependence of the allo-HLA crossreactivity from viral specific memory T-cells is reinforced by differing potency of the alloreactivity exerted by virus specific T-cells against different cell targets. For example, a VZV specific HLA-A2 restricted T-cell clone recognizes allogeneic HLA-B*57:01 expressing EBV LCLs, PHA Blasts and monocyte derived DCs, but does not recognize HLA-B*57:01 expressing B-cells, T-cells, monocytes nor fibroblasts (25) (Chapter 3). Therefore allo-HLA expression is not solely sufficient to elicit target killing. Presumably the cell types that are not recognized do not present the relevant self-peptide.

In contrast to allogeneic HLA-B*44:02$^+$ EBV LCLs and SALs, allogeneic HLA-B*44:02$^+$ proximal tubular epithelial cells (PTECs) are poor targets for EBV EBNA3A specific CD8 T cells (Chapter 4). However the specific lysis of HLA-B*44:02 expressing PTECs was greatly increased by exogenous EEY peptide loading. HLA-B*44:02 expressing HUVECs were only killed by an EBV EBNA3A clone when loaded with exogenous EEY peptide. This confirms that kidney specificity of the alloresponse from the EBV EBNA3A specific T-cell is dependent on endogenous self-peptide processing and presentation.

Peptide dependent alloreactivity suggests that immunomodulating techniques could be used to inhibit these harmful T-cell clonotypes, as suggested by Burrows (30). Further studies are required.

HLA alloreactivity by viral specific memory T-cells can be tissue specific
Tissue specific alloresponses by viral specific memory T-cells are described in chapters 3,4 and 5. Differences in peptide antigen processing and presentation could account for this tissue specific alloreactivity. For example, EBV LCLs, PHA Blasts and K562 cells constitutively express the immunoproteosome which may generate novel antigenic allopeptides.

To further investigate tissue specificity by EBV EBNA3A specific T-cells, long peptides from the ABCD3 protein containing the EEYLQAFTY epitope were generated. Cleavage products from these long peptides were compared following immunoproteosome vs constitutive proteosome digestion, using mass spectrometry analysis. Results of the proteosomal digestion are shown in tables 1a and b.

These results are unexpected given the tissue specificity reported in chapter 4 of this thesis. EBV LCL and PHA blasts were efficiently targeted by EBV EBNA3A specific T-cells, whereas endothelial and epithelial cells were poor targets. If these differences were attributable to proteosome peptide processing then theoretically the immunoproteosome (present in EBV LCLs and PHA Blasts) should generate relatively more EEYLQAFTY peptide, as compared to the constitutive proteosome. In fact the epitope is a target of proteosomal cleavage and there may therefore be little EEYLQAFTY peptide available for presentation on the cell surface in all cell lines. Alternatively the EEYLQAFTY peptide may not be the natural ligand. Nonetheless, these results nicely demonstrate how proteosomal digestion could alter the self-peptide (allopeptide) repertoire presented on allo-HLA molecules. Further antigenic processing studies are clearly warranted.

Alternatively, differences in expression of a protein that contains a peptide capable of competing with an antigenic peptide for the peptide-binding groove of the allogeneic molecule could also cause the tissue specific alloreactivity reported in chapter 4. HLA-B*44:02 is a highly tapasin-dependent HLA molecule (34-35) and therefore limited tapasin expression in PTECs and/or HUVECs could decrease EEY peptide presentation in these cell lines. However tapasin mRNA is strongly induced in endothelial cells following IFNγ treatment (36), and IFNγ treatment did not increase the targeting of HUVECs in our assays despite inducing elevated HLA-B44 expression.

Table 1a. EEYLQAFTYYKMGN peptide digestion

Res	i-prot(%)	c-prot(%)	Peptide
1--14	50.1	44.2	E E Y L Q A F T Y Y K M G N
1--4	13.5	16.0	E E Y L
1--6	4.1	5.1	E E Y L Q A
1--9	4.5	3.0	E E Y L Q A F T Y
1--10	8.2	8.9	E E Y L Q A F T Y Y
2--11	3.7	3.9	E Y L Q A F T Y Y K
5--10	6.9	7.1	Q A F T Y Y
6--10	4.7	5.3	A F T Y Y
7--10	4.0	5.3	F T Y Y

Table 1a & 1b: Immunoproteosome vs. constitutive proteosome digestion of ABCD3 protein.
Long peptides from the ABCD3 protein, containing the EEYLQAFTY epitope, were generated and then digested using the two different proteosomes. Peptide products were analysed using mass spectrometry, after 4 hours incubation. (Table 1a) Long peptide EEYLQAFTYYKMGN - The EEYLQAFTY epitope was generated by both proteosomes however accounted for less than 5% of the long peptide cleavage product. Multiple cleavage points were found within the epitope. (Table 1b) Long peptide TKYLYEEYLQAFTYYKMGN – The long peptide was completely degraded and the EEYLQAFTY epitope was not generated. The EEYLQAFTY epitope was a cleavage target for both proteosomes with all peptide products generated from cleavage of the long peptide within the epitope (Residues 6-14). Res= Residues. i-prot=Immunoproteosome. c-prot=constitutive proteosome. %= Results expressed as percentage of total detected peptide products.

Table 1b: TKYLYEEYLQAFTYYKMGN peptide digestion

Res	i-prot(%)	c-prot(%)	Peptide
1--19	0	0	T K Y L Y E E Y L Q A F T Y Y K M G N
1--8	15.3	10.2	T K Y L Y E E Y
1--9	22.2	26.8	T K Y L Y E E Y L
1--11	0	5	T K Y L Y E E Y L Q A
2--6	2.8	4.6	K Y L Y E
2--8	3.9	0	K Y L Y E E Y
2--9	0	3.8	K Y L Y E E Y L
2--11	1.9	0	K Y L Y E E Y L Q A
3--10	3.9	0	Y L Y E E Y L Q
6--14	0	0	E E Y L Q A F T Y
7--15	2.1	0	E Y L Q A F T Y Y
8--14	5.2	6.2	Y L Q A F T Y
9--19	12.7	5.5	L Q A F T Y Y K M G N
10--14	2.2	1.8	Q A F T Y
10--15	5.1	10.4	Q A F T Y Y
10--18	6.2	4.2	Q A F T Y Y K M G
10--18	9.0	6.0	Q A F T Y Y K M G
11--16	5.1	10.4	A F T Y Y K
12--15	2.1	4.8	F T Y Y

Viral specific T-cell responses may not give predictable allo-HLA crossreactivity
Unlike the public BV6S2 TCR response against FLR peptide presented on HLA-B8, immune responses against other common pathogens are not so immunodominant and memory CD8 T-cells generated following viral infections often demonstrate a wide diversity of Vβ usage and therefore allo-HLA crossreactivity. Several examples of differing alloresponses from T-cell clones with the same viral peptide/HLA restriction are reported in this thesis (25) (Chapters 3, 5 and 7).

Variable allo-HLA crossreactivity by T-cell clones sorted from the same individual with the same specificity, but different TCR Vβ usage, was also reported in this thesis (25) (Chapter 3). Single cell sorting of VZV IE62 specific T-cells from an individual with VZV infection generated three different clones with usage of Vβ 21.3, Vβ 14 and an undetermined Vβ. These T-cell clones cross-reacted against allo HLA-A*02:05, HLA-B*55:01 and HLA-B*57:01 respectively. Demonstrating how a single viral peptide/HLA restricted immune response can generate different clonotypes with differing allo-HLA crossreactivity within the same individual.

It is currently not known if viral specific T-cells from different individuals with the same specificity and the same Vβ usage will always demonstrate similar allo-HLA crossreactivity. This knowledge is essential in order to be able to predict (un)acceptable mismatches based on donor-recipient HLA mismatches and immunological history of the recipient. At the current point in time functional assays, such as those described in this thesis, are required to determine if a certain HLA mismatch is a target for memory T-cells in a given individual. HLA matching remains the best predictor of long-term renal graft survival.

HLA alloreactivity likely occurs via molecular mimicry
The multiple mechanisms of T-cell receptor crossreactivity have been reviewed extensively by others (37-40). Despite peptide/HLA diversity and TCR plasticity, these T-cell responses always exhibit exquisite HLA and peptide specificity.

Work presented here strongly supports molecular mimicry, but not structural degeneracy, as the mechanism of TCR crossreactivity from viral specific memory T-cells. Differing potency of the virus specific T-cells against different cell targets, as reported in chapters 3, 4 and 5, is consistent with a TCR specifically crossreacting against single (or limited) self-peptide(s) presented on an allo-HLA molecule. If a viral specific TCR was crossreactive against an allo-HLA molecule via structural degeneracy then the peptides presented via the allo-HLA molecule should be irrelevant and the T-cell would recognize all allo-HLA expressing tissue cells equally.

Previous viral (pathogen) infection is critical to induction of the alloreactive T-cells
In this thesis we show that virus specific memory T-cells can demonstrate immediate cytolytic effector function against allogeneic HLA molecules in cytotoxicity assays (25-26) (Chapter 2,

3 and 5). A CCR7^{+} CD45Ra^{+} naive T-cell with the same TCR (e.g. from a seronegative individual), upon first contact with alloantigen, will secrete only IL-2, is not cytolytic and requires CD4 T-cell and B-cell help within the germinal centre to initiate an immune response. In a pilot study we found that stimulation of HLA-B8^{+} B44^{-} cord blood T-cells with HLA-B*44:02^{+} irradiated blood cells does not result in an alloresponse by HLA-B8/FLR specific naïve T-cells. Naïve T-cells recognizing an alloantigen without the appropriate co-stimulatory signals and T-cell help may gain regulatory function, be deleted or become anergic (41-42). This illustrates the critical importance of previous viral infection to the activation of alloreactive T-cells.

Consistent with this theory cord blood T cells are less able to mediate GvHD than marrow derived T-cells because of their naïve status (41,43).

Selective therapies to inhibit alloreactive memory T-cells are required

Renal transplantation is a life saving procedure for end stage renal disease and generally short-term transplantation outcome is excellent. The introduction of calcineurin inhibitor therapy has been critical for the prevention of acute rejection and improved one-year graft survival, although any beneficial effect on long-term graft survival is small. "Memory" is a critical barrier to long-term transplantation outcome and tolerance induction (1), therefore, the effect of newer immunosuppressive drugs on alloresponses by viral specific memory T-cells may be critical to graft survival and/or tolerance induction and should be studied further.

Selective therapies at the time of transplantation may allow inhibition of allo-HLA crossreactivity from pre-existing memory T-cells while still allowing de-novo naïve responses against viral antigens. For example, selective blockade of ICOSL and CD86 which represent two major co-stimulatory signals for the activation of resting peripheral blood memory T-cells (2,44) may still allow immune responses via the CD40/CD154 and/or CD70/CD27 co-stimulatory pathways which are important for naïve T-cell activation. While the effect of immunosuppressive drugs on allo-HLA crossreactivity from viral specific T-cells has not been studied, the calcineurin inhibitors are able to inhibit proliferation and cytokine production from effector CD4 memory T cells (27). Unfortunately leukocyte depleting therapies such as antithymocyte globulin and alemtuzumab are less able to diminish the memory T-cell pool (27).

Results presented in this thesis also suggest caution is warranted when interpreting tolerance protocols studied in pathogen free animals.

Adoptive transfer of pathogen specific T-cells could be complicated by GvHD disease

Adoptive transfer of virus or fungal specific T-cells offers an effective option for the management of specific immune defects in an immune compromised host (45), particularly following allogeneic BMT. However given the high frequency of allo-HLA crossreactivity from viral specific T-cells, it is not surprising that adoptive transfer has already been associated

with GvHD. For example, adoptive transfer of CMV specific T-cells to nine recipients after allogeneic BMT resulted in three cases of GvHD, including one patient who died (46). Similarly, TCR gene transfer to induce anti-leukaemia reactivity is associated with α and β chain rearrangements and therefore the formation of mixed dimer TCRs (47), which could also be alloreactive. Screening of adoptively transferred antigen or leukaemia specific T-cells for allo-HLA crossreactivity may help prevent GvHD.

HLA alloreactivity could be useful to conversely stimulate a human cytolytic viral specific T-cell responses using allogeneic cell therapy

Finally in chapter 7 of this thesis we provide evidence that allogeneic cell therapy may be useful to conversely stimulate a beneficial anti-viral cytolytic effector response for treatment of viral infection. We demonstrated that human viral specific memory T-cells gain cognate viral antigen specific cytolytic effector function following stimulation with allogeneic HLA molecules against which they are crossreactive. This proof-of-principle technique could provide important future options for the treatment of viral infections. This approach should be investigated further.

CONCLUSION

An essential feature of the T-cell response is the ability to recognize a diverse array of potentially unlimited antigens, necessitating that the TCR be inherently crossreactive. The memory T-cells that are specific to previously encountered pathogens accumulate following repeated infectious exposure and have low activation thresholds. Experiments presented in this thesis reveal that these viral specific memory T-cells are commonly crossreactive with allo-HLA molecules in a self-peptide specific manner. Thus, getting a certain infection in an individual with a certain HLA type might have significant adverse consequences in the event of organ or marrow transplantation. Human ex-vivo studies are clearly warranted. We suggest that current research objectives should focus on the human in-vivo relevance of allo-HLA crossreactivity from viral specific memory T-cells, and specifically how self-peptide dependent allorecognition from viral specific T-cells alters tissue specificity. Allo-HLA crossreactivity could also have serious adverse effects in the setting of adoptive transfer and TCR transfection of viral specific T-cells. New understandings of the origin of alloreactivity may lead to an era whereby donor suitability is defined not only by HLA typing but also using immunological history, and hopefully toward successful donor (antigen) specific transplantation tolerance.

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Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

De beste behandeling voor patiënten met eindfase nierfalen is een niertransplantatie. Het probleem na transplantatie is echter dat de nier door het immuunsysteem van de patiënt herkend wordt als lichaamsvreemd. De T-cellen (witte bloedcellen) van de patiënt herkennen de donorcellen als niet-eigen (allogeen) en veroorzaken afstoting van het orgaan. Om dit proces zo veel mogelijk te voorkomen dient de patiënt voor de rest van zijn leven immunosuppressieve medicijnen te gebruiken.

Iedereen heeft unieke eiwitten van het immuunsysteem, HLA moleculen (humaan leukocyten antigenen) genaamd, op het oppervlak van zijn cellen. Afstoting kan optreden wanneer T-cellen vreemde HLA moleculen herkennen op de cellen van het getransplanteerd orgaan.

Het is al langer bekend dat virale infecties een nadelige invloed hebben op de uitkomst van niertransplantatie. Infecties die opgetreden zijn vóór de transplantatie kunnen in verband gebracht worden met afstoting na transplantatie. Het mechanisme waardoor een in het verleden doorstane virusinfectie de afstoting van een getransplanteerde nier kan veroorzaken was nog onduidelijk. Eén hypothese is dat de T-cellen die geactiveerd worden door de infectie langere tijd kunnen overleven en op een later moment afstoting van het getransplanteerde orgaan kunnen veroorzaken. Echter, deze hypothese vereist dat dezelfde T-cel twee volstrekt verschillende vreemde antigenen kan herkennen, namelijk zowel het virus als het vreemde HLA op de allogene cellen van het transplantaat. In het verleden werd aangenomen dat deze kruisreactiviteit zelden voorkomt.

Het doel van het onderzoek beschreven in dit proefschrift was om vast te stellen of deze kruisreactiviteit van virusspecifieke T-cellen de verklaring is voor de aanwezigheid van donor specifieke T-cellen in patiënten die niet eerder getransplanteerd zijn. Indien dit wordt bevestigd, is het volgende doel om de frequentie van het optreden van deze kruisreactiviteit vast te stellen. Voor het bepalen van virusspecifieke T-cellen, die tevens kruisreageren tegen getransplanteerde cellen, hebben wij een nieuwe techniek ontwikkeld. Het is mogelijk om een enkele T-cel, ontstaan na een normale virusinfectie, te identificeren en te isoleren. Deze enkele T-cel werd gestimuleerd om te delen en uit te groeien tot vele miljoenen identieke T-cellen (T-cel kloon) die gebruikt konden worden voor experimenten. Daarna is getest of deze individuele T-cel kloon ook de verschillende celtypes kon herkennen die aanwezig zijn in een getransplanteerde nier.

Deze techniek bleek de basis te zijn voor een effectief systeem om te bevestigen dat door een virusinfectie geactiveerde T-cellen in staat zijn om afstoting van allogene cellen te veroorzaken. Tevens hebben we bevestigd dat afstoting kan worden veroorzaakt door dezelfde T-cel die ook een virus kan doden. Daarnaast laten we zien dat dit veel frequenter voorkomt dan algemeen werd aangenomen.

Hier volgt een overzicht van de nieuwe bevindingen op het gebied van transplantatie waartoe het onderzoek dat beschreven wordt in dit proefschrift heeft geleid:

Hoofdstuk 1 is een inleiding op het proefschrift. Het geeft een overzicht van wat tot nu toe bekend is over de normale immuunrespons tegen virussen en over de immunologische afstoting van getransplanteerde cellen.

Hoofdstuk 2 beschrijft een nieuwe techniek voor het isoleren en opkweken van vele identieke T-cellen uit een individuele virusspecifieke T-cel om de immuunreactie tegen allogene cellen te kunnen bepalen. In de appendix van hoofdstuk 2 wordt uitgebreid de methode beschreven die is gebruikt.

In hoofdstuk 3 is nagegaan hoe vaak kruisreactiviteit van T cellen, geactiveerd door een virusinfectie, optreedt. Hiervoor hebben we meerdere virusspecifieke T cellen van verschillende personen getest. Er is aangetoond dat één-en-dezelfde T-cel zowel het originele virus als bepaalde HLA moleculen op de allogene cellen herkent.

Hoofdstuk 4 laat zien dat de kruisreactiviteit gebaseerd is op het feit dat de T-cel een lichaamseigen eiwit herkent dat gepresenteerd wordt op het celoppervlak van allogene cellen.

In hoofdstuk 5 wordt aangetoond dat niet alleen virale infecties, maar ook anti-virale vaccinaties, T-cellen kunnen activeren die mogelijk in staat zijn afstoting van niertransplantaten te veroorzaken. Er is een patiënt bestudeerd die op de wachtlijst stond voor niertransplantatie, en die een vaccinatie had gekregen tegen het varicella-zoster (waterpokken) virus. Het bleek dat de T-cellen die geactiveerd werden door de vaccinatie in staat waren om cellen te vernietigen die normaliter aanwezig zijn in een getransplanteerde nier.

De huidige stand van zaken wat betreft kruisreactiviteit van virusspecifieke T-cellen tegen allogene menselijke cellen wordt besproken in hoofdstuk 6. Dit hoofdstuk geeft een samenvatting van onze eigen bevindingen, en ook van eerder onderzoek van anderen met betrekking tot virusspecifieke T-cellen die afstoting van niertransplantaten kunnen veroorzaken.

Hoofdstuk 7 laat zien dat, andersom, stimulatie van bloedcellen met allogene cellen bruikbaar kan zijn om een virusspecifieke T-cel reactie te versterken bij patiënten met een infectie. De mogelijke klinische toepassingen worden besproken.

In hoofdstuk 8 worden alle bevindingen samengevat en in een klinisch perspectief geplaatst. Verder wordt bediscussieerd hoe onze bevindingen gebruikt zouden kunnen worden om afstoting bij toekomstige niertransplantaties te voorkomen.

Curriculum vitae

CURRICULUM VITAE

Lloyd Joseph Andrew D'Orsogna werd op 16 juni 1976 geboren te Perth, Australië. Op vrijdag 20 augustus 1976 kreeg hij zijn eerste DKT (Difterie, Kinkhoest, Tetanus) vaccinatie en in 1994 kreeg hij de ziekte van Pfeiffer (EBV mononucleosis infectiosa). De middelbare schooltijd heeft hij doorlopen op het Trinity College in Perth, waarna hij de studie Geneeskunde volgde aan de University of Western Australia vanaf 1994.

In 1999 behaalde hij zijn medische graad (doctoraal examen) en werkte vervolgens als dokter bij het Sir Charles Gairdner Hospital en het Royal Perth Hospital. Zijn co-schappen heeft hij voltooid in Kalgoorlie en Alice Springs, Australië en in Glasgow, Schotland. In 2005 begon hij zijn opleiding tot Klinisch Immunoloog onder supervisie van Prof. Frank Christiansen. Tijdens deze periode schreef hij verschillende wetenschappelijke publicaties over immunodeficienties, orgaan transplantatie en beenmerg transplantatie.

In Oktober 2007 startte zijn promotie onderzoek onder leiding van Prof Frans Claas, Prof Ilias Doxiadis en Dr Dave Roelen bij de afdeling Immunohematologie en Bloedtransfusie van het Leids Universitair Medisch Centrum te Leiden, Nederland. De resultaten van dit onderzoek worden in dit proefschrift gepresenteerd. In Leiden werd hij een expert in virus specifieke T-cell clonering en Salsa dansen. In augustus 2011 zal Lloyd D'Orsogna, na voltooiing van het laatste immunopathology examen, geregistreerd worden als Klinisch Immunoloog en als "Fellow" worden toegelaten tot the "Royal Australian College of Physicians" en "Royal College of Pathologists of Australia".

Op 8 september 2011 zal Lloyd D'Orsogna om 11.30h in het huwelijk treden met Martina Cocco in "La Trinita" kerk te Chieti, Abruzzo, Italië.

CURRICULUM VITAE

Lloyd Joseph Andrew D'Orsogna was born on the 16th June 1976 in Perth, Australia. On Friday 20th August 1976 he received his first DTP (Diptheria-Tetanus-Pertussis) vaccination and in 1994 suffered acute EBV infectious mononucleosis. He attended secondary school at Trinity College in Perth and after graduating he started medical training at the University of Western Australia in 1994.

He obtained his medical degree in 1999 and subsequently worked as a resident and registrar at both Sir Charles Gairdner Hospital and Royal Perth Hospital. As part of medical training he also completed clinical rotations in Kalgoorlie and Alice Springs, Australia and Glasgow, Scotland. In 2005 he started his training as a clinical immunologist at the department of clinical immunology and immunogenetics under the supervision of Professor Frank Christiansen. During this period he published research papers on immunodeficiency diseases, solid organ transplantation and bone marrow transplantation.

From October 2007 until December 2010 he worked as a PhD researcher under the supervision of Prof Frans Claas, Prof Ilias Doxiadis and Dr Dave Roelen at the department of immunohematology and blood transfusion at the Leiden University Medical Centre, the Netherlands, where the research presented in this thesis was performed. In Leiden he became an expert in viral specific T-cell cloning and salsa dancing. Lloyd D'Orsogna will be registered as a clinical immunologist and admitted as a fellow of the Royal Australian College of Physicians and Royal College of Pathologists of Australasia in August 2011, following completion of the final immunopathology examination.

At 11:30am on the 8th September 2011 Lloyd D'Orsogna will marry Martina Cocco at "La Trinita" Church in Chieti, Abruzzo.

List of publications

LIST OF PUBLICATIONS

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List of abbreviations

LIST OF ABBREVIATIONS

aa	Amino acid
ABCD3	ATP-binding cassette sub-family D member 3
Ag	Antigen
APC	Antigen presenting cell and Allophycocyanin
bp	Base pair
CCR7	CC chemokine receptor 7
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EBV LCL	EBV transformed B-cell
EBNA3A	Epstein-Barr virus nuclear antigen 3A
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cell
ICP47	Infected cell peptide 47
IE62	Immediate early protein 62
IFNγ	Interferon gamma
IL	Interleukin

IMDM	Iscove's modified dulbecco's media
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
mRNA	Messenger RNA
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PTEC	Proximal tubular epithelial cell
PTLD	Post-transplant lymphoproliferative disorder
RNA	Ribonucleic acid
RT-AMV	Reverse transcriptase-avian myeloblastosis virus
SAL	Single antigen cell line (Single HLA transfected K562 cell)
SD	Standard deviation
SIV	Simian immunodeficiency virus
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TNFα	Tumour necrosis factor alpha
UCB	Umbilical cord blood
Vpu	Viral protein U
VZV	Varicella-zoster virus