Heterologous immunity in organ transplantation

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Heleen van den Heuvel

Colophon

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ISBN: 978-94-6375-333-3

The research described in this thesis was performed at the department of Immunohematology and Blood Transfusion at the Leiden University Medical Center, the Netherlands.

Financial support for the publication of this thesis was kindly provided by: The Dutch Kidney Foundation, de Nederlandse Transplantatie Vereniging, Pfizer B.V. and U-CyTech Biosciences.

Cover design and layout and by Eduard Boxem, persoonlijkproefschrift.nl.
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Printed by Ridderprint BV | www.ridderprint.nl

Heterologous immunity in organ transplantation

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op donderdag 25 april 2019

klokke 15.00 uur

door

Heleen van den Heuvel

geboren te Amsterdam

in 1986

Il y a plus de philosophie dans une bouteille de vin que dans tous les livres - Louis Pasteur (1822-1895)

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Leden promotiecommissie

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CHAPTER

1

GENERAL INTRODUCTION

A BRIEF HISTORY OF TRANSPLANTATION

Organ transplantation has long captured humans' minds, reflected by the fact that archaeological finds dating as far back as to the ancient Egypt, China and India already showed evidence of experimental transplantation (1). However, substantial progress has only been made in the past decades, not in the least due to religious constrictions that left the field untouched for centuries.

The first official reports on transplantation date back to the Renaissance in the 16th Century, when the famous Italian surgeon Gasparo Tagliacozzi reconstructed noses and ears using skin grafts of the upper arm. Initially, both patient-derived (autologous) and donor-derived (allogeneic) grafts were used, but it soon appeared that the latter was rather unsuccessful. Tagliacozzi was one of the first to become aware that individual differences could severely hamper allogeneic transplantation: in his treatise De Curtorum Chirurgia per Insitionem ("On the Surgery of Mutilation by Grafting") dated to 1597 he mentioned that "*... the singular character of the individual entirely dissuades us from attempting this work on another person. For such is the force and power of individuality, that if anyone should believe that he could achieve even the least part of the operation, we consider him plainly superstitious and badly grounded in physical science*". This belief, in addition to heavy criticism by prominent writers, including in later times Voltaire, on the unethical donation procedure (that was devoid of donation consent and donors were often slaves), led to the abolishment of allogeneic transplantation.

It took a few centuries for allogeneic transplantation to gain appeal again, when slowly evidence started to accumulate that allograft transplantation could hold potential after all. One of the first memorable reports of a successful allogeneic skin transplantation involved the then 23-year-old Sir Winston Churchill, who at that time was a war correspondent during the Sudanese / Boer War in 1898. When a befriended officer got wounded, he donated a piece of his skin and documented the experience: "*... he (i.e. the surgeon) then proceeded to cut a piece of skin and some flesh about the size of a shilling from the inside of my forearm. This precious fragment was then grafted onto my friend's wound. It remains there to this day and did him lasting good in many ways. I for my part keep the scar as a souvenir*". Whether the graft was truly accepted long-term remains under debate (after all immunosuppressive drugs did not exist at that time), however it was a first sign that allogeneic transplantation could be successful after all (1).

Shortly thereafter, in 1905, the first successful allogeneic corneal transplantation was performed, and around that same time surgeons even started experimenting with transplanting animal

kidneys to patients with renal failure. The results were poor however, and patients did not survive beyond a few days. Similarly, the first properly documented human-to-human kidney transplantation performed in 1936 was rejected quickly and the recipient died shortly after transplantation. It was not until 1954 that the first successful human kidney transplantation took place, performed by Murray and Hartwell Harrison on identical twins. For his merits, Murray received the Nobel prize in 1990.

The field took a great leap forward during the Second World War. Many soldiers suffered from severe skin wounds and were in great need of allogeneic skin transplantation, and hence the British Medical Council asked the young Sir Peter Medawar to investigate allogeneic skin graft rejection. Fortunately so, for his research brought great new insights and he is now by many regarded as the Founding Father of transplantation immunology. Being one of the first to acknowledge the immune system in rejection, Medawar discovered that secondary transplantations were rejected much more quickly compared to primary transplantations – which he attributed to "actively acquired immune reactions". In the 1950s, he and others revealed the importance of donor leukocytes in transplant immunity and rejection, by performing adoptive transfer experiments (although the exact mechanisms still remained elusive at that time). In 1960, he shared the Nobel Prize with Sir Frank Macfarlane Burnet for their work on "acquired immunological tolerance", the "unresponsiveness" of the immune system to foreign (allogeneic) antigens. Their studies were seminal to understand the dichotomy between allograft rejection and acceptance.

Meanwhile, one started to understand that certain molecules expressed on the cell surface could be involved in anti-donor immune responses. These molecules were named "major histocompatibility antigens" (MHC), or more specifically "human leukocyte antigen" (HLA) molecules in humans. Together, the HLA antigens form a system called the HLA system, which is highly polymorphic between individuals. George Snell discovered the genes of the MHC system in mice.

The vital role of the HLA system in dictating human allograft acceptance versus rejection was demonstrated in the 1950s by Jean Dausset, Rose Payne and Jon van Rood. Jean Dausset found that sera from patients who had undergone blood transfusion could agglutinate white blood cells, and he identified the first leukocyte specificity (now known as HLA-A2). Rose Payne and Jon van Rood independently discovered that "agglutinating antibodies" were also present in women that had given birth to multiple children. The leukocyte agglutinating antibodies were then used to group the leukocyte antigens, for which purpose Jon van Rood designed the first

computer programs. Based on these findings, correlations could be made between HLA and transplantation outcome, and quickly the vital importance of HLA matching became apparent. In 1967, Jon van Rood founded Eurotransplant (an international organization that matches patients and donors for solid organ transplantation), followed by Europdonor (the Dutch stem cell bank specialized in matching hematological patients with stem cell donors, now known as Matchis) in 1970, and Bone Marrow Donors Worldwide (the worldwide database with > 30 million HLA-typed stem cell donors and cord blood samples) in 1988. These organizations have saved millions of lives by optimal matching of donors and recipients. For the discovery of the HLA system George Snell, Baruj Benacerraf and Jean Dausset won the Nobel Prize in 1980, although many still wonder why Jon van Rood was not among them.

For a long time, it was assumed that only humoral (antibody-mediated) immunity was involved in allograft rejection. However, during the 1960s, one started to realize that lymphocytes could also exert direct cytotoxicity towards allogeneic donor cells: revealing the important role for cellular (cell-mediated) immunity. This led to the development of the classical cell-mediated lympholysis (CML) assay by Brunner and Cerrottini: an assay that, until this day, has been widely used to investigate the effector function of cytotoxic immune cells. In addition, the mixed lymphocyte culture (MLC) assay developed by Bain, Vas and Lowenstein, enabled the detection of proliferation of alloreactive cells and was implemented to determine donor-recipient compatibility in bone marrow transplantation for years. In 1964, Bach and Hirschhorn refined the assay by specifically detecting the proliferative response of patient lymphocytes, while donor lymphocytes were chemically treated to prevent proliferation. Nowadays, except CML for anti-donor cytotoxicity testing for bone marrow and hematopoietic stem cell transplantation, these assays are no longer standard protocol.

Although standardization of HLA matching between donor and recipient and preclinical testing led to a vivid increase in transplantation success, it was not until the third milestone, the discovery of immunosuppressive drugs, that success rates really took off. Immunosuppressive drugs enabled the suppression of anti-donor immune responses (alloresponses) and thus remarkably expanded the possibilities and implications of allogeneic transplantation. Transplant recipients showed significantly less allograft rejection and much prolonged allograft survival. In particular the discovery of cyclosporine in the 1970s defined a turning point, for it generated a great boost to transplantation success from the 1980s onwards. Today, success rates are higher than ever, but unfortunately this comes with a price: broad-spectrum immunosuppression often leads to severe side effects, such as increased susceptibility to infections and cancer.

Attenuating dose-intake and identification of more refined immunosuppressive drugs are therefore key topics in current research (2).

GENERAL IMMUNOLOGY

The recipient's immune system is key to allograft acceptance and rejection, as it is trained to distinguish between "self" and "non-self". When immune cells mount a response against the donor, they are called "alloreactive" (in contrast to "autoreactive", meaning they recognize "self"). When immune cells are not responsive towards a donor, they are called "tolerant". Tolerance is the Holy Grail of transplantation, as it would obviate the use of potentially harmful immunosuppression.

Innate and adaptive immunity

When a pathogen tries to invade the body, it is subjected to several host-defence mechanisms. After anatomical barriers, the first line of defence it encounters is the innate immune system, one of the oldest defence mechanisms conserved throughout evolution. In fact, in addition to humans and other vertebrates, it can also be found in insects, plants and fungi. Innate immunity generates immediate defence and thereby quick and efficient protection. However, cells of the innate immune system cannot generate long-lasting protective immunity and their reactivity is not antigen-specific. Phagocytes for example, recognize pathogens by general pathogen-specific markers and natural killer (NK) cells elicit a response against any cell that does not express self-HLA.

In the far majority of cases, these aspecific defence mechanisms are sufficient to control and clear pathogens. However, when the innate immune system is unable to control the infection, a second immune strategy is summoned: the adaptive immune system. Unlike innate immunity, adaptive immunity provides long-lasting protection. Immunological memory ensures that upon repeated exposure to the same pathogen, the immune response is quick and efficient and progression to disease is prevented. It is therefore not surprising that the acquisition of immunological memory forms the basis for active vaccination strategies. Another contrast to innate immunity is that adaptive immunity is specific: adaptive immune cells (white blood cells called "lymphocytes") are trained to specifically recognize only certain pathogens. B lymphocytes (or simply "B cells") provide humoral immunity by producing antibodies, while T lymphocytes (or simply "T cells") provide direct cell-mediated immunity. T cells can further be divided into CD4-expressing ($CD4^+$) and CD8-expressing ($CD8^+$) subsets: of which $CD4^+$ T cells mainly perform helper functions and $CD8^+$ T cells mainly exhibit cytotoxicity.

Furthermore, subsets of both B and T cells have the capacity to regulate the immune response ("Bregs" and "Tregs"), and thereby keep immune responses in check.

Both B and T cells express highly specialized receptors that recognize so-called "antigens", which are usually parts of proteins (or sometimes polysaccharides). Such antigens can originate from the host itself ("self-antigens") or from foreign sources ("non-self-antigens"). Non-self-antigens can be derived from bacteria, viruses and other pathogens, but also from food, and in light of transplantation, from organ donors. The main role of the immune system is to distinct self from non-self, and while self-antigens usually do not trigger immune responses, non-self-antigens often do.

B cells recognize antigens by membrane-bound antibodies called B-cell receptors (BCRs), and T cells by T-cell receptors (TCRs). The part of the antigen that binds to the BCR or TCR is called the "epitope". BCRs can recognize epitopes on an antigen directly, whereas TCRs recognize an epitope as a peptide presented within a major histocompatibility complex (MHC) molecule (FIGURE 1).

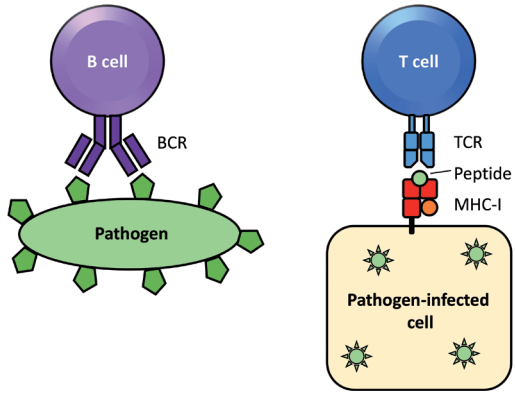


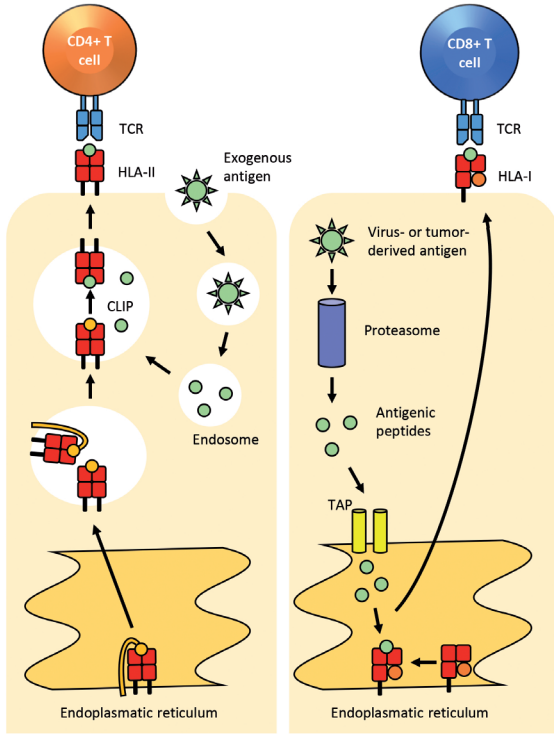
Figure 1. Recognition by B-cell receptors and T-cell receptors. B cells recognize antigens by membrane-bound antibodies called B-cell receptors (BCRs), and T cells by T-cell receptors (TCRs). The part of the antigen that binds to the BCR or TCR is called the "epitope". BCRs can recognize epitopes on an antigen directly, whereas TCRs recognize an epitope as a peptide presented within a major histocompatibility complex (MHC) molecule.

Since B and T cells generally only express receptors with a single specificity per cell (although exceptions do exist – see section "T-cell development", p.20) and epitope recognition is highly stringent, a vast number of cells is needed to provide protection against all pathogens. On average, the human body contains around 2×10^{12} lymphocytes of which 2% is found in the peripheral blood circulation - the remainder reside within tissues and lymph.

MHC molecules

The MHC complex is vital for immunological defence. It encodes MHC class I and MHC class II molecules, that are specialized in the presentation of peptides derived from intracellular and extracellular pathogens to immune cells respectively. All nucleated cells express MHC class I on their cell surface, whereas MHC class II is only expressed on antigen-presenting cells (APCs; typically dendritic cells, macrophages and B cells; atypically endothelial, epithelial and stromal cells) and activated T cells. Of note, amid the MHC class I and II genes reside the MHC class III genes, which encode for immune proteins such as components of the complement cascade (e.g. C2, C4, factor B), cytokines (e.g. TNF-α and -β) and heat shock proteins (e.g. HSP70). Since these proteins are not involved in antigen presentation itself, this section will focus on MHC class I and II.

MHC class I molecules present endogenous peptides to $CD8^+$ T cells; whereas MHC class II molecules present exogenous peptides to $CD4^+$ T cells (FIGURE 2). Although exceptions can occur, peptides presented in MHC class I molecules are generally restricted in size by the binding cleft (8-10 amino acids), while peptides presented in MHC class II molecules are not (15-24 amino acids). Teleologically, endogenous peptides are derived from proteins produced within the cell - and are therefore usually self-proteins involved in normal physiology. These should not trigger a defensive immune response for this would result in autoimmunity. On the other hand, MHC class I also presents endogenous peptides derived from intracellular viruses and bacteria, and such peptide:MHC-I complexes should trigger an immune response to kill the infected cells. Exogenous peptides are derived from proteins outside of the cell. APCs can take up foreign antigens, process them intracellularly into peptides and present those in the MHC class II molecules to $CD4^+$ T cells. When $CD4^+$ T cells recognize a specific peptide:MHC-II complex, they become activated and produce cytokines, which are small molecules that induce inflammation and attract and activate other immune cells to the site of infection.



Adapted from: Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. Nat Rev Immunol 2012

Figure 2. CD8⁺ and CD4⁺ T cells recognize HLA class I and class II presenting endogenous and exogenous peptides respectively. Exogenous antigens are taken up by the host cell (either by endocytosis, phagocytosis or pinocytosis) and degraded into peptides in so-called endosomes. The latter fuse with specialized vesicles that contain HLA class II molecules (initially loaded with a CLIP peptide to prevent binding of endogenous/self-peptides). Subsequently, the exogenous peptides are loaded onto the HLA class II molecules, and the resulting peptide:HLA complexes are transported to the cell surface. Endogenous antigens are degraded into peptides by the intracellular proteasome, whereupon they enter the endoplasmatic reticulum (ER) via the TAP transporter. In the ER, the endogenous peptides are loaded onto HLA class I molecules and the peptide:HLA complexes are subsequently transported to the cell surface.

In humans, the MHC system is known as the human leukocyte antigen (HLA) system. At the genomic level, the HLA system consists of over 200 genes, which are located closely together on chromosome 6. The most important HLA class I genes are HLA-A, -B, -C, while HLA class II is divided into 3 major HLA class II genes HLA-DR, -DQ, and -DP (FIGURE 3). Each individual expresses 12 HLA-isoforms: HLA-A, -B, -C, -DR, -DP and -DQ on two haplotypes; one inherited from the mother and the other from the father. To date, 12,893 HLA class I and 4,802 HLA class II alleles have been identified (3), and this number is still growing. Years of intensive

international collaboration were needed to unravel the alleles – and even today new HLA alleles are described on a regular basis. Not surprisingly, the HLA system is the most polymorphic region of the entire human genome.

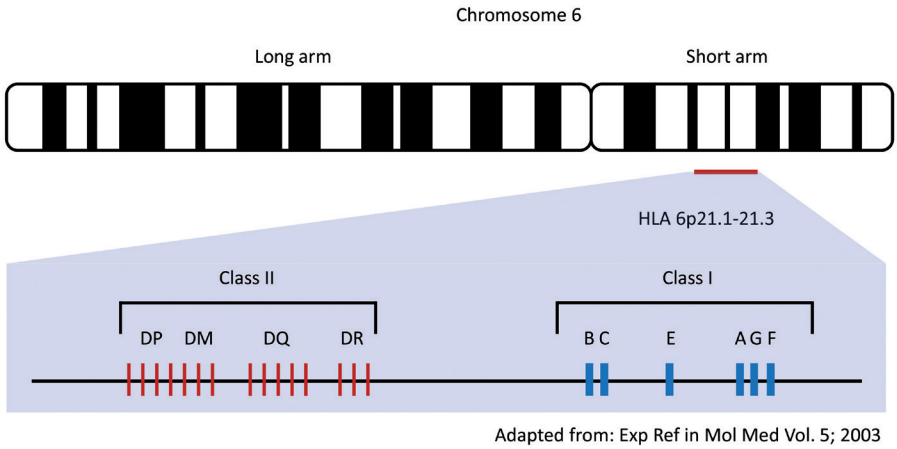


Figure 3. The different HLA class I and II genes and their position on chromosome 6. The HLA class I genes encode for the classical HLA-A, -B, and -C, and the non-classical HLA-E, -F, and -G antigens. The HLA class II genes encode for the classical HLA-DR, -DQ, and -DP, and the non-classical HLA-DM, and -DO antigens.

From an evolutionary point of view, great diversity in HLA molecules throughout a population is beneficial to the survival of the species, for it increases the chance of survival from infectious diseases: different HLA molecules can present different pathogen-derived peptides, increasing the chance that a TCR will recognize the complex. However, in transplantation it has been an eyesore for decades. The great variety in HLA molecules makes complete HLA matching between an unrelated donor and a recipient extremely difficult (if not impossible) and mismatched HLA molecules can be highly immunogenic. Anti-donor-HLA responses could lead to severe complications and even graft loss in the transplanted patient.

T-CELL IMMUNITY

T cells recognize their epitopes, peptide:MHC complexes (pMHC), by interaction with their TCR. This interaction determines whether the intracellular signalling pathways downstream of the TCR are triggered and the T cell becomes activated. In addition, TCRs provide the fundamental basis for T-cell selection to become part of the T-cell repertoire. It is needless to say that T-cell immunity relies heavily on the TCR, but other factors should not be neglected. These topics will be discussed in the following section.

T-cell receptors

The TCR is a heterodimer and consists of two chains: either an α and a β chain (αβ T cells), or a γ and a δ chain (γδ T cells). The genes for these receptors are located on chromosomes 7 (β/γ chain) and 14 (α/δ chain). The vast majority of T cells express αβTCRs, whereas only a small subset (approximately 5%) express a γδTCR. Although structurally similar, it is apparent that αβ T cells and γδ T cells are functionally different. Whereas αβ T cells are widely expressed, γδ T cells mainly reside in the gut mucosa. But more importantly, γδ T cells are not MHC-restricted, and rather recognize lipid structures, (phosphorylated) non-peptidic antigens or stress-related antigens. Due to this non-pathogen-specific character, they are proposed to reside at the border of the innate and adaptive immune system, although their exact function is still under investigation. This thesis addresses αβ T cells, and from here onwards "TCR" refers to the αβTCR. Both the α and β TCR chains consist of two extracellular domains, the constant (C) and variable (V) domains. The constant domain shows little variability; its main purpose is to anchor the TCR in the membrane and to bind the two TCR chains together via disulphide bonds. The variable domain instead shows great diversity, for this is the part of the TCR that interacts with the peptide:MHC complex and is the main determinant of T-cell specificity. The variable domains are characterized by three complementarity determining regions (CDRs), which form loops that interact with the peptide:MHC complex.

At the genetic level, the TCR Vα chain is generated by V-J recombination, and the TCR Vβ chain by V-D-J recombination (FIGURE 4). CDR1 and CDR2 loops are encoded within the V region, whereas the CDR3 loop is encoded at the junctions between the segments. The nucleotide composition at these junctions is arbitrary and orchestrated by palindromic (P) and random non-templated nucleotide (N) insertions and/or nucleotide deletions. Consequently, the CDR3 loops show extensive variation, resulting in a vast number of different TCR clonotypes.

So just like the massive variation in HLA molecules, there is enormous diversity in TCR usage. In theory, it has been estimated that an individual could generate over a quintillion (short scale: 10^{18}) unique TCRs, which leaves 10^{16} unique TCRs after they have been through a thorough selection to enter the periphery (thymic selection, the thorough selection process of TCRs that are allowed to enter the periphery; discussed in the next paragraph) (4). In practice, this number appears to be lower but is still estimated to be around 10^{8} (5).

Given this enormous variation, the chance that two or more unrelated individuals at random generate an identical TCR is almost zero. Yet defying the odds, T cells that express (nearly) identical TCR usage have been identified in multiple unrelated individuals.

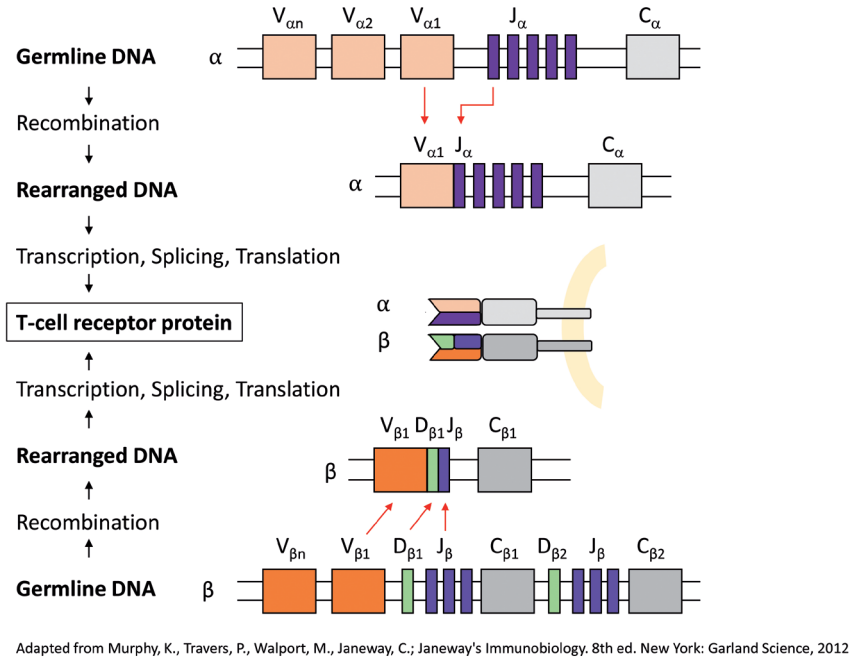


Figure 4. TCR rearrangement of the Vα and Vβ chain. The genetic landscape encoding the TCRα- and β-chains is divided into V, (D), J and C regions. The mRNA for the TCRα chain is generated by rearrangement of a V_{α} gene segment with a J_{α} gene segment, and subsequent transcription and splicing to the C_{α} gene segment. Translation of the mRNA results in the TCRα-chain protein. The TCRβ chain is generated similarly, but contains an additional segment (D) that makes up the variable domain. After translation into protein, the TCRα and TCRβ chains pair and form the TCR heterodimer.

Such TCRs are called "public" TCRs, in contrast to "private" TCRs that are uniquely expressed within individuals. Whereas it is assumed that most TCRs are private, an increasing amount of evidence suggests that public TCRs may be more common than anticipated. Provided that individuals share the HLA allele presenting the viral peptide, public TCRs have been described for several viruses including cytomegalovirus (CMV) (6), Epstein-Barr virus (EBV) (7), human immunodeficiency virus (HIV) (8), and influenza (FLU) (9).

Public TCR usage can (partly) be explained by immunodominance. Pathogens are sufficiently large to form hundreds to thousands of antigenic peptides that can be presented by all HLA alleles, so in theory, the T-cell response can be extremely broad. In practice however, T-cell responses are often dominated by T cells directed against only a few viral peptides presented in specific HLA alleles, so-called "immunodominant epitopes". This skewing of the T-cell repertoire is also called immunodominance (10). Immunodominant epitopes can vary between

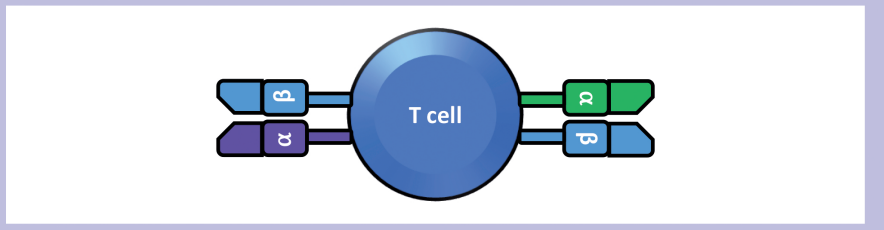
individuals, but they can also be the same ("common immunodominant epitopes"). In the latter case, it is hypothesized that the viral peptides are critical for the survival of the pathogen and therefore cannot be eliminated or altered for viral escape. Although the mechanisms behind immunodominance are not well understood, TCRs that have a strong interaction with the peptide:HLA complex ("TCR affinity") are more prone to be immunodominant: plausibly the associated enhanced T-cell survival and proliferation enable them to "outgrow" the other T-cell responses. Immunodominance thus creates a bias for public TCR usage, and for some viral specificities, public TCRs are present in almost 100% of individuals who express the relevant HLA and have encountered the virus of interest (11, 12).

T-cell development

Both T and B cells are generated from hematopoietic stem cells in the bone marrow. B cells subsequently migrate to the spleen for maturation, while T-cell progenitors migrate to the thymus. Here, they undergo strict positive and negative selection largely (though not solely) based on TCR affinity for the self-peptide:HLA complexes (13). Positive selection ensures that TCR affinity is sufficient to recognize self-HLA, whereas negative selection ensures that it is not too strong to avoid T-cell activation upon recognition of a self-peptide. To ensure optimal expression of self-peptides present throughout the body and not just the thymus, the thymic stroma has the ability to express tissue-specific peptides through an enzyme called autoimmune regulator (AIRE). The result of positive and negative selection is that merely 3% of T cells survive thymic selection and enter the periphery, generally expressing TCRs with low affinity for self-HLA. Furthermore, the majority of T cells express TCRs of a single TCR specificity, although T cells with two different TCRs can emerge during thymic selection (BOX 1).

When the T cells that survived thymic selection leave the thymus, they are in a so-called "naïve" state; meaning they have not encountered their specific antigen yet. They circulate through the body in the peripheral blood and lymph, and when they enter the lymph nodes and meet a professional antigen-presenting cell (APC; most often referring to the dendritic cell) that presents their specific antigen, they become activated and start to proliferate. Only those T cells that efficiently recognize the peptide:HLA complex will undergo proliferation, resulting in a large population of T cells expressing the same unique TCR (a "T-cell clone"). This process is called clonal expansion.

Box 1.



- It is estimated that up to 30% of T cells express two different TCRs, bearing the same TCRβ chain but different TCRα chains (2).
- Such "dual" TCR expression is a remnant of ongoing TCRα chain rearrangement during thymic selection.
- During positive selection, T cells that express a TCR that suboptimally binds self-MHC can be rescued if the second TCRα chain sufficiently improves binding. Subsequently, TCRs expressing both TCRα chains can be expressed on the cell surface.
- During negative selection, T cells that express a TCR that vigorously binds self-MHC can also be rescued by a second TCRα chain. As a result, T cells expressing highly self-reactive TCRs survive, and autoreactive T cells may enter the circulation and generate autoimmune responses (3).

From here on, the T cells are no longer naïve but turn into effector T cells, dedicated to directly eliminate pathogens, and memory T cells, the facilitators of long-lived immunological memory. When effector T cells have completed their job, they undergo apoptosis to maintain homeostasis in the T-cell compartment (14). Memory T cells survive, and because they have less activation requirements compared to their naïve counterparts, are able to quickly differentiate into effector T cells upon re-infection.

T-cell subsets

Functionally, T cells come in different flavours. As briefly mentioned in paragraph 2.1 "Innate and adaptive immunity", the main subsets of αβ T cells are divided based on their co-receptor usage: $CD4^+$ and $CD8^+$ T cells.

CD4⁺ T cells recognize MHC class II molecules and are therefore specialized in the recognition of exogenous pathogens. They are so-called "helper T cells" and orchestrate the immune response mainly by the production of cytokines. Well-known examples of helper T cells are T helper 1 (Th1) and T helper 2 (Th2) cells, of which Th1 cells stimulate antibody production by B cells, and Th2 cells aid the clearance of parasites by eosinophils and facilitate isotype switching of B cells. An additional important subset of CD4⁺ T cells on the contrary regulate the immune response, and these cells are hence named regulatory T cells. They hamper immune responses to prevent their "overshooting" and associated damage to the host.

CD8⁺ T cells recognize MHC class I molecules and are therefore specialized in the recognition of endogenous pathogens, primarily viruses. CD8⁺ T cells are most often cytotoxic T cells (CTLs), which induce cell death of pathogen-infected host cells by producing cytokines and cytotoxins. Perforin and granulysin generate pores in the plasma membrane of the infected cell, whereupon granzymes (and granulysin) can enter the cell to induce apoptosis.

T-cell activation

In order to perform effector functions, the T cell needs to be activated. The first step of T-cell activation is binding of the TCR to its ligand: the peptide:MHC complex. Generally, a TCR docks onto a peptide:MHC complex in a conserved diagonal and orthogonal orientation, wherein the CDR1 and CDR2 loops bind to the MHC and the CDR3 loop to the peptide (although exceptions do exist; for example CDR1α can interact with the N-terminus and CDR1β with the C-terminus of the peptide) (FIGURE 5).

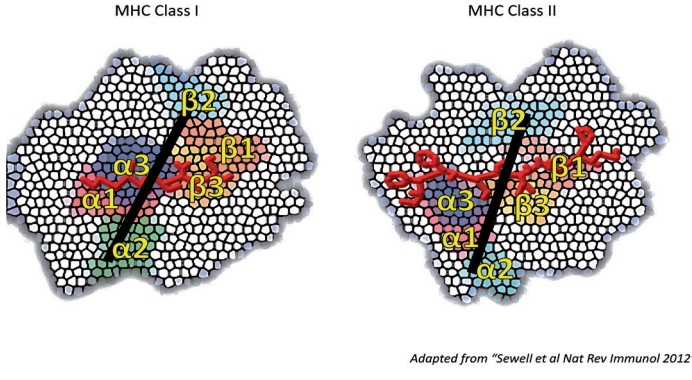


Figure 5. Docking mode of a TCR onto a peptide:MHC complex. A TCR docks in a conserved diagonal and orthogonal orientation, wherein the CDR1 and CDR2 loops bind to the MHC and the CDR3 loop to the peptide (although exceptions do exist; for example CDR1α can interact with the N-terminus and CDR1β with the C-terminus of the peptide).

To support signalling from the TCR to within the cell, the TCR forms a complex with CD3 molecules, of which CD3δ/ε and CD3γ/ε protrude extracellularly and CD3ζ projects intracellularly. The transmembrane parts of these CD3 subunits express residues that contain tyrosines, which are key to the phosphorylation cascade that enables downstream gene transcription for T-cell activation.

In addition, binding of the co-receptors CD4 (to the β2 chain of MHC class II) and CD8 (to the α3 chain of MHC class I) to the MHC stabilizes the TCR:pMHC interaction (FIGURE 6). Stabilization is needed, since TCR affinity for peptide:self-HLA is generally low as a result of thymic selection. CD4 and CD8 co-receptors increase cell-cell interactions, which allows for prolonged T-cell signalling, and facilitate CD3 signal transduction within the cell. Thereby T-cell sensitivity is increased and the threshold for T-cell activation is lowered.

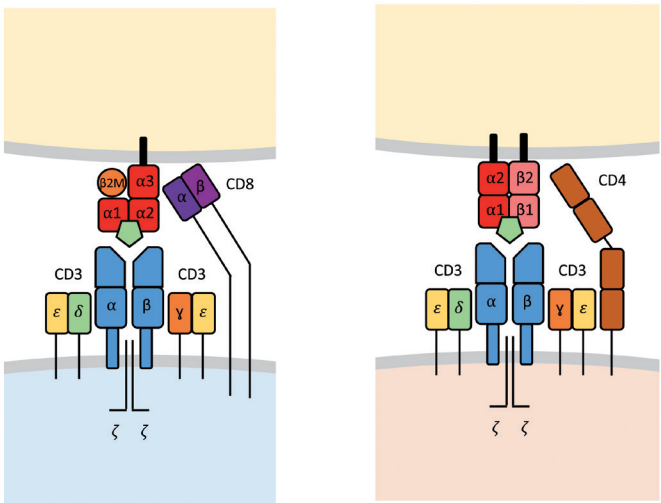


Figure 6. Structural overview of the TCR complex. The TCR forms a complex with CD3 molecules, of which CD3δ/ε and CD3γ/ε protrude extracellularly, and CD3ζ projects intracellularly. CD8 binds to the δ/ε domain of the HLA-I molecule.

In addition, binding of the costimulatory molecule CD28 on the T-cell surface to the membrane protein B7 (subtypes CD80 and CD86) on the surface of APCs is required for priming of naïve T cells. In fact, when naïve T cells solely undergo TCR:pMHC interactions without co-stimulation, they may even become anergic: a state of unresponsiveness that acts as a safety precaution to prevent autoimmunity. Memory T cells on the other hand, are known to be largely co-stimulation independent, so do not need co-stimulation binding for activation. This explains

why immunosuppressive drugs that tackle co-stimulatory molecules are effective against naïve, but not memory T cells. Finally, the formation of microclusters of TCRs on the T-cell surface and cell adhesion molecules such as ICAM-1 leads to the generation of a signalling unit called the "immunological synapse" that furthermore facilitates T-cell activation.

The cumulative binding strength of all non-covalent binding interactions (TCR affinity, co-receptor binding, cell adhesion molecules, costimulatory molecules) is called TCR avidity. Especially when TCR affinity is very low, TCR avidity can compensate for the weak signal strength to induce T-cell activation.

When the sum of all interactions reaches the activation threshold, T-cell activation is induced by intracellular signalling downstream of the TCR. Phosphorylation of downstream proteins generates a cascade of events by enzymes, co-receptors, adaptor molecules that facilitate protein-protein interactions, and ultimately transcription factors that affect gene transcription in the nucleus (signal transduction). The main transcription factors that are activated upon T-cell recognition and are needed for T-cell activation are "activator protein 1" (AP-1; downstream the MAPK pathway, involved in apoptosis, survival and vital for IL-2 production), "nuclear factor kappa-light-chain-enhancer of activated B cells" (NF-κB; essential for T-cell activation and the induction of inflammatory responses), and "nuclear factor of activated T cell" (NFAT; activated by the protein phosphatase calcineurin and again vital for IL-2 production) (FIGURE 7). The process of T-cell activation is tightly regulated and, particularly in the case of priming, depends on the mode and strength of the TCR:ligand interaction (signal 1), co-stimulation (signal 2) and environmental cytokines (signal 3).

T-CELL IMMUNITY IN TRANSPLANTATION

Alloreactive T cells can play an important role in both solid organ transplantation and allogeneic hematopoietic stem cell transplantation (HSCT) rejection.

In allogeneic HSCT, T-cell alloreactivity is directed against recipient cells and can lead to graft-versus-host disease (GVHD). GVHD inflicts severe damage to mucosa, connective tissues and exocrine glands, and primarily involves the skin (dermatitis), liver (hepatitis), and gastrointestinal tract (enteritis) - although at chronic stages it can involve almost any organ. Around 35-50% of allogeneic HSCT recipients will develop a form of GVHD, and depending on its grade and responsiveness to immunosuppression, symptoms range from mild to life-threatening. Interestingly however, T-cell alloreactivity may also benefit allogeneic HSCT:

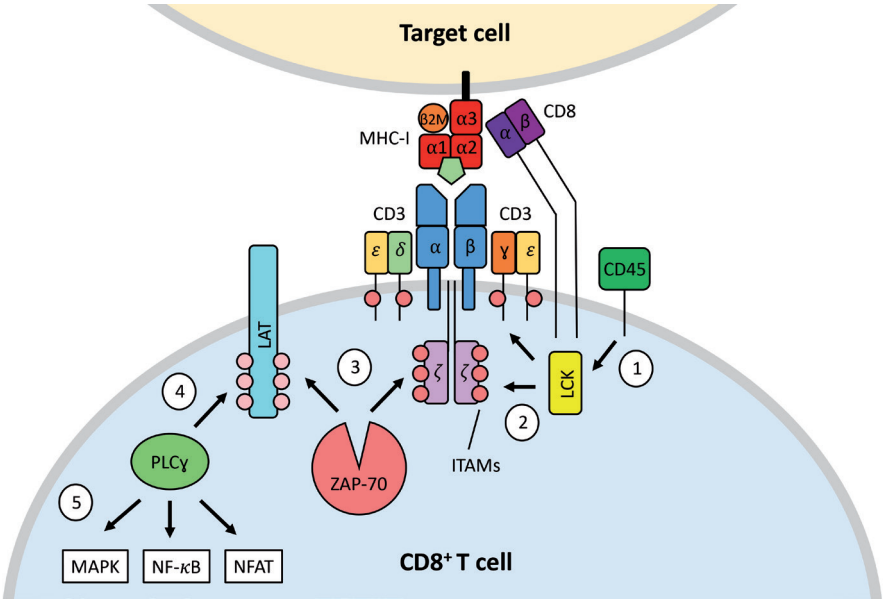


Figure 7. Simplified schematic overview of intracellular signaling downstream of the TCR leading to T-cell activation. When the threshold for T-cell activation is reached, a cascade of biochemical events take place. First, the protein tyrosine phosphatase CD45 activates Src family kinases (e.g. Lck or Fyn) that were recruited by CD8 (or CD4) co-receptors. They subsequently phosphorylate tyrosines on so-called immunoreceptor tyrosine-based activation motifs (ITAMs) present on the intracellular parts of CD3 and the ζ-chains. Thereupon, ZAP-70, a cytoplasmic Syk kinase, binds the phosphorylated ITAMs and phosphorylates tyrosines on the adaptor protein linker for activation of T-cells (LAT). Consequently, LAT recruits the enzyme PLC-γ that triggers the MAPK, NF-κB and NFAT pathways: important regulators of gene transcription involved in T-cell activation.

when HSCT is implemented as therapy for leukemia treatment, alloreactive T cells can mount a response against residual leukemic cells and thereby reduce the risk of leukemia relapse. This is known as the "graft-versus-leukemia" (GVL) effect. Mild T-cell alloreactivity is therefore desired when allogeneic HSCT is applied for leukemia treatment. However, finding the delicate balance between risk of GVHD versus retaining the benefits of GVL can be difficult (15).

In solid organ transplantation, T-cell alloreactivity is involved in acute and chronic allograft rejection (as further discussed in paragraph 4.1). Roughly 10% of all (first) kidney transplant recipients encounter an episode of T-cell mediated rejection. Fortunately, most of these episodes can be resolved by immunosuppression, but when immunosuppression fails, the damage inflicted by alloreactive T cells can result in chronic allograft nephropathy (also known as interstitial fibrosis/tubular atrophy; IF/TA) and graft loss becomes a serious risk (16).

Of note, when addressing allogeneic HSCT and solid organ transplantation, the allo-versus-self orientation is reversed: in allogeneic HSCT immune cells are donor-derived and the recipient is considered allogeneic, whereas in solid organ transplantation immune cells are recipient-derived and donor cells are allogeneic. For clarity, this thesis primarily addresses T-cell alloreactivity in light of solid organ transplantation; with a special focus on kidney (*renal*) transplantation.

Acute versus chronic T-cell-mediated rejection

Roughly, there are three different types of solid organ transplant rejection: hyperacute, acute and chronic.

Hyperacute rejection occurs, as the name implies, quickly upon transplantation. Within minutes, pre-existing anti-HLA donor-specific antibodies (DSA) of the recipient bind to the donor graft and initiate humoral rejection by activation of the complement system. The resulting inflammation and irreversible graft damage quickly lead to graft loss. Being driven by antibodies, this type of rejection is a form of "antibody-mediated rejection" (ABMR). Hyperacute rejection could easily result in systemic inflammation, and therefore intervention consists of graft removal. Fortunately, cross-matching and HLA compatibility screening has drastically reduced the incidence of hyperacute rejection over the years, and nowadays it is practically eliminated.

Acute rejection usually occurs in the first few months and within 12 months after transplantation. It can be antibody-mediated (acute ABMR) or T-cell mediated (acute TCMR, also known as acute cellular rejection). Acute ABMR and TCMR can occur separately but also simultaneously, although especially acute TCMR occurs frequently and when untreated, has poor clinical outcome. In renal transplantation, acute rejection manifests itself by organ dysfunction (decreased urine production and proteinuria) and microvascular pathology.

Finally, chronic rejection is often a combination of antibody-mediated (chronic ABMR) and T-cell mediated (chronic TCMR) rejection. Chronic rejection is initiated by alloreactive B and T cells but is furthermore facilitated by injury and repair mechanisms; leading to damage of the graft vessels (chronic allograft vasculopathy, CAV) and ischemic injury. In renal transplantation, chronic rejection manifests itself by the fibrosis of blood vessels, gradually leading to a loss of kidney function and overall graft loss.

However, the diagnosis of acute and chronic rejection can be complex and requires the continuous adjustment of diagnostic criteria. For renal transplantation, these criteria are set at the Banff Conference for Allograft Pathology (17).

Direct versus indirect allorecognition

Alloreactive T cells can recognize alloantigen in a direct, indirect or semi-direct way.

Direct allorecognition occurs when recipient T cells directly recognize donor MHC antigens expressed on donor-derived APCs. These donor-derived APCs are typically carried along with the allograft as "passenger cells". Allorecognition via the direct pathway is often robust, and is, aided by the large precursor frequency of alloreactive T cells (18), considered to be the instigator of acute T-cell mediated rejection (19). Donor-derived dendritic cells will however disappear within a few weeks after transplantation, and therefore direct allorecognition (in particular of HLA class II) is considered to be mainly involved in acute rejection.

When T cells recognize alloantigen via the indirect pathway, allo-derived peptides are presented by self-MHC molecules on the cell surface of recipient APCs. $CD4^+$ T cells are notorious for exerting alloreactivity via the indirect pathway, and their involvement in chronic rejection of heart, kidney, liver and skin transplants has long been described (20-23).

Finally, the semi-direct pathway is a combination between the direct and indirect pathways: in this pathway recipient APCs take up donor-derived exosomes and thereupon express donor MHC on their cell surface, thereby enabling direct recognition of donor MHC by recipient alloreactive T cells.

Of note, these pathways are by no means exclusive, and rejection is inevitably the result of a complex interplay between the different forms of allorecognition.

Heterologous immunity

Old scholar textbooks state that a TCR only recognizes one specific peptide:MHC complex, and thus has a single TCR specificity. Although TCRs are considered highly specific, it is now known that T cells are able to recognize multiple antigens through cross-reactivity of their TCR. In fact, it is estimated that in theory, a single TCR could recognize over a million peptides (24, 25). In Figure 8, a range of different docking modes are depicted by which TCRs can cross-react.

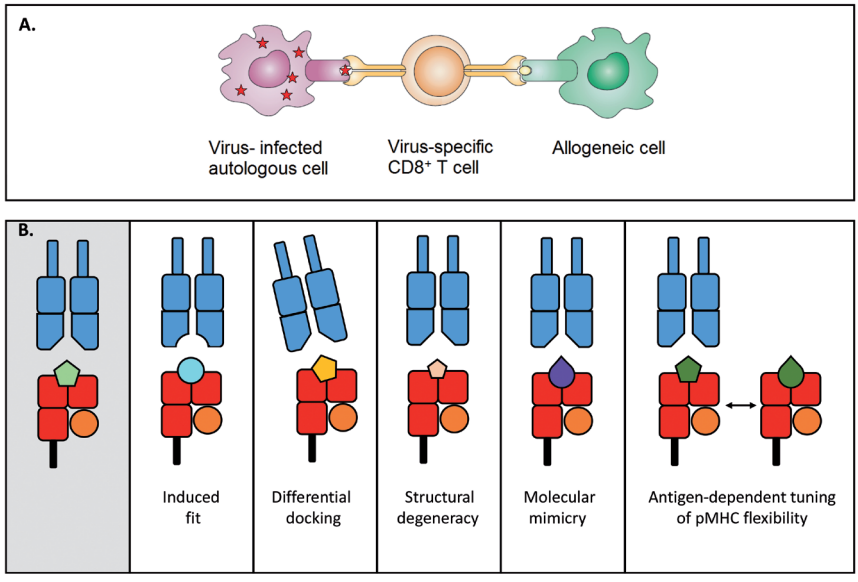


Figure 8. Different docking modes by which TCRs can cross-react. A) Allo-HLA cross-reactivity of virus-specific T cells. B) Proposed mechanisms of TCR cross-reactivity: TCR adaptation by induced fit, differential docking of the TCR onto the pMHC, structural degeneracy, molecular mimicry and antigen-dependent tuning of pMHC flexibility (54). Molecular mimicry is the most well-described example of an underlying mechanism for TCR cross-reactivity and is a form of TCR degeneracy: flexibility in TCR antigen recognition whereby a TCR can recognize multiple ligands. Especially the CDR loops are extremely flexible, and TCR cross-reactivity is mainly determined by conformational changes in the CDR3 loops (55, 56).

From an evolutionary point of view, TCR cross-reactivity is advantageous: in theory it would not be possible to generate a specific TCR for all pathogen-derived peptides, for the body cannot possibly accommodate such a large number of unique T-cell clonotypes. Besides, cross-reactive T cells with a memory phenotype can quickly respond towards a range of pathogenic antigens and thereby provide protection against different pathogens - even without previous encounter.

Immunity for a pathogen that is developed by exposure to another pathogen is called “heterologous immunity”. Several examples are known of T cells that, by means of TCR cross-reactivity, provide protection against a different virus from the one they were initially primed for. (The term “virus-specific” is therefore misleading – yet this terminology is still most commonly used to refer to virus-induced T cells). For example, cross-reactive virus-specific $CD8^+$ T cells have been described that recognize both the influenza virus and hepatitis C virus (26), or both the influenza virus and Epstein-Barr virus (EBV) (27). Also, FLU-specific A2/GIL

CD8$^+$ T cells can cross-react with an HIV peptide (SLYNTVATL) (28). Although T-cell responses against this immunodominant HIV epitope cannot prevent HIV infection, they are associated with control of viremia and slower disease progression (29, 30). It should be noted, however, that the extent of heterologous immunity between viruses in humans is still a matter of debate, and recent research was unable to detect any cross-reactivity of EBV memory T cells against CMV or Influenza A viral epitopes (31).

In addition, T-cell cross-reactivity can occur between pathogen-derived peptides and self-peptides, which could lead or contribute to autoimmune disorders. In fact, the estimation that a single TCR can recognize more than a million peptides has been verified by a diabetes type I autoimmune TCR (25). If self-peptides and pathogen-derived peptides appear structurally similar ("molecular mimicry"), recognition of self-peptides could elicit an immune response, which is suggested to play a role in multiple sclerosis (32, 33).

Moreover, virus-specific T-cell receptors do not only have the ability to cross-react with multiple peptides, but also with allogeneic HLA (allo-HLA). Especially cross-reactive virus-specific T cells with a memory phenotype could pose a threat to transplantation: since they have fewer activation requirements, immunosuppression with co-stimulatory inhibitors is only marginally, if at all, effective. Besides, their activation is much more rapid, and their effector function is greatly enhanced compared to naïve cells (34). Indeed, the potential threat of memory cells is illustrated by the first transplantation studies in mice, which surprisingly barely showed any rejection episodes in contrast to humans and non-human primates. This remarkable finding is explained by the sterile environment in which laboratory mice grow up: as a result of the lack of pathogen encounter, their memory T cell counts remain low. Upon adoptive transfer of virus-specific memory T cells, transplantation success dropped drastically (35).

Virus-specific memory T cells with cross-reactivity against MHC could thus hamper successful transplantation, and several in vivo mouse studies have shown that they can indeed result in allograft rejection (35-37). In humans, only a few studies have addressed the impact of allo-HLA cross-reactivity of virus-specific T cells on transplantation outcome so far (38-41). However, it is clear that also human virus-specific T cells are able to cross-react to allo-HLA (39, 42, 43). The most prominent example is the cross-reactivity of EBV B8/FLR-specific CD8$^+$ T cells against allo-HLA-B*44:02, which can be explained by molecular mimicry (despite large structural differences between HLA-B*08:01 and HLA-B*44:02) (11, 44). This cross-reactivity is of special interest as it is mediated by a public TCR and therefore shared by multiple individuals. If such "public cross-reactivity" would hamper successful transplantation, alloreactivity may thus be

predicted for specified patient-donor combinations. The clinical relevance of the EBV B8/FLR public cross-reactivity is however still under investigation (40). Moreover, the identification of novel public allo-HLA cross-reactive virus-specific T cells could further broaden the potential for making donor-patient risk estimations. More research is therefore needed to determine whether a) additional public allo-HLA cross-reactivities of virus-specific T cells can be identified that b) could affect transplantation outcome.

Immunosuppression

While evaluating the potential impact of cross-reactive virus-specific T cells on transplantation outcome, a quintessential factor to keep in mind is the impact of immunosuppression. Without immunosuppression allografts are easily rejected, so as a consequence, solid organ transplantation recipients are subjected to life-long intake of immunosuppressive drugs. Today, standard maintenance immunosuppression consists of induction therapy by CD25-blocking antibodies followed by a triad of calcineurin inhibitors (CNI; usually low-dose tacrolimus), mycophenolate mofetil (MMF) and corticosteroids (45). Although CNIs are very effective in hampering memory T-cell responses and are unmistakably associated with improved allograft survival, they are also associated with severe side effects such as nephrotoxicity, neurotoxicity, diabetes, metabolic syndrome and increased risk of viral infections (46-50). Therefore, attempts are being made to identify immunosuppressive drugs that specifically hamper alloresponses but maintain anti-viral immunity, and as an example, co-stimulation blockade is often suggested. Co-stimulation is required for full activation of naïve T cells, yet it is no longer (or significantly less) needed for activation of memory T cells. It thereby prevents priming of donor-specific naïve T cells but does not (or only to a minor extent) affect virus-specific memory T cells. Yet, an immunosuppressive regimen that fully replaces CNI by co-stimulation blockade does not hamper virus-specific memory T cells with cross-reactivity towards allo-HLA and may therefore prevent successful transplantation (51-53).

AIM OF THIS THESIS

In vivo mouse models have clearly shown the negative impact of cross-reactive virus-specific memory T cells on transplantation outcome. However, the human situation is much more complex, and the exact role of cross-reactive virus-specific T cells on allograft survival remains elusive. The aim of this thesis is to gain more insight into the role of allo-HLA cross-reactive virus-specific (or perhaps rather virus-*induced*) memory $CD8^+$ T cells in transplantation. Questions that will be addressed are: what is our current understanding of T-cell alloreactivity in transplantation (**Chapter 2**)? What is the potency and what are the flaws of current experimental techniques for the detection of allo-HLA cross-reactive virus-specific T cells in vitro (**Chapter 3**)? What is the impact of a single viral infection on the allo-HLA cross-reactive T-cell repertoire (**Chapter 4**)? Is it possible to stimulate allo-HLA cross-reactive virus-specific T cells ex vivo to boost anti-virus immunity (**Chapter 5**)? What is the likelihood that virus-specific T cells of unrelated individuals express identical allo-HLA cross-reactive TCRs (**Chapter 6**)? And is the interaction of cross-reactive virus-specific TCRs with allogeneic ligands just as efficient as with viral ligands (**Chapter 7**)? Finally, the results, pitfalls and suggestions for future research are discussed in the Summary & General discussion **(Chapter 8**).

CHAPTER

2

T-CELL ALLOREACTIVITY AND TRANSPLANTATION OUTCOME - A BUDDING ROLE FOR HETEROLOGOUS IMMUNITY?

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Curr Opin Organ Transplant 2015 Aug;20(4):454-60

ABSTRACT

Purpose of the review

Despite the association between alloreactive T cells and poor graft survival, the mechanisms behind T-cell-mediated rejection are still under investigation. In this review, we will discuss the latest insights into the impact of T-cell alloreactivity on solid organ transplantation and hematopoietic stem cell transplantation (HSCT), with special emphasis on the potential impact of heterologous immunity.

Recent findings

A large part of the memory T-cell repertoire is induced upon viral infections, and evidence for a role of T-cell receptor cross-reactivity of virus-induced memory T cells against allogeneic human leukocyte antigen (HLA) is accumulating in experimental and clinical solid organ transplantation studies. In HSCT, strong alloreactive potential of naïve T cells causes concerns for graft-versus-host disease while additional HLA-DP matching is suggested to prevent $CD4^+$ alloreactivity. Furthermore, virus-induced memory T cells hamper mixed chimerism induction, pointing once more towards a role for heterologous immunity.

Summary

Both memory and naïve T cells contribute to the alloimmune response after transplantation. Monitoring for T-cell phenotypes could help predict rejection episodes and/or graft-versus-host disease, allowing timely intervention. Tailoring donor lymphocyte infusions and additional HLA matching could prevent strong alloreactivity in HSCT. Furthermore, the potential role of heterologous immunity in T-cell alloreactivity and transplantation is gaining interest.

INTRODUCTION

T-cell-mediated alloreactivity is often involved in clinical complications after solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT). Whereas in SOT alloreactivity by T cells other than regulatory T cells (Tregs) is considered detrimental, the outlook on HSCT is more delicate. HSCT is frequently applied to cure hematological cancers, and a certain extent of T-cell alloreactivity is desired in order to attack residual malignant cells. This is known as the graft-versus-leukemia (GVL) effect. However, vigorous T-cell alloreactivity poses a risk for developing graft-versus-host disease (GVHD).

Both naïve and memory T cells have alloreactive potential. In HSCT, both are indispensable for proper functioning of the donor-derived immune system, although alloreactivity mediated by either subset can contribute to GVHD (57). In SOT, patients are treated with immunosuppressive drugs. Standard maintenance immunosuppressive therapy in SOT consists of a triad of calcineurin inhibitors (CNI; most commonly low-dose tacrolimus), mycophenolate mofetil (MMF) and corticosteroids. Together with induction therapy by CD25-blocking antibodies, this regimen is most potent compared to other current immunosuppressive regimens (45). Although effective in terms of patient survival and allograft rejection, treatment with CNI is associated with severe side effects such as nephrotoxicity (46), neurotoxicity (47), new-onset diabetes (48), metabolic syndrome (49) and increased susceptibility to viral infections (50, 58), which necessitate the identification of novel immunosuppressive drugs that specifically target alloreactive cells without hampering anti-viral responses. Hereto, costimulation blockade is suggested to be a promising strategy, and several studies have investigated the therapeutic potential of belatacept (a cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-specific fusion protein blocking CD28-B7 costimulation) as an alternative to CNI (59). Immunosuppression by costimulation blockade has the advantage of selectively targeting naïve T cells, leaving memory T cells largely unaffected as they are less dependent on costimulation to exert effector functions. Virus-specific memory T cells are therefore only minimally hampered by costimulation blockade, however, the same applies to alloreactive memory T cells. Furthermore, memory T cells have the capacity to exert more vigorous immune responses upon antigen recognition compared to naïve T cells, and therefore alloreactive memory T cells are considered more prone to impact SOT outcome compared to naïve T cells (60).

Interestingly, alloreactive memory T-cell responses can be mounted without prior encounter of alloantigen. This is explained by heterologous immunity of virus-induced memory T cells, in which cross-reactivity of the T-cell receptor (TCR) enables the recognition of allogeneic human

leukocyte antigen (HLA) (Figure 1). The cross-reacting TCR has never been trained to recognize allogeneic HLA by positive and negative selection in the thymus, which suggests that cross-reactive T cells simply mistake unknown allogeneic cells for virus-infected autologous cells. As cross-reactivity is an intrinsic feature of TCRs (24), one could envision that a substantial part of virus-induced memory T cells are cross-reactive. Indeed, 80% of all virus-induced T-cell lines and 45% of all virus-induced T-cell clones are reported to exert cross-reactivity against allogeneic HLA (43). Given the myriad of viral infections that are encountered throughout life, it is therefore likely to assume that all individuals harbor a large repertoire of virus-induced memory T cells with cross-reactive potential to allogeneic antigens. In vivo animal models have shown that virus-induced cross-reactive memory T cells hamper successful transplantation (35, 36, 61), but their impact on clinical transplantation remains elusive.

In this review, we will discuss the latest insights into T-cell alloreactivity and its impact on solid organ and HSCT outcome. Special emphasis will be given to the role of heterologous immunity of virus-induced T cells.

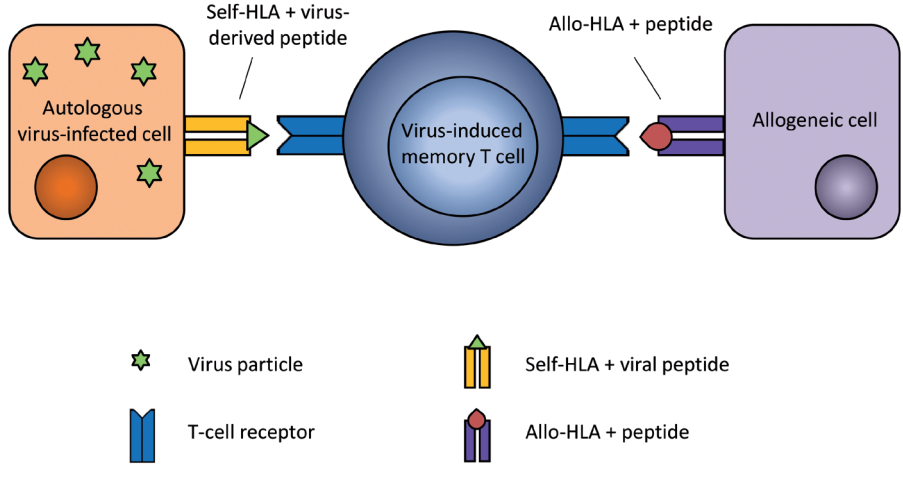

Figure 1. Virus-induced memory T cells can recognize allogeneic HLA + peptide by means of heterologous immunity. Through TCR cross-reactivity, a single TCR can recognize multiple antigens. In this schematic overview, virus-induced memory T cells recognize both self-HLA + viral peptide and allo-HLA + peptide using the same TCR. HLA, human leukocyte antigen; TCR, T-cell receptor.

SOLID ORGAN TRANSPLANTATION

Transplantation is the most desired treatment for end-stage organ failure. Despite drastically improved graft outcomes over time, rejection remains a major threat to successful transplantation. Alloreactive T cells play a pivotal role in SOT rejection, although their mode of action is not always fully understood. However, recent experimental and human studies have generated new and relevant data on this topic.

Solid organ transplantation: experimental studies

$CD4^+$ T cells play a prominent role in both humoral and cellular alloreactivity, by providing help to alloreactive B cells and $CD8^+$ cytotoxic T cells respectively. The latter requires that alloantigens are recognized by both $CD4^+$ and $CD8^+$ T cells: both as processed peptides in self-HLA as well as intact allogeneic HLA class I molecules. Accordingly, Sivaganesh et al. (62) showed that unprimed alloreactive $CD8^+$ T cells directly recognized intact allogeneic HLA class I antigens taken up and presented by recipient dendritic cells, provided that alloantigen peptides were also presented in self-HLA-class II. The authors therefore hypothesized that direct pathway $CD8^+$ alloreactive T cells depend on indirect pathway $CD4^+$ alloreactive T-helper cells for their activation (62). According to this model, matching for HLA class I alone would be sufficient to prevent $CD8^+$ T-cell activation. Yet, a recent study by Ishii et al. (63) suggested that $CD8^+$ T-cell responses could also be elicited upon sole HLA class II mismatching. In a kidney transplant model, $CD8^+$ T cells recognized peptides from mismatched MHC class II molecules presented by matched MHC class I, which led to allograft rejection (63). Thus, MHC class II peptides may serve as minor histocompatibility antigens (mHAg) that could trigger $CD8^+$ T-cell alloreactivity. This implies that matching for HLA class II may not only prevent $CD4^+$, but also $CD8^+$ T-cell allorecognition.

As an induction therapy prior to transplantation, T cells can be depleted by lymphocyte-depleting antibodies, such as antithymocyte globulin (ATG) or alemtuzumab. However, lymphocyte depletion can provoke a disturbed balance in T-cell subsets as a result of homeostatic proliferation in favor of memory T cells. To prevent this, Mai et al. (64) studied interleukin (IL)-7 receptor blockade following lymphodepletion. IL-7 is a central regulator of both $CD4^+$ and $CD8^+$ T-cell survival and homeostasis, and IL-7 receptor blockade indeed prevented memory T-cell proliferation and promoted allograft survival in both pancreatic island and skin transplantation mouse models (64). Alternatively, selective depletion of $CD2^{hi}CD8^+$ effector memory T cells by targeting CD2 has been shown to promote immunosuppression-free renal

allograft survival in nonhuman primates who were tolerized by a donor bone marrow transplant (65).

Apart from traditionally primed memory T cells (either by viral infection or alloantigen exposure), naturally occurring memory T cells with unknown priming history also exist. These may be primed by endogenous signals or even emerge spontaneously from naïve T cells without passing the effector phase (66, 67). Several studies on unprimed mice housed in pathogen-free conditions have indicated that these endogenous memory T cells could be alloreactive (57, 68). Recently, Su et al. (69) demonstrated that endogenous memory T cells could mediate cardiac allograft rejection. Graft infiltration, activation and enhanced effector function of these endogenous memory $CD8^+$ T cells were directly linked to prolonged cold ischemia times, suggesting that these cells may respond to danger signals, which has been suggested previously (70, 71).

Yet, not all memory T cells are detrimental, and distinctive memory T-cell phenotypes may in fact have antagonistic effects on transplantation outcome. Whereas effector memory $CD8^+$ T cells are mainly described to have a detrimental impact on transplant outcome, it appears that central memory $CD8^+$ T cells may instead promote allograft acceptance. Indeed, Krupnick et al. (72) showed that central memory $CD8^+$ T cells infiltrating into lung allografts were crucial for their acceptance. These cells had potent immunoregulatory properties, and were able to down-regulate $CD4^+$ and $CD8^+$ T-cell alloresponses (72). The observed regulation was attributable to nitric oxygen (NO) production by graft-infiltrating central memory $CD8^+$ T cells that were abundantly present in accepted lung allografts. In contradiction to this study, central memory T cells have also been described to mediate graft rejection in experimental skin transplantation (35). This discrepancy may be explained by the unique physiology of the lungs to limit pulmonary inflammation (73), or because nonvascularized skin allografts are less susceptible to tolerogenesis (74). The impact of central memory T cells on transplantation outcome may therefore vary depending on the transplanted organ.

A common feature of memory T-cell differentiation is the gradual loss of CD28, which can have direct implications for transplantation, since (alloreactive) memory T cells that have lost CD28 expression are less dependent on costimulatory signals for their activation. In addition, CD28 loss renders them insusceptible to costimulation blockade by belatacept (53). Therefore, costimulation blockade alone may be insufficient to suppress the activity of progressively matured alloreactive $CD28^{null}$ T cells. Indeed, a large phase III clinical trial (Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial; BENEFIT) investigating

immunosuppression by belatacept as an alternative to cyclosporine, showed that allograft rejection was significantly higher in the belatacept versus the cyclosporine group (17-22% vs 7% respectively) (75). Surprisingly, a recent nonhuman primate renal transplantation model with belatacept in combination with the mammalian target of rapamycin (mTOR) inhibitor sirolimus, found no necessity for memory T-cell depletion to ensure allograft survival (76). A subsequent human study conducted by the same group supported these data (77), indicating that the combination of costimulation and mTOR inhibition could be sufficient to hamper memory T cell responses. The findings remain to be confirmed in larger human studies.

In addition to belatacept resistance, Demmers et al. (78) demonstrated that terminally differentiated $CD4^+CD28^{null}$ effector memory T cells can also be unresponsive to tacrolimus and everolimus. In addition, the authors showed that these terminally differentiated $CD4^+CD28^{null}$ effector memory T cells were able to proliferate in response to allogeneic renal tubular epithelial cells (TECs). Although terminally differentiated $CD4^+CD28^{null}$ effector memory cells are uncommon in healthy individuals, they are found during end-stage renal disease and are associated with cytomegalovirus (CMV) infection (79, 80).

Interestingly, the humoral arm of the immune system may also affect T-cell mediated alloimmunity, as was recently described by Jane-Wit et al. (81). They showed that alloantibody and complement deposition on the cell surface of allogeneic endothelial cells upregulated the transcription of pro-inflammatory genes, leading to recruitment and activation of alloreactive $CD4^+$ T cells and resulting in cardiac allograft vasculopathy in mice (81). Additionally, the group of Heeger described a direct interaction of complement and T cells. Naïve $CD4^+$ T cells were shown to express complement receptors C3aR and C5aR, which upon activation induced type 1 helper T cell (Th1) maturation (82). Recently, they found that blockade of the complement receptors also significantly enhanced the stability of alloantigen-induced Tregs (83). These findings create a therapeutic potential for C3aR and C5aR antagonists, as they could simultaneously hamper alloreactive T cells and promote alloantigen-induced Treg stability.

Solid organ transplantation: clinical studies

Cytomegalovirus (CMV) infection is negatively associated with graft outcome, and recent clinical studies highlight the potential role of the anti-CMV response. Donckier et al. (84) found that strong effector memory $CD8^+$ T-cell expansion induced by lymphodepletion impeded early immunosuppression withdrawal in cadaver liver transplant recipients. The expansion mainly involved CMV- and Epstein-Barr virus (EBV)-directed T cells, and correlated with the occurrence of acute rejection. The authors therefore suggested a model in which virus-induced effector memory $CD8^+$ T cells homeostatically proliferated upon lymphodepletion, and

subsequently cross-reacted to the donor tissue: creating a causal link between graft rejection and heterologous immunity. Likewise, a role for heterologous immunity in graft rejection was suggested by Roux et al. (85), who performed a longitudinal study on lung transplant recipients. It was shown that memory $CD8^+$ T-cell responses directed against CMV comprised a large proportion of the intra-graft immune infiltrate and could therefore be involved in transplant rejection (85). Moreover, Nguyen et al. (86) found cross-reactive CMV-directed memory $CD8^+$ T cells in a lung-transplant recipient. Interestingly, despite increased numbers of highly activated cross-reactive $CD8^+$ T-cells prior to and during CMV reactivation, there was no negative correlation with long-term transplantation outcome. This finding is in concordance with their previous findings on cross-reactive EBV-induced $CD8^+$ T cells, which were also detected in human lung allografts without exerting a negative effect on transplant outcome (40). The latter two studies may therefore seem to argue against a role for cross-reactive virus-induced T cells in transplant outcome. However, one should keep in mind that the donor used in the CMV study did not express the cross-reacting HLA-B27 subtype, which could explain for the lack of detrimental effect on transplantation outcome. Furthermore, the cross-reactive response described in the EBV study is shown to be highly peptide-dependent and as a consequence is tissue-specific (87). The cross-reactive T cells specifically recognize a peptide derived from the ATP-binding cassette sub-family D member 3 (ABCD3) protein, and although lung tissue cells can express ABCD3, this study also showed that ABCD3 expression does not, as per definition, lead to surface presentation of the appropriate cross-reacting peptide (87). This cross-reactive response should therefore ideally also be investigated in other human transplantation types.

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) can be employed for both malignant and nonmalignant hematological diseases. Whereas HSCT for malignant diseases aims at inducing GVL by moderate alloreactivity, HSCT for nonmalignant hematological diseases aims at full donor-specific tolerance. The latter can be achieved by inducing a state of mixed hematopoietic chimerism, in which there is a stable balance between immune cells of the donor and the recipient (88).

Hematopoietic stem cell transplantation: experimental studies

Recent experimental studies on T-cell alloreactivity in HSCT mainly focus on alloreactivity directed against malignant cells. Although entering the field of tumor immunology is beyond the scope of this review, lessons can be learned regarding the alloreactive potential of donor-derived T cells. For instance, Binsfeld et al. (89) developed a murine model to investigate

the curative properties of allogeneic SCT in multiple myeloma, and revealed that especially alloreactive $CD8^+$ T cells are potent mediators of the graft-versus-myeloma (GVM) effect.

Westerhuis et al. (90) recently studied the effect of heterologous immunity on the induction of mixed chimerism. In an experimental mouse model, the persistence of mixed hematopoietic chimerism was hampered by recipient $CD8^+$ T cells induced upon vaccination with the vaccinia virus (90). The authors therefore hypothesize that heterologous immunity of vaccinia-induced $CD8^+$ T cells directed against allogeneic antigens interfered with the mixed chimerism induction. This study is one of the first describing heterologous immunity in HSCT. Since patients undergoing HSCT are highly immunocompromized, infections are a regular complication (91), potentially interfering with mixed chimerism induction or contributing to GVHD.

Hematopoietic stem cell transplantation: clinical studies

In essence, replacing the recipient's immune system by that of a donor results in the presence of donor-derived naïve T cells in the recipient. Unfortunately, naïve $CD4^+$ T cells can be highly alloreactive and may predispose for developing acute GVHD, which was recently confirmed by Chérel et al. (92). Analyzing HSCT in HLA-identical twins of different sex, they found that naïve $CD4^+$ T cells exerted vigorous alloreactivity towards the H-Y minor antigen (92). Selective depletion of naïve $CD4^+$ T cells from HSCT allografts may therefore prevent GVHD. This is in line with data by Distler et al., who showed that CD45RA-negative T cells exert only limited alloreactivity (93), and therefore assessed the functional properties of CD45RA-depleted donor leukapheresis products. When naïve $CD4^+$ and $CD8^+$ T cells were depleted from leukapheresis products by using CD45RA, they indeed observed reduced alloreactive $CD8^+$ T cell numbers, without effecting responses against pathogens. Unexpectedly, alloreactive $CD4^+$ T-cell numbers were not reduced (94). The authors ascribe this to the completely HLA-mismatched setting and emphasize this is not representative for clinical HSCT. In addition, CD45RA targeting may deplete late-differentiated memory T cells and naïve Tregs. Depletion of late-differentiated memory T cells may hamper anti-virus responses, but could also be beneficial, given their high alloreactive potential as previously discussed (78). The depletion of Tregs, on the contrary, does raise concerns as they are potent tolerogenic cells and are even employed for tolerance induction (95). Inevitably, the forthcoming multicenter pilot trial using CD45RA-depleted donor lymphocyte infusions (DLI) will give more insights into the overall effects of CD45RA depletion.

Currently, HLA matching for HSCT does not include HLA-DP, and mismatching of HLA-DP has previously been suggested to selectively induce GVL (and not GVHD) in T-cell-depleted allo-HSCT patients (96). However, the immunogenicity of HLA-DP is still under investigation, and

recent studies call for awareness of $CD4^+$ reactivity against HLA-DP. For instance, Stevanovic et al. (97) described two patients who underwent severe acute GVHD after prophylactic $CD4^+$ DLI, induced by alloreactive $CD4^+$ T cells directed against mismatched HLA-DP on epithelial cells. Interestingly, both patients suffered from CMV reactivation shortly after HSCT. A likely explanation is therefore that anti-viral inflammatory responses induced HLA class II expression on colonic epithelial cells, enabling the recognition of HLA-DP by alloreactive $CD4^+$ donor T cells and the establishment of severe acute colonic GVHD. Whereas prophylactic $CD4^+$ T-cell DLI is an effective treatment of residual malignant hematopoietic cells, this study calls for caution of performing this procedure in the presence of concurrent viral infections. Interestingly, a great effort is put into the understanding of the molecular mechanisms behind anti-HLA-DP cross-reactivity (98, 99), which recently resulted in the development of a prediction algorithm (100, 101). The algorithm accurately predicted known and unknown allo-HLA-DP $CD4^+$ T-cell cross-reactivity *in vitro* (100), and thereby creates a valuable tool for risk prediction in unrelated HSCT.

Combining these data, it appears that there is an association between virus infection, T-cell cross-reactivity against allogeneic HLA and GVHD. Plausibly, the majority of these alloreactive cells are primed by direct alloantigen recognition, and inflammation induced by viral infection simply upregulates allogeneic HLA on the donor cells. Yet, as suggested by the experimental study by Westerhuis et al., virus-induced T cells may also affect HSCT outcome. Research into heterologous immune responses in HSCT is of special interest at present, as progress is being made into the employment of adoptive transfer of virus-induced T cells to treat viral infections in HSCT patients (102).

CONCLUSION

Recent literature describes the alloreactive potential of both naïve and memory T-cells. Research on HSCT revealed that especially naïve $CD4^+$ T cells have high alloreactive potential. Tailoring donor-lymphocyte infusions by partial selection of naïve alloreactive T cells and additional HLA-DP matching to prevent strong $CD4^+$ alloreactive responses are therefore promising therapeutic strategies. Whereas in SOT, naïve T cells are efficiently hampered by immunosuppression, memory T cells could escape suppression and provide a barrier to successful transplantation. A large part of the memory T-cell compartment is induced by viral infection, and heterologous immunity of virus-induced memory T cells directed against donor-HLA demands attention in both solid organ transplantation and HSCT. However, much is still unknown with regard to their impact on clinical transplantation outcome, and additional research is strongly urged.

CHAPTER

3

DETECTION OF VIRUS-SPECIFIC CD8$^+$ T CELLS WITH CROSS-REACTIVITY AGAINST ALLOANTIGENS: POTENCY AND FLAWS OF PRESENT EXPERIMENTAL METHODS

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Transplant Direct. 2015 Nov 4;1(10):e40

ABSTRACT

Background

Virus-specific T cells have the intrinsic capacity to cross-react against allogeneic HLA antigens, a phenomenon known as heterologous immunity. In transplantation, these cells may contribute to the alloimmune response and negatively impact graft outcome. This study describes the various techniques that can be used to detect heterologous immune responses of virus-specific $CD8^+$ T cells against allogeneic HLA antigens. The strengths and weaknesses of the different approaches are discussed and illustrated by experimental data.

Methods

Mixed lymphocyte reactions (MLRs) were performed to detect allo-HLA cross-reactivity of virus-specific $CD8^+$ T cells in total peripheral blood mononuclear cells. T-cell lines and clones were generated to confirm allo-HLA cross-reactivity by IFNγ production and cytotoxicity. In addition, the conventional MLR protocol was adjusted by introducing a 3-day resting phase and subsequent short restimulation with alloantigen or viral peptide, whereupon the expression of IFNγ, interleukin-2 (IL-2), CD107a and CD137 was determined.

Results

The accuracy of conventional MLR is challenged by potential bystander activation. T-cell lines and clones can circumvent this issue, yet their generation is laborious and time-consuming. Using the adjusted MLR and restimulation protocol, we found that only truly cross-reactive T cells responded to re-encounter of alloantigen and viral peptide, while bystander-activated cells did not.

Conclusions

The introduction of a restimulation phase improved the accuracy of the MLR as a screening tool for the detection of allo-HLA cross-reactivity by virus-specific $CD8^+$ T cells at bulk level. For detailed characterization of cross-reactive cells, T-cell lines and clones remain the golden standard.

INTRODUCTION

Viral infections are a common complication after transplantation and are associated with rejection and decreased graft survival (103). Viruses may cause transplant injury directly by infecting cells of the graft, or indirectly by activating innate and adaptive immune responses. Local viral infections, for instance initiated by BK virus in kidney transplantation or by airborne viruses in lung transplantation, may harm the graft by lytic viral replication within epithelial cells and immune cell-mediated (bystander) injury (104, 105). In addition, viral infections can alter the cytokine milieu inside the graft or even systemically, affecting the differentiation and function of lymphocytes including alloreactive T cells. For example, cytomegalovirus (CMV) infection induces a systemic immune activation characterized by increased levels of Th1-associated cytokines in both healthy individuals and kidney transplant recipients (106).

The role of viruses in alloimmune responses is illustrated by experimental murine studies. Whereas transplantation tolerance is easily achieved in pathogen-free mice, it is far more difficult to achieve in humans and nonhuman primates. As humans and nonhuman primates are continuously exposed to bacteria and viruses, this suggests that pathogens and acquired immunological memory may affect alloresponses. Indeed, studies using pathogen-free versus pathogen-experienced mice showed that the latter were significantly less susceptible to the induction of tolerance (35). Interestingly, viral infections may affect transplant outcome even if viremia has been resolved long before transplantation, and virus-specific $CD8^+$ T cells may directly contribute to graft rejection (37), suggesting a role for memory T cells induced by viral exposure (35, 107).

A significant part of virus-specific memory $CD8^+$ T cells can recognize allogeneic human leukocyte antigens (allo-HLA) (108). This is due to cross-reactivity of their T-cell receptor (TCR), enabling the recognition of different epitopes by the same TCR. This phenomenon is known as heterologous immunity. Heterologous immunity often occurs in a physiological setting and creates an evolutionary benefit by enhancing the protection against (un)related pathogens. Cross-reactivity is essential for organisms that encompass only a restricted number of T cells and is an intrinsic feature of all TCRs (24). Therefore, it is not surprising that the vast majority of virus-specific $CD8^+$ T cells in healthy individuals can cross-react to 1 or multiple allo-HLA antigens *in vitro* (43).

Compared to naïve T cells, memory T cells tend to be less sensitive to immunosuppressive drugs (109, 110). Therefore, memory T cells that cross-react to donor alloantigens may play a role in T

cell-mediated allograft rejection (111-114). Several studies in heart, kidney, and liver transplant recipients demonstrate a distinct correlation between the frequency of donor-reactive memory T cells before and the incidence and severity of rejection episodes after transplantation (115, 116). Indeed, cross-reactive virus-specific memory T cells have been found in allografts of lung transplant recipients (39, 40, 86).

Clinical studies on cross-reactive virus-specific memory T cells in transplantation are limited, and additional studies are required. A potential obstacle facing these studies is the complex detection of truly cross-reactive responses. Here, we have described the strengths and weaknesses of various approaches that can be used to detect and functionally analyze virus-specific $CD8^+$ T cells with cross-reactivity to allo-HLA antigens. We compared current experimental methods, divided into bulk culture and clonal analyses, for their accuracy, potential applications and limitations. Furthermore, we suggest an altered protocol to more accurately distinguish true cross-reactivity from bystander-activation at bulk level.

MATERIALS AND METHODS

Collection of responder and target cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals and anonymous donors (Buffy coats, Sanquin Blood Supply, The Netherlands) after informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated by standard density gradient centrifugation and cryopreserved. Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated by incubating PBMCs with supernatant of the EBV-producing marmoset cell line B95.8 for 1.5 hours at 37°C, and additional culture in RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine and 10% fetal calf serum (FCS). Single-antigen-expressing cell lines (SALs) were generated as described previously (117). HLA typing was performed by sequence-specific oligonucleotide (SSO) or sequence-specific primer (SSP) genotyping at the Tissue-typing laboratory (Leiden University Medical Center (LUMC), Leiden, The Netherlands).

Generation of virus-specific $CD8^+$ T-cell lines and clones

$CD8^+$ memory T-cell lines and clones were generated from individuals 1 and 2 by fluorescence-activated cell sorting (FACS Aria; BD), as previously described (118). PBMC were stained with phycoerythrin (PE)-labeled viral tetramers CMV pp65(417-426) HLA-B*07:02/TPRVTGGGAM (CMV B7/TPR), EBV EBNA-3A(379-387) HLA-B*07:02/RPPIFIRRL (EBV B7/RPP), and EBV EBNA-3A(458-466) B*35:01/YPLHEQHGM (EBV B35/YPL) (Protein facility, LUMC) and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) against CD4, CD19, CD45-RA,

CD14, CD40, CD16 and CD56. The FITC channel (FL1) served as a dump channel, as concurrent CD8 mAb and major histocompatibility complex (MHC)-tetramer staining may trigger TCR internalization. T-cell receptor (TCR) usage was determined by DNA sequencing using TCR-specific polymerase chain reaction (PCR) primers (119).

Mixed lymphocyte reaction

Responder PBMC ($5x10^5$ cells) were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, 5mM), and co-cultured for 6 days with irradiated stimulator PBMC (3000 Rad, responder:stimulator ratio 1:1) in a 24-well flat bottom plate at a slant. Culture medium consisted of either RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine and 15% human serum (HS) or Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with 10% HS, penicillin/streptomycin and 0.00036(v/v)% β-mercaptoethanol. For culture beyond 6 days, medium was supplemented with IL-2 (10 U/mL) to ensure T-cell survival. Cells were stained with fluorescence-labeled CD8 and CD3 antibodies, a viability dye (fixable viability dye eFluor 506, eBioscience) and viral tetramer conjugated with PE or allophycocyanin (APC) (Protein facility, LUMC, or Sanquin, Amsterdam, The Netherlands). Flow cytometric analyses were performed on FACS Calibur and FACS CANTO (BD Biosciences).

Proliferation assay with correction for bystander activation: Mixed lymphocyte reaction followed by restimulation

After 6 days of MLR with unmanipulated responders and allogeneic stimulators (see above), medium was replaced with culture medium containing 10 U/ml IL-2, and cells were cultured for additional 3 days to allow downregulation of activation markers. Importantly, addition of viral peptide during the first MLR is discouraged because this will lead to preferential expansion of T-cell clones with a high affinity for the viral peptide. Next, the cells were taken up in stimulation medium (IMDM + 10% HS + βME + P/S + a-CD28 (2 µg/ml) + a-CD29 mAb (1 µg/ml)) and restimulated with: PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) (TCR-independent positive control), the original allogeneic stimulators ($2x10^6$), autologous cells ($5x10^5$) loaded with 10-100 ng viral peptide (TCR-dependent positive control), or co-stimulation alone (negative control) in a non-tissue-culture-treated round-bottom 96-wells plate. Stimulator cells were labeled with Celltracker Violet BMQC (Invitrogen) to allow discrimination between responders and stimulators. Costimulation through anti-CD28 and anti-CD29 antibody binding was provided to ensure optimal responses (120). The kinetics of the functional markers were previously analyzed: cytokine production and CD107a exposure peaked after 6 hours of restimulation, while the induction of CD137 and other activation markers was most prominent after 24 hours (data not shown). a-CD107a-PE antibody (BD Pharmingen) was added during

the 6-hour restimulation and after 1 hour monensin (0.7 µg/ml; GolgiStop, BD Pharmingen) and brefeldin A (10 µg/ml; Invitrogen) were administered to inhibit protein secretion. Next, the cells were harvested and stained intracellularly for IL-2 (IL-2-PE-Cy7, BioLegend) and IFNγ (IFNγ-allophycocyanin (APC)-eFluor 780, eBioscience). CD137 was measured by cell-surface staining (CD137-PE, BD Pharmingen) at 24 hours after restimulation (without addition of monensin and brefeldin A). All parameters were analyzed by flow cytometry (FACS CANTO; BD Biosciences).

Cytokine production assay

IFNγ levels were measured in a standard enzyme-linked immunosorbent assay (ELISA), performed according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, the Netherlands). $CD8^+$ T-cell lines and clones were stimulated by a panel of 11 EBV-LCLs or 6 SALs. $5x10^3$ $CD8^+$ T cells were incubated with $5x10^4$ EBV-LCLs or SALs in triplicate wells for 24 hours at 37°C in IMDM supplemented with penicillin/streptomycin, glutamine, 5% FCS, 5% HS, and IL-2 (10 U/mL), after which supernatants were collected.

Cytotoxicity assay

For optimal culture conditions, $CD8^+$ T-cell lines and clones were cultured with irradiated PBMCs (4000 Rad) from anonymous buffy coats 8 days prior to cytotoxicity testing. Cytotoxic capacity was assessed by ^{51}Chromium-release (^{51}Cr) assay (121). Serial dilutions (responder/stimulator ratio 30:1; 10:1; 1:1; 0.1:1) of responder $CD8^+$ T-cell lines and clones were stimulated with ^{51}Cr-labeled EBV-LCLs and/or phytohaemagglutinin (PHA) blasts in round-bottom 96-wells plates for 4 hours at 37°C, in IMDM, penicillin/streptomycin, glutamine, 5% FCS, 5% HS, and IL-2 (10 U/mL). PHA blasts were generated by incubation of $1x10^6$ cells PBMCs with PHA (0.8mg/mL; Murex Biotec Limited). Supernatants were collected for analysis on a γ-counter (PerkinElmer 2470 Wizard^2), and specific lysis was determined by the following calculation: *(Experimental ^{51}Cr release -Spontaneous ^{51}Cr release)/(Maximum ^{51}Cr release - Spontaneous ^{51}Cr release) x100*. Maximum ^{51}Cr release of the target cells was determined in PBS 1% Triton X-100, and spontaneous ^{51}Cr release in medium. Values for specific ^{51}Cr lysis represent the mean ± standard deviation of triplicate wells.

RESULTS

Techniques to assess virus-specific T cells with cross-reactivity to alloantigen in bulk cultures

Cross-reactivity of virus-specific T cells can be assessed in bulk cultures using PBMCs. PBMCs are easily obtained from blood samples, do not need pre-culturing, and are considered to be a fair representation of the immune repertoire.

Mixed lymphocyte reaction: a tool to screen for cross-reactivity and determine precursor frequencies of cross-reactive T cells

A widely used method to determine alloreactive lymphocytes in vitro at bulk level is the mixed lymphocyte reaction (MLR). Responder PBMCs are cultured with irradiated allogeneic stimulator PBMCs, whereupon proliferation and expression of activation markers can be assessed. Figure 1 shows how MLR can be used to determine proliferation of cross-reactive $CD8^+$ T cells that recognize both viral and alloantigen epitopes.

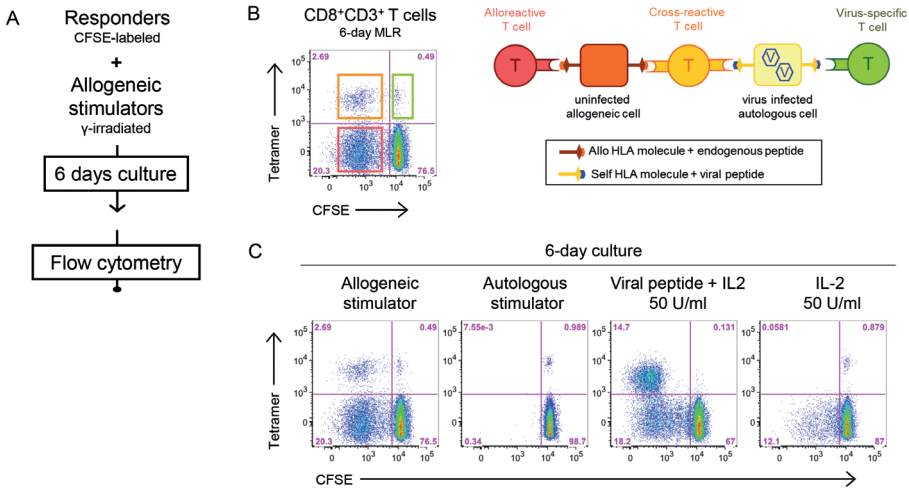
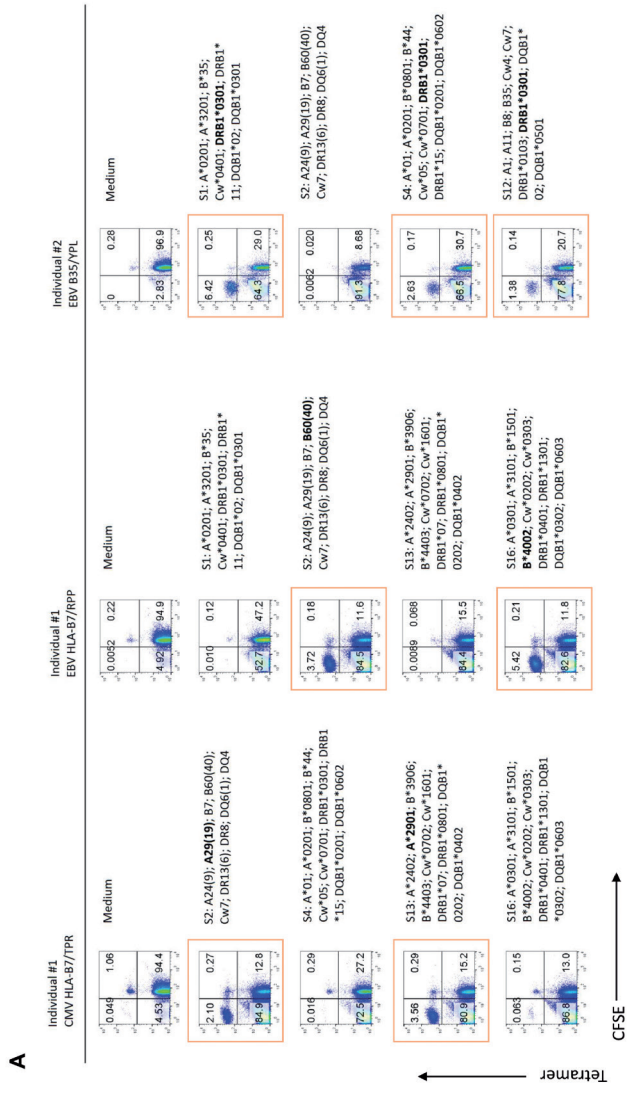


Figure 1. Identification of virus- and alloantigen cross-reactive $CD8^+$ T cells by combining MLR with viral tetramer staining. A) Flow chart of the experimental setup for a standard MLR. B) Flow-cytometric analysis of $CD8^+$ T cells after a 6-day MLR. Plotting viral tetramer against CFSE can distinguish between virus-specific cells ($CFSE^+tetramer^+CD8^+CD3^+$ T cells, green), cross-reactive cells ($CFSE^{dim}tetramer^+CD8^+CD3^+$ T cells, orange), and alloreactive cells ($CFSE^{dim}tetramer^-CD8^+CD3^+$ T cells, red). Schematic overview of allo-, virus- and cross-reactive T cells (right panel) C) Overview of different FACS plots after a 6-day MLR showing from left to right: a proliferative response of tetramer-positive and tetramer-negative cells upon allogeneic stimulation; no proliferative response of tetramer-positive and tetramer-negative cells upon autologous stimulation; a proliferative response of tetramer-positive and tetramer-negative cells upon viral peptide + IL-2 stimulation; a proliferative response of tetramer-negative cells but no proliferative response of tetramer-positive cells upon IL-2 stimulation alone.

We composed a panel of 16 different HLA-typed stimulator PBMCs, which covered the most common HLA class I molecules in the Western European population (>5%) (Table S1, SDC, http://links.lww.com/TXD/A15). The PBMCs of two HLA-typed healthy individuals were screened against this panel in MLR. In both individuals, $CD8^+$ T cells directed against different viral epitopes proliferated upon encounter with 1 or more stimulator targets (Table S2, SDC, http://

links.lww.com/TXD/A15). CMV B7/TPR and EBV B7/RPP T-cell responses of individual 1, as well as EBV B35/YPL T-cell responses of individual 2, revealed potential cross-reactivities to allo-HLA antigen (Figure 2A). Additional MLRs were performed, which confirmed allo-HLA cross-reactivity (Figure 2B). CMV B7/TPR T cells proliferated strongly in response to stimulators expressing HLA-A29, whereas EBV B7/RPP T cells responded toward HLA-B40-expressing stimulators. EBV B35/YPL T cells appeared to recognize the HLA class II molecule HLA-DRB1*03:01.



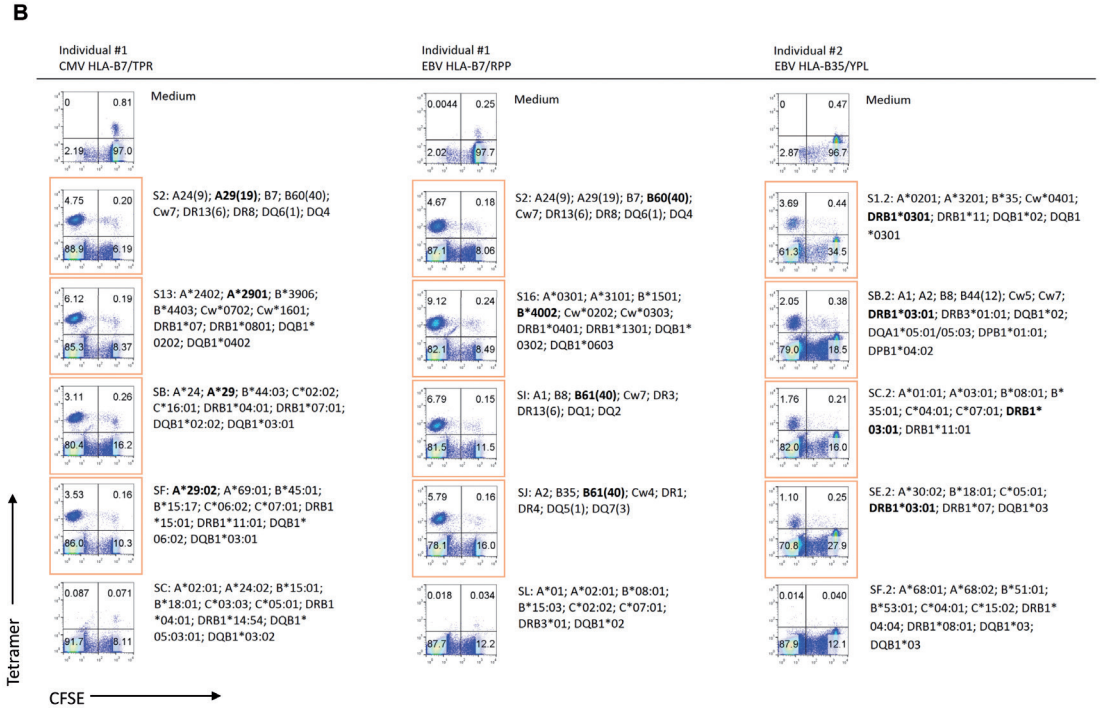


Figure 2. Cross-reactivity of CMV- and EBV-specific T cells to multiple allogeneic stimulators. A) FACS plots showing proliferation of CMV B7/TPR-, EBV B7/RPP- and EBV B35/YPL-specific CD8⁺ T cells following MLRs against the HLA-typed stimulator PBMC panel (n = 16). Illustrated are proliferative responses against stimulators S1, S2, S4, S12, S13 and S16, and presumed cross-reactive HLA antigens are indicated in bold. B) FACS plots showing proliferation of CMV B7/TPR-, EBV B7/RPP- and EBV B35/YPL-specific CD8⁺ T cells after MLRs against stimulator PBMCs derived from healthy donors expressing the presumed cross-reactive HLA antigens (indicated in bold). Plots are gated on CD8⁺ lymphocytes. Left column: responder R1 CMV B7/TPR. Middle column: responder R1 EBV B7/RPP. Right column: responder R2 EBV B35/YPL. X-axis: CFSE; Y-axis: tetramer-PE.

Responder HLA typing:
R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01;
R2: A*02; A*03; B*07; B*35.

Proliferating alloreactive T cells produce IL-2 and additional cytokines that can promote T-cell activation and proliferation in an antigen-independent manner. Consequently, it is difficult to determine which responses are truly cross-reactive. IL-2-mediated bystander proliferation is illustrated by CMV B7/TPR T cells of individual 3 in Figure 3B. To get an impression of potential bystander activation, the extent to which virus-specific T cells proliferate in response to IL-2 can be assessed (Figure 1). However, unresponsiveness to IL-2 alone does not exclude bystander activation and proliferation in response to IL-2 alone does not exclude true cross-reactivity toward alloantigen. The probability of bystander activation can be assessed by performing additional MLRs with various HLA-typed stimulators. Overall, these findings demonstrate that performing MLRs against a broad panel of HLA-typed targets can aid in identifying HLA class I and II antigens recognized by cross-reactive virus-specific CD8⁺ T cells, yet one should be aware of bystander activation.

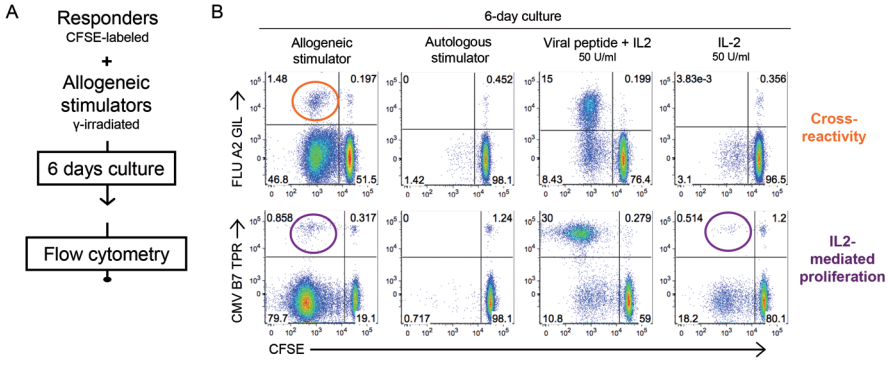


Figure 3. Potential cross-reactivity of virus-specific CD8⁺ T cells can be misinterpreted due to bystander activation in a mixed lymphocyte reaction. A) Flow chart of a 6-day MLR. B) FACS plots depicting the extent of proliferation and tetramer-reactivity of CD8⁺ T cells after 6-day culture with (from left to right): allogeneic stimulators, autologous stimulators (negative control), viral peptide and IL-2 (positive control) or IL-2 alone (cytokine-mediated bystander activation).

Responder HLA typing:
R3 (FLU A2/GIL): A1, A2, B8, B44(12), Cw5, Cw7, DR1, DR4
R4 (CMV B7/TPR): A2, B7, remainder unknown
Allogeneic stimulator HLA typing:
FLU A2/GIL: A2, A19, B7, B16, DR2, DR6
CMV B7/TPR: A1, A31(19), B8, B51(5), DR13(6), DR3

MLR with restimulation: identification of cross-reactive T cells in total PBMC, an optimized protocol to detect true cross-reactivity at bulk level

Performing multiple MLRs is time-consuming, and it would thus be beneficial to rule out bystander activation in a single experiment. This could be achieved by combining a primary MLR with a subsequent short restimulation with the same allogeneic responder (122). This approach ensures a more accurate and sensitive detection of alloreactivity due to clonal expansion and diminished activation requirements of prestimulated alloreactive cells. Cross-reactive cells responding during the primary MLR will respond quickly against the original stimulator cells, yet only modestly to other allogeneic stimulator cells (123).

We introduced a 3-day resting period and restimulation phase following the conventional MLR to identify truly cross-reactive T cells and simultaneously elucidate their function by assessment of cytokine production, exposure of the degranulation marker CD107a, and expression of the activation marker CD137 (124, 125). The FLU A2/GIL and CMV B7/TPR responses of the responder-stimulator combinations that were previously investigated in conventional MLR (Figure 3B) were now investigated in MLR with restimulation. Proliferating FLU A2/GIL T cells expressed comparable levels of interferon γ (IFNγ), IL-2, CD107a and CD137 upon restimulation with either TCR-independent stimulus (PMA-ionomycin), autologous cells pulsed with viral peptide, or allogeneic stimulator cells. This indicated true cross-reactivity (Figure 4B). In contrast, CMV B7/TPR cells showed no IFNγ, IL-2, CD107a and negligible CD137 expression levels upon allogeneic restimulation compared to TCR-independent stimulus and autologous cells with viral peptide, suggesting that the CMV B7/TPR T cells indeed proliferated in an alloantigen-independent manner (as indicated by IL-2-mediated proliferation; Figure 3B) and thus were not truly cross-reactive. When cross-reactive or bystander-activated cells were not restimulated, they expressed no or very little functional and activation markers. These findings were reproduced in independent experiments with the same responder-stimulator pairs. The examples depicted in Figure 4 are representative for n = 15 responses of T cells specific for epitopes of CMV, EBV and FLU. The altered MLR with restimulation protocol is thus a suitable tool to identify true cross-reactivity at bulk level.

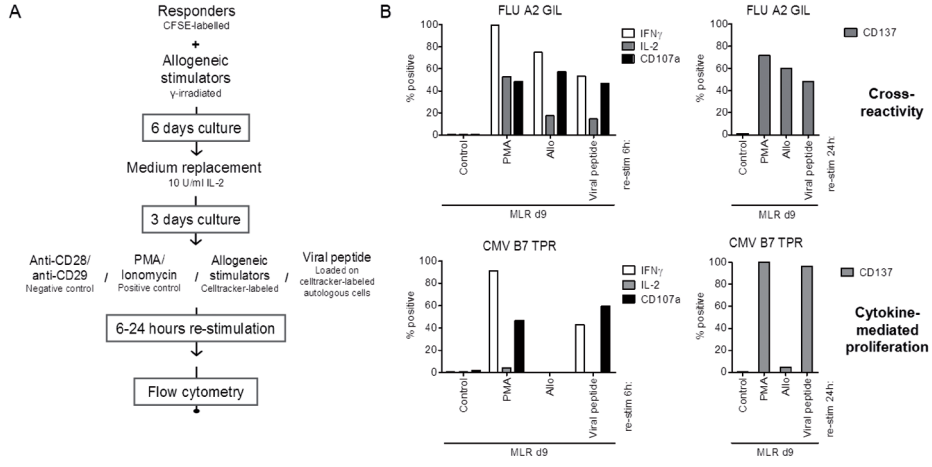


Figure 4. Restimulation after an MLR can help to discriminate true cross-reactivity from bystander activation. A) Flow chart of the 9-day MLR followed by 6-24 hr restimulation. B) Expression of IFNγ, IL-2, CD107a and CD137 in FLU A2/GIL$^+$ T cells that cross-react to alloantigen, and CMV B7/TPR$^+$ T cells that have proliferated independent of alloantigen. Responder and stimulator cells correspond to the ones used in Figure 3B. The examples of true cross-reactivity and bystander activation are representative for n = 15 responses for epitopes of CMV, EBV and FLU.

Responder HLA typing:
R3 (FLU A2/GIL): A1, A2, B8, B44(12), Cw5, Cw7, DR1, DR4
R4 (CMV B7/TPR): A2, B7, remainder unknown
Stimulator HLA typing:
FLU A2/GIL: A2, A19, B7, B16, DR2, DR6
CMV B7/TPR: A1, A31(19), B8, B51(5), DR13(6), DR3

Techniques to assess virus-specific T-cells with cross-reactivity toward alloantigen using T-cell lines and clones

To determine in-depth characteristics and cytotoxicity of cross-reactive CD8$^+$ T cells, CD8$^+$ T-cell lines and clones are recommended. In addition, they can be used to support MLR findings.

T-cell lines and clones: accurate detection and in-depth characterization of TCR cross-reactivity at clonal level

To confirm allo-HLA cross-reactivity of CMV- and EBV-specific T cells, we generated CD8$^+$ T-cell lines and clones of the following viral specificities: CMV B7/TPR, EBV B7/RPP, and EBV B35/YPL. Homogeneity of the lines and clones was confirmed by TCR usage (Table S3, SDC, http://links.lww.com/TXD/A15). The T-cell lines and clones were first stimulated with a panel of HLA-typed immortalized EBV-LCLs, whereupon IFNγ production was determined by ELISA (Figure 5A, 5B).

Significant amounts of IFNγ were produced by EBV B35/YPL T-cell line 2A6 upon recognition of HLA-DRB1*03:01+ EBV-LCLs and by CMV B7/TPR T-cell line 5A1 upon recognition of HLA-A29+ EBV-LCLs. The EBV B7/RPP T-cell clones 9G6 and 10D8 produced moderate levels of IFNγ upon recognition of HLA-B40+ EBV-LCLs. T-cell lines and clones with similar viral specificity but different TCR usage did not produce IFNγ in response to the same allo-HLA molecules, demonstrating that cross-reactivity is mediated by a subpopulation of virus-specific T cells with defined TCR usage. In contrast to EBV-LCLs, SALs did not induce significant IFNγ production, suggesting that the recognized endogenous peptide might not be expressed by these cells (Figure 5C). This highlights the importance of testing cross-reactivity with different cell types.

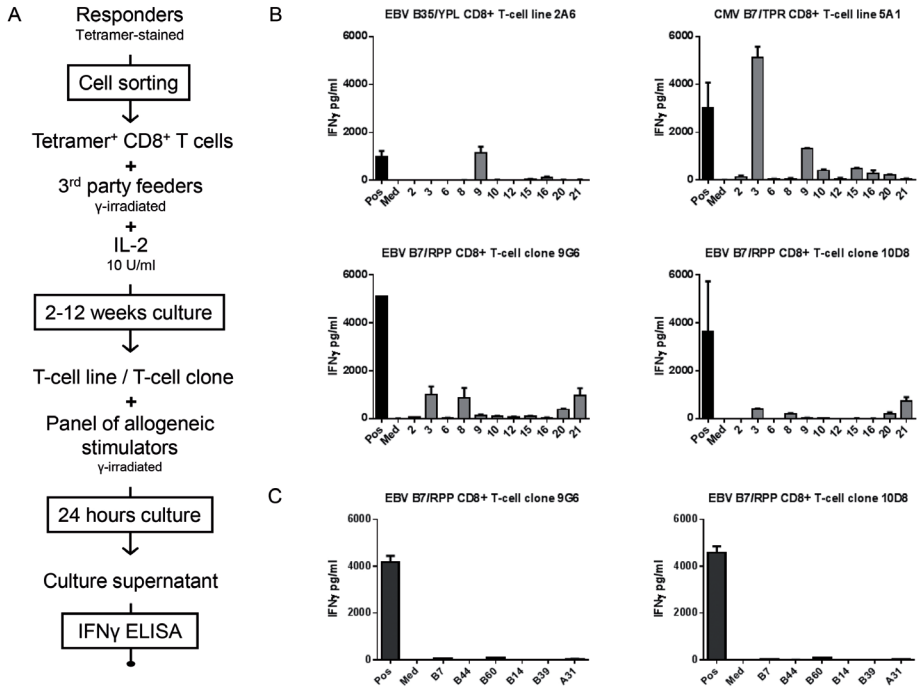


Figure 5. Generation of virus-specific T-cell lines and clones followed by analysis of cross-reactivity based on IFNγ secretion. A) Flow chart of the procedure to generate CD8+ T-cell clones and subsequent measurement of IFNγ production upon stimulation with allogeneic cells by ELISA. B) IFNγ production by CD8+ T-cell lines 2A6 (EBV B35/YPL; R2) and 5A1 (CMV B7/TPR; R1) and CD8+ T-cell clones 9G6 and 10D8 (both EBV B7/RPP; R1) upon stimulation with HLA-typed EBV-LCLs. EBV B35/YPL T-cell line 2A6 responded to EBV-LCL 9 (HLA-DR3), CMV B7/TPR T-cell line 5A1 to EBV-LCL 3 and 9 (both HLA-A*29:02), and EBV B7/RPP T-cell clones 9G6 and 10D8 responded to EBV-LCL 3 (HLA-B60), 8 (HLA-B*40:01), 21 (HLA-B61). Positive control: EBV-LCL sharing the autologous HLA antigen (B35/B7) loaded with viral peptide. C) IFNγ production of EBV B7/RPP T-cell clones 9G6 and 10D8 upon stimulation with SALs expressing depicted HLA molecules. All bars represent the mean of duplicates.

Responder HLA typing:

R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01

R2: A*02; A*03; B*07; B*35

Stimulator HLA typing:

EBV-LCL 2: A*24:02; A*33:01:01; B*14:02:01; C*02:02/02:32; C*08:02/08:29; DR1; DQ*05:01; DP1; DP4

EBV-LCL 3: A*03:01/03:22; **A*29:02/29:09**; B*07:02/07:61/07:114; B*44:03/44:105; C*07:02:01; C*16:01:01; DR2

EBV-LCL 6: A*01:01:01:01; A*26:01:01; B*08:01:01; B*49:01:01; C*07:01:01; DR01; **DR*03:04**; DQ*03:04; DQ*05:04

EBV-LCL 8: A*02:03:01; A*24:02; B*38:02:01; **B*40:01:02**; C*03:04:01; C*07:02:01; DR2; DR5

EBV-LCL 9: **A*29:02:01**; A*31:01:02; B*18:01/18:17N; B*58:01:01; C*05:01; C*07:18/07:01; **DR3**; DR8; DQ2

EBV-LCL 10: A*24:03:01; B*51:01:01; C*15:02:01; DR*11:04; DQ*03:01; DP*04:02

EBV-LCL 12: A*24:02:01; A*30:01:01; B*51:01:01; B*58:01:01; C*01:02:01; C*03:02:02; DR1; DR7; DQ1; DQ2

EBV-LCL 15: A*24:02; A*31:01:02; B*39:01; B*55:01:01; C*03:03:01; C*12:03:01; DR13; DQ1; DP2; DP4

EBV-LCL 16: A*30:01:01; A*68:02:01; B*42:01:01; C*17:01:01; **DR*03:02**; DQ*04:02; DP*01:01; DP*04:02

EBV-LCL 20: A31; A*24:02; B7; Cw4; Cw7; DR12; DR15

EBV-LCL 21: A*02:10; A30; B13; **B61**; Cw6; DR7; DR9

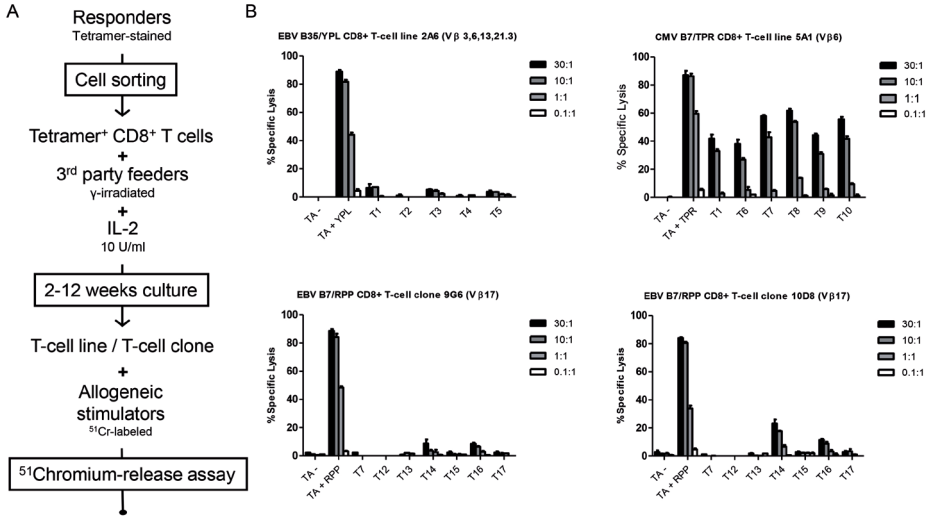


Figure 6. Cytotoxic potential of cross-reactive T-cell lines and clones. A) Flow chart of the generation of CD8+ T-cell clones and subsequent measurement of cytotoxicity toward allogeneic cells in a 51Chromium-release assay. B) Percentage specific lysis of 51Chromium-labeled target cells by CD8+ T-cell lines 2A6 (EBV B35/YPL; R2) and 5A1 (CMV B7/TPR; R1) and CD8+ T-cell clones 9G6 and 10D8 (both EBV B7/RPP; R1). Negative control: autologous PHA blasts without peptide (TA-). Positive control: autologous PHA blasts loaded with viral peptide (TA + YPL, TA + TPR, TA + RPP). All bars represent triplicate wells with standard deviation.

Responder HLA typing:
R1: A*02; A*03; B*07; B*35; C*04; C*07; DRB1*01; DRB1*08; DQB1*04:02; DQB1*05:01
R2: A*02; A*03; B*07; B*35
Stimulator HLA typing:
T1: EBV-LCL 9: **A*29:02:01**; A*31:01:02; B*18:01/18:17N; B*58:01:01; C*05:01; C*07:18/07:01; **DR3**; DR8; DQ2
T2: PHA-blast S1.2 Figure 2B: A*02:01; A*32:01; B*35; Cw*04:01; **DRB1*03:01**; DRB1*11; DQB1*02; DQB1*03:01
T3: PHA blast SB.2 Figure 2B: A1; A2; B8; B44(12); Cw5; Cw7; DRB1*03:01; **DRB3*01:01**; DQB1*02; DQA1*05:01/05:03; DPB1*01:01; DPB1*04:02
T4: PHA blast SC.2 Figure 2B: A*01:01; A*03:01; B*08:01; **B*35:01**; C*04:01; C*07:01; **DRB1*03:01**; DRB1*11:01
T5: PHA blast SE.2 Figure 2B: A*30:02; B*18:01; C*05:01; **DRB1*03:01**; DRB1*07; DQB1*03
T6: EBV-LCL 3: A*03:01/03:22; **A*29:02/29:09**; B*07:02/07:61/07:114; B*44:03/44:105; C*07:02:01; C*16:01:01; DR2
T7: PHA blast S2 Figure 2B: A24(9); **A29(19)**; B7; **B60(40)**; Cw7; DR13(6); DR8; DQ6(1); DQ4
T8: PHA blast S13 Figure 2B: A*24:02; **A*2901**; B*39:06; B*44:03; Cw*07:02; Cw*16:01; DRB1*07; DRB1*08:01; DQB1*02:02; DQB1*04:02
T9: PHA blast SE: A2; **A29(19)**; B57(17); B55(22); Cw3; Cw6; DR14(6); DR7; DQ5(1); DQ9(3)
T10: PHA blast SF Figure 2B: **A*29:02**; A*69:01; B*45:01; B*15:17; C*06:02; C*07:01; DRB1*15:01; DRB1*11:01; DQB1*06:02; DQB1*03:01
T12: EBV-LCL 8: A*02:03:01; A*24:02; B*38:02:01; **B*40:01:02**; C*03:04:01; C*07:02:01; DR2; DR5
T13: PHA blast SG: A*01:01; A*02:01; B*08:01; **B*40:01**; C*03:04; C*07:01; DRB1*03:01; DRB1*13:02; DQB1*06:04; DQB1*02:01
T14: EBV-LCL 21: A*02:10; A30; B13; **B61**; Cw6; DR7; DR9
T15: PHA blast S16 Figure 2B: A*03:01; A*31:01; B*15:01; **B*40:02**; Cw*02:02; Cw*03:03; DRB1*04:01; DRB1*13:01; DQB1*03:02; DQB1*06:03
T16: PHA blast SI Figure 2B: A1; B8; **B61(40)**; Cw7; DR3; DR13(6); DQ1; DQ2
T17: PHA blast SJ Figure 2B: A2; B35; **B61(40)**; Cw4; DR1; DR4; DQ5(1); DQ7(3)

Furthermore, the cytotoxic capacity of the T-cell lines and clones was determined in a ^{51}Chromium-release assay, which is the golden standard for measuring cytotoxicity of cross-reactive T cells (126). CMV B7/TPR T-cell line 5A1 efficiently lysed HLA-A*29:01^{+} EBV-LCLs, whereas EBV B35/YPL T-cell line 2A6 and EBV B7/RPP T-cell clones 9G6 and 10D8 were not cytotoxic toward the EBV-LCLs that induced IFNγ production (Figure 6). This is in concordance with previous data, demonstrating a similar discrepancy of cross-reactive CD8^{+} T cells that produce IFNγ, but lack cytotoxic capacity in response to alloantigen (127).

DISCUSSION

This article summarizes the advantages, limitations and applications of commonly used experimental methods for the detection of virus-specific $CD8^+$ T cells with cross-reactivity to allogeneic HLA antigen (see overview in Table S4, SDC, http://links.lww.com/TXD/A15).

We conclude that MLR can be a useful tool to screen for cross-reactivity of virus-specific T cells against alloantigen at bulk level. By using determined responder-stimulator combinations, donor-specific cross-reactivity can be identified and characterized. Furthermore, precursor frequencies of cross-reactive T cells can be calculated and unknown allo-HLA cross-reactivity can be identified by using a broad array of HLA-typed targets. Accordingly, we were able to identify allo-HLA specificity in conventional MLR: CMV B7/TPR $CD8^+$ T cells proliferated in response to HLA-$A29^+$, EBV B7/RPP T cells to HLA-$B40^+$ and EBV B35/YPL $CD8^+$ T cells proliferated in response to HLA-DRB1*03:01 stimulators. Interestingly, the latter alloresponse was mediated by $CD8^+$ T cells cross-reacting toward HLA class II alloantigen. Recognition of an HLA-DRB1*03:01-derived peptide within an HLA class I molecule was unlikely as not all stimulators shared HLA class I molecules. Although cross-reactivity of virus-specific $CD8^+$ T cells toward HLA class II molecules is rare, it has been reported previously for CMV-reactive T cells (43, 128). Therefore, when evaluating MLR results, one ought to keep in mind that TCR cross-reactivity is not restricted by the rules of cognate pMHC recognition.

Although MLR provides a suitable tool for cross-reactivity screening, it has limitations. First, a vast amount of both responder and stimulator cells is needed. Second, due to the usage of bulk PBMC cultures, high-affinity alloresponses may dominantly overgrow low-affinity alloresponses, leading to an underestimation of the latter. Third, cross-reactive cells with a low precursor frequency may not, or only incidentally, be detectable at bulk level depending on the number of analyzed responder cells. Fourth, the composition of cell types within the PBMC compartment may differ between stimulators, which could lead to further overestimation or underestimation of alloantigen recognition. MLRs are thus insufficient in detecting the full spectrum of cross-reactivity, resulting in only a moderate sensitivity of the assay.

In addition, one should keep in mind that allo-HLA cross-reactivity is directed against the combination of allogeneic HLA and endogenous peptide (129). Because cells derived from different individuals may differ in HLA expression levels and/or the ability to present cross-reactive peptides, the strength of an alloresponse could vary between individuals (Figure 2A). Moreover, tissue-specific expression of endogenous peptides could influence alloreactivity

(130, 131), and thereby affect transplantation outcome of different organs. Indeed, tissue-specific cross-reactivity of virus-specific T cells has been described: cross-reactive EBV-induced $CD8^+$ T cells showed decreased cytotoxic capacity toward epithelial and endothelial target cells compared to PBMCs due to poor presentation of the cross-reactive peptide in these cell types (87). Because MLR uses PBMCs as targets, potential recognition of tissue-specific peptides remains unnoticed. The latter could be overcome by modifying the protocol using different stimulator cell types such as epithelial and endothelial cells.

Finally, one of the major issues affecting the accuracy of MLR is bystander activation. In our experience, a vigorous proliferative response against allogeneic stimulator cells by virus-specific T cells in MLR gives a fair indication for cross-reactivity. On the contrary, less pronounced responses are more difficult to interpret. These responses could represent truly cross-reactive responses with low TCR affinity and/or low precursor frequencies, but they may also be the result of cytokine-mediated bystander activation. Hereto, a resting period and short restimulation phase was introduced in the conventional MLR protocol. We have shown that this experimental approach accurately identifies truly cross-reactive T cells based on proliferative capacity, cytokine production, degranulation and activation state upon encounter of alloantigen or viral peptide.

Cross-reactive T cells may respond differently to restimulation with alloantigen, depending on the TCR affinity and the levels of allo-HLA and peptide presented on the stimulator cells. The extent of the response may be less reproducible when studying cross-reactive responses with a very low precursor frequency. The number of cross-reactive T cells at the start of each MLR could differ by chance, and this difference is enlarged during the 9-day culture period. This protocol should therefore not be used for determining the strength of an alloresponse, but rather as a quick tool to discriminate cross-reactivity from bystander activation.

Compared to bulk level protocols, T-cell lines and clones can provide more detailed characterization and do not suffer from bystander activation and dominant overgrowth of TCR cross-reactivity. T-cell lines and clones can be screened against large panels of different cell types that express a wide array of HLA antigens (87, 117, 121, 132), and because they constitute a homogeneous population, the cross-reactive HLA antigen and peptide can be identified (44). Also, the mechanism underlying TCR cross-reactivity can be investigated. Molecular mimicry and alternate TCR docking modes have been identified as mechanisms for TCR cross-reactivity (44, 133-135). Moreover, T-cell lines and clones can be used to determine cytotoxicity of cross-reactive T cells, which is important because differences in effector function can influence the

impact on transplantation outcome. Our data show that cross-reactive T cells producing IFNγ upon alloantigen encounter are not always cytotoxic toward the same targets and highlight the importance of performing multiple functional assays for proper characterization of the cross-reactive response.

Despite these advantages, T-cell lines and clones have limitations as well. First, their generation is labor-intensive and time-consuming and they only represent part of the total T-cell repertoire generated against a specific viral epitope. Moreover, the precursor frequency, which has prognostic value for the impact of alloreactive T-cell responses on transplantation outcome (136), cannot be determined.

A major limitation of all techniques is the availability of viral peptide-HLA tetramer complexes. Although available tetramers are believed to cover the most dominant epitopes in individuals with the corresponding HLA type, it is not possible to address the total impact of all virus-specific $CD8^+$ T cells. Unfortunately, it is thereby impossible to uncover all cross-reactivities of virus-specific T cells. Moreover, limited availability of HLA class II tetramers impairs the analysis of the cross-reactive potential of virus-specific $CD4^+$ T cells, leaving their role in heterologous immune responses underexposed. Finally, current methods studying cross-reactive responses are labor-intensive and costly, which might hamper large scale screening of transplant recipients required to address the impact of cross-reactive T-cell responses on transplant function and outcome.

In conclusion, the cross-reactive potential of virus-specific T cells against allogeneic HLA antigen can be studied by using the techniques discussed, provided that one should be aware of their limitations. Depending on the research question and the availability of cells and resources, one can apply bulk MLR cultures for fast broad-spectrum screening, or T-cell lines and clones for in-depth characterization of heterologous immune responses.

ACKNOWLEDGMENTS

Financial support by the Dutch Kidney Foundation (Project CP09.04) is gratefully acknowledged. Furthermore, we would like to thank Prof. R.A.W. van Lier (Sanquin, Amsterdam, The Netherlands) for valuable advices and constructive criticisms.

SUPPLEMENTARY MATERIAL

Table S1. HLA class I antigens represented in the HLA-typed stimulator panel composed of 16 different stimulators.

HLA-A	HLA-B	HLA-C
A1	B7	Cw1
A2	B8	Cw2
A3	B13	Cw3
A11	B14	Cw4
A24	B18	Cw5
A25	B35	Cw6
A26	B38	Cw7
A29	B37	Cw8
A30	B39	Cw12
A31	B41	Cw14
A32	B44	Cw15
A33	B51	Cw16
A*68:01	B55	Cw17
A*68:02	B57	
A66	B58	
	B60	
	B61	
	B62	

Table S2. Virus-specific $CD8^+$ T cells in two healthy individuals show proliferation upon encounter with one or more allogeneic stimulators[1,2]

	CMV	EBV	FLU
R1	B7/TPR B35/IPS	B7/RPP	A2/GIL
R2		A2/GLC B35/EPL B35/YPL	A2/GIL

[1] Two healthy individuals (R1 and R2) selected on the presence of $CD8^+$ T cells directed against CMV, EBV and/or FLU were tested in mixed lymphocyte reactions against a panel of HLA-typed stimulators and analysed by flow cytometry.

[2] The viral epitopes that showed cross-reactivity to 1 or more allogeneic stimulators, defined as cells that bound indicated viral tetramers and proliferated in the MLR, are depicted.

Table S3. TCR usage of the generated CD8+ T-cell lines and clones[1]

	Name	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β
B35/YPL	1C1	TRAV21*01/02	TRAJ13*01/02	C A V L F W G Y Q K V T L	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
	2A6	TRAV8-2	TRAJ23	C V V S D G W D R G G K L I F	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
		TRAV12-1	TRAJ7	C V V N P Y Y G N N R L A F	TRBV6-1*01	TRBJ2-7*01	C A S S L V L A G E E T Q Y F
					TRBV11-2*01	TRBJ2-5*01	C A S S L V L A G E E T Q Y F
	3A6	TRAV8-4	TRAJ42	C A V S D Y G G S Q G N L I F	TRBV7-8*01	TRBJ2-1*01	C A S S L A G G G G N E Q F F
		TRAV21*01/02	TRAJ13*01	C A V L F W G Y Q K V T F	TRBV6-2*01/3*01	TRBJ2-1*01	C A S S S R T S G V P L G D E Q F F
B7/TPR	**5A1**	TRAV2*01/02	TRAJ12*01	C A V E P R M D S S Y K L S S	TRBV7-9	TRBJ2-7*01	C A S S L P D G A N Y E Q Y F
	5A3	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
	5A4	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K F S S	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
	5B2	TRAV17*01	TRAJ12*01	C A T E R R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S S R D V G A Y E Q Y F
	5B8	TRAV14/DV4*02	TRAJ12*01	C A M R E G G D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S S H D S T G F N S P L H F
	5D3	TRAV2*01	TRAJ12*01	C A V E P R M D S S Y K L I F	TRBV7-9	TRBJ2-7*01	C A S S L R D G A N Y E Q Y F
B7/RPP	9A12	TRAV38-2DV8	TRAJ54	C A Y T A G A Q K L V F	TRBV4-1*01	TRBJ2-7*01	C A S S Q E A Y N Y E Q Y F
		TRAV24*01	TRAJ24*01	C A F F T T D S W G K F Q L	TRBV25-1*01	TRBJ2-1*01	C A S S D M A G V G N E Q F F
					TRBV6-6*01/03	TRBJ2-5*01	C A S S L W V G Q Q E T Q Y F
					TRBV2*01/02	TRBJ1-3*01	C A S S D H S G G D G N T I Y F
	9G6	TRAV14/DV14*01	TRAJ9*01	C A M R D D T G G F K T I F	TRBV19*01/02/03	TRBJ2-7*01	C A S S I S S G V A Y E Q Y F
	10D8	TRAV14/DV14*01	TRAJ9*01	C A M R D D T G G F K T I F	TRBV19*01/02/03	TRBJ2-7*01	C A S S I S S G V A Y E Q Y F

[1]Cross-reactive T-cell lines and clones are indicated in bold

Table S4. Overview of strengths and limitations of experimental protocols to detect cross-reactivity

Detection method	Advantages	Disadvantages	Applications
Mixed Lymphocyte Reaction (bulk PBMC level)	+ Information on precursor frequency and clonality + Functional characterization + Fast results + Simultaneous analysis of multiple viral epitopes + Analysis of multiple allogeneic stimulators	- Vast amount of cells needed - Potential selective outgrowth of high-affinity responses - Moderate sensitivity - No cytotoxicity assay possible - Variation in target cells: composition of PBMC compartment and HLA expression - Bystander activation bias	> Screening for presence of cross-reactivity to a specific donor > Screening against HLA-typed targets > Analysis of frequency of cross-reactivity > Analysis of multiple viral epitopes > Analysis of clonality of cross-reactive responses > Identification of public cross-reactivities
Mixed Lymphocyte Reaction followed by restimulation (bulk PBMC level)	+ Information on precursor frequency and clonality + Functional characterization + Fast results + Simultaneous analysis of multiple viral epitopes + Analysis of multiple allogeneic stimulators + Discrimination of bystander activation versus cross-reactive responses	- Vast amount of cells needed - Potential selective outgrowth of high-affinity responses - Moderate sensitivity - No cytotoxicity assay possible - Variation in target cells: composition of PBMC compartment and HLA expression	> More accurate screening for true cross-reactive responses compared to MLR > Functional characterization of cross-reactivity > Screening against HLA-typed targets > Analysis of frequency of cross-reactivity > Analysis of multiple viral epitopes > Analysis of clonality of cross-reactive responses > Identification of public cross-reactivities

Table S4. Continued

Detection method	Advantages	Disadvantages	Applications
T-cell lines	+ Cytokine production by ELISA + Cytotoxicity assays + Screening against target cells of various origins + Minimal bystander activation	- Labor-intensive - Time-consuming - No information about precursor frequency - Selection bias - Potential selective outgrowth - Restricted to a single viral epitope	> Screening against HLA-typed targets > Analysis of cross-reactivity within a single viral epitope > Characterization of the effector function of cross-reactive T cells
T-cell clones	+ (Allo-HLA) specificity of a single TCR can be determined + Cytokine production by ELISA + Cytotoxicity assays + Screening against target cells of various origins + No bystander activation + Functional read-out specific for single clone + Inclusion of low affinity, low proliferating cells	- Labor-intensive - Time-consuming - No information about precursor frequency - Selection bias - Restricted to a single viral clone	> Screening against HLA-typed targets > Characterization of the effector function of cross-reactive T cells > Identification of the recognized allo-antigen > Affinity/avidity analyses

CHAPTER

4

INFECTION WITH A VIRUS GENERATES A POLYCLONAL IMMUNE RESPONSE WITH BROAD ALLOREACTIVE POTENTIAL

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Hum Immunol 2019 Feb;80(2):97-102

ABSTRACT

Virus-specific T cells have been shown to cross-react with allogeneic HLA (allo-HLA) at a clonal level. However, the impact of a single virus on the allorepertoire has never been investigated at the polyclonal level. We made an inventory of the incidence and specificity of allo-HLA-cross-reactive-virus-specific $CD8^+$ T cells in 24 healthy individuals. T cells were stained for 25 virus-specific tetramers, and mixed lymphocyte reactions were performed against a panel of HLA-typed allostimulators. Allospecificity was confirmed by IFNγ-ELISA using T-cell clones against a panel of HLA-typed cell-lines. The polyclonal immune repertoire directed against CMV alone was associated with a memory response against six allo-HLA molecules. Besides, a single allostimulator activated memory T-cell responses with multiple viral specificities. Concluding, a single virus can substantially broaden the allo-HLA memory T-cell repertoire. This study only looked at CMV- and EBV-specific T cells, whereas the immune repertoire consists of T cells directed against many different viruses. Hence, transplant patients receiving an HLA-mismatched graft may already express a polyclonal repertoire of anti-donor-memory T cells before transplantation.

INTRODUCTION

As a result of the inherent capacity of T-cell receptors (TCRs) to cross-react to multiple antigens, T cells can express memory phenotypes even for antigens they have never been exposed to. Virus-specific TCRs have been shown to commonly cross-react to allogeneic HLA (allo-HLA), and as a result, an alloreactive memory T-cell pool may exist without prior interaction with allogeneic HLA. This is of particular interest to the field of transplantation, where memory T-cell responses directed against donor cells pose a threat to transplant tolerance (60). Compared to naïve cells, memory T cells have a stronger effector potential, improved survival capacities and upregulated cell adhesion molecules that enable binding to and entering of inflammation sites. In addition, they have lower activation requirements as they do not rely on co-stimulation for their activation. Co-stimulation blockade is an important factor in routine immunosuppressive regimens and is very effective in preventing the activation of naïve T cells, but not of memory T cells. Calcineurin inhibitors (CNI) effectively suppress the activity of both phenotypes (137), but as they are extremely potent and non-specific, they come at the price of increased susceptibility to opportunistic infections (138). In addition, they have severe toxic side effects such as chronic nephrotoxicity and neuropathy (46, 47). In the quest for finding alternative immunosuppressive agents, a major focus lies on co-stimulation blockade, thereby leaving the memory compartment largely unaffected (59, 75-77). A recent report of a randomized clinical trial comparing the CNI tacrolimus to the CD28-CD80/86 co-stimulation inhibitor belatacept in kidney transplant recipients however shows that the acute rejection rate was significantly higher and more severe in the belatacept-treated versus the tacrolimus-treated group (139). Potentially, virus-specific memory T cells with cross-reactivity to donor HLA may have played a role in these rejections.

Several research groups have examined the potential cross-reactivity of virus-specific memory T cells toward allo-HLA. However, so far, studies primarily focused on the identification and characterization of individual allo-HLA-reactive virus-specific memory $CD8^+$ T-cell clones, whereas a viral infection generally induces a polyclonal immune response. The latter is comprised of T cells expressing a broad range of TCRs with different epitope specificities and large variation in TCR affinity and avidity for their epitopes. As TCR cross-reactivity of virus-specific T cells occurs in 45% of virus-specific T-cell clones and 80% of virus-specific T-cell lines (43), polyclonal immune responses that are generated in response to just a single virus are likely to induce many memory T cells that are able to cross-react to different allogeneic HLA molecules. The impact of such a broad polyclonal virus-induced immune response on the allorepertoire within an individual has not yet been determined. In this report, we made an

inventory of polyclonal anti-virus immune responses and their impact on the allorepertoire in healthy individuals.

MATERIALS AND METHODS

Collection of responder and target cells

Peripheral blood mononuclear cells (PBMCs) were derived from healthy individuals of both male and female origin with informed consent conform the Declaration of Helsinki. Standard density gradient centrifugation (Ficoll-Isopaque separation) was performed to isolate PBMCs from whole blood. PBMCs were cryopreserved prior to usage.

Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated from PBMCs by incubation with supernatant of the EBV-producing marmoset cell line B95.8 for 1.5 hours at 37°C. Culturing was done in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with penicillin/streptomycin (Gibco), glutamine and 10% fetal calf serum (FCS).

Generation of virus-specific $CD8^+$ T-cell clones and lines

$CD8^+$ memory T-cell clones and lines were generated by fluorescence-activated cell sorting (FACS Aria; BD) (118). PBMCs were stained with phycoerythrin (PE)-labeled viral tetramers (Table 1) (Leiden University Medical Center Protein facility, Department of Immunohematology and Blood Transfusion, the Netherlands) and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) for CD4, CD19, CD45-RA, CD14, CD40, CD16 and CD56 (BD Pharmingen). FL1 was used as a dump channel to avoid TCR internalization as a result of simultaneous CD8 mAb and major histocompatibility complex (MHC)-tetramer staining. $CD8^+$ memory T-cell clones were generated by sorting 1 cell per well^{96} and $CD8^+$ memory T-cell lines by sorting 10 cells per well^{96}. TCR usage was assessed by antibody staining against the TCR Vb (IO Test Vbeta TCR repertoire kit, Beckman Coulter, USA). $CD8^+$ memory T-cell clones and lines were cultured in the presence of irradiated allogeneic PBMCs (4000 Rad) from anonymous buffy coats (Sanquin, Leiden, the Netherlands) for 8 days prior to experimental testing to achieve optimal conditioning.

HLA typing of responder and target cells

HLA typing was achieved by sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) genotyping, at the European Federation of Immunogenetics (EFI)-accredited national reference laboratory for histocompatibility testing at the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands.

Mixed lymphocyte reactions

To assess proliferation of cross-reactive viral tetramer-positive $CD8^+$ T cells in response to the most commonly occurring HLA class I alleles in the Western population (>5%), PBMCs of healthy donors positive for multiple CMV and/or EBV tetramers were labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with irradiated allogeneic PBMCs (3000 Gy) in mixed lymphocyte reactions (MLRs) against a panel of 16 HLA-typed stimulators. MLRs were performed in Roswell Park Memorial Institute medium (RPMI) supplemented with penicillin/streptomycin (Gibco), glutamine, 15% human serum (HS) and 10 CU/ml IL-2. Upon 8 days, proliferation of tetramer-positive cells was measured by flow cytometry as identified by the $tetramer^+CFSE^{low}CD8^+$ subset. MLRs were first performed against stimulator pools (4x4), and subsequently against individual stimulators of the pool(s) of interest.

Cytokine production assays

Virus-specific $CD8^+$ T-cell clones and lines were stimulated with a panel of allogeneic EBV-LCLs (E:T 1:10; triplicate wells) for 24 hours at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% fetal calf serum (FCS; Lonza), 5% human serum (HS), and IL-2 (10 CU/mL). The panel was designed to cover the most commonly occurring HLA class I alleles in the Western population (>5%). Interferon γ (IFNγ) production was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, the Netherlands).

RESULTS

For an overview of the experimental procedure, a flowchart is added in the supplementary material (Supplemental Figure 1).

The polyclonal CD8$^+$ T-cell response directed against a single virus has the potential to recognize multiple allogeneic stimulators

First, an inventory was made of the incidence and specificity of allo-HLA cross-reactive virus-specific CD8$^+$ T cells in a cohort of 30 healthy individuals. PBMCs were stained with a panel of CMV (n = 13) and EBV (n = 12) tetramers (Table 1).

Healthy donors that stained positive for multiple tetramers directed against the same virus (n = 24) were screened for alloreactivity in mixed lymphocyte reactions (MLRs), which were performed against a panel of allogeneic cells (n = 16) designed to express the most common HLA class I antigens (>5%) in the Western population (Table 2).

Table 1. Panel of 25 CMV- and EBV-specific tetramers directed against public viral epitopes[a]

CMV			EBV		
HLA	Peptide	Origin	HLA	Peptide	Origin
A1	VTEHDTLLY	pp65	A2	GLCTLVAML	BMLF1
A1	YSEHPTFTSQY	pp65	A3	RLRAEAQVK	EBNA3A
A2	NLVPMVATV	pp65	A3	RVRAYTYSK	BRLF1
A2	VLEETSVML	IE-1	A3	KHSRVRAYTYSK	BRLF1
A3	TVYPPSSTAK	pp150	B7	RPPIFIRRL	EBNA3A
A11	GPISGHVLK	pp65	B8	FLRGRAYGL	EBNA3A
A24	QYDPVAALF	pp65	B8	RAKFKQLL	BZLF1
B7	RPHERNGFTVL	pp65	B35	EPLPQGQLTAY	BZLF1
B7	TPRVTGGGAM	pp65	B35	HPVGEADYFEY	EBNA-1
B8	ELRRKMMYM	IE-1	B35	MGSLEVMPM	LMP2A
B8	ELKRKMIYM	IE-1	B35	YPLHEQHGM	EBNA3A
B8	QIKVRVDMV	IE-1	B35	AVLLHEESM	EBNA3B
B35	IPSINVHHY	pp65			

[a]All tetramers are phycoerythrin (PE)-labelled

Table 2. Panel of HLA-typed allogeneic stimulator PBMCs, designed to cover the most commonly occurring HLA-I antigens in the Western population (>5%)

HLA ALLELES REPRESENTED IN THE STIMULATOR PANEL				
HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A1	B7	Cw1	DR1	DQ1
A2	B8	Cw2	DR4	DQ2
A3	B13	Cw3	DR7	DQ4
A11	B14	Cw4	DR8	DQ5
A24	B18	Cw5	DR10	DQ6
A25	B27	Cw6	DR11	DQ7
A26	B35	Cw7	DR13	DQ8
A29	B37	Cw8	DR15	
A30	B38	Cw9	DR16	
A31	B39	Cw10	DR17	
A32	B41	C*12		
A33	B44	C*14		
A66	B51	C*15		
A68	B55	C*16		
	B57	C*17		
	B58			
	B60			
	B61			
	B62			

HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF THE STIMULATOR PANEL										
Stimulator	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ	
1	A*02:01	A*32:01	B*35		C*04:01		DRB1*03:01	DRB1*11	DQB1*02	DQB1*03:01
2	A24(9)	A29(19)	B7	B60(40)	Cw7		DR13(6)	DR8	DQ6(1)	DQ4
3	A*02:01	A*11:01	B*07:02	B*13:02	C*06:02	C*07:02	DRB1*15	DRB1*07	DQB1*02	DQB1*06:02
4	A*01	A*02:01	B*08:01	B*44	C*05	C*07:01	DRB1*03:01	DRB1*15	DQB1*02:01	DQB1*06:02
5	A*02:01	A*30:01	B*07:02	B*13:02			DRB1*04:03	DRB1*15:01	DQB1*03:02	
6	A2	A33	B44	B14	Cw5	Cw8	DR1	DR4	DQ5	DQ8
7	A2	A26	B38	B55	Cw1		DR13		DQ1	
8	A*26	A*68	B*51		C*15		DRB1*04:04	DRB1*13:01	DQB1*03:02	DQB1*06:03
9	A1	A3	B55	B37	Cw3	Cw6	DR16	DR13	DQ6	
10	A1	A31	B62	B57	Cw3	Cw6	DR15	DR11	DQ1	DQ7
11	A*01:01	A*25:01	B*18:01	B*58:01	C*03:02	C*12:03	DRB1*08:01	DRB1*13:01	DQB1*04:02	DQB1*06:09
12	A1	A11	B8	B35	Cw4	Cw7	DRB1*01:03	DRB1*03:01	DQB1*02	DQB1*05:01
13	A*24:02	A*29:01	B*39:06	B*44:03	C*07:02	C*16:01	DRB1*07	DRB1*08:01	DQB1*02:02	DQB1*04:02
14	A*02:05	A*66:01	B*41:02	B*58:01	C*07:01	C*17:01	DRB1*13:03	DRB1*07:01	DQB1*02:01	DQB1*03:01
15	A*03:01	A*31:01	B*51:01	B*18:01	C*07:01	C*14:02	DRB1*10		DQB1*05:01	
16	A*03:01	A*31:01	B*15:01	B*40:02	C*02:02	C*03:03	DRB1*04:01	DRB1*13:01	DQB1*03:02	DQB1*06:03

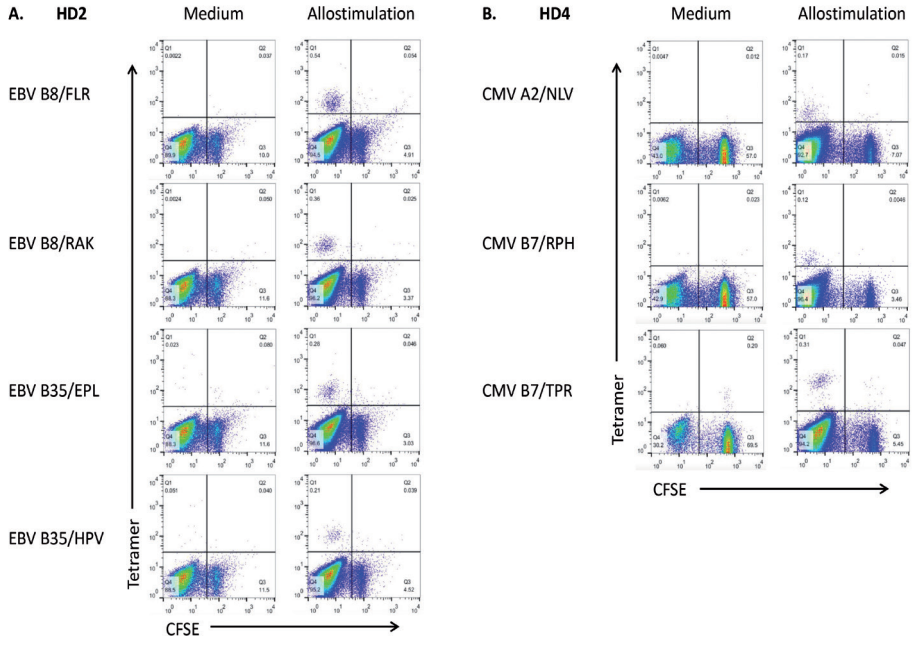


Figure 1. Multiple virus-specific CD8⁺ T cells of the same individual proliferate in response to allostimulation. A) Example of individual HD2 showing alloreactivity of the polyclonal immune response against EBV. Plots show: EBV B8/FLR x Pool 1 (stimulator 1-4); EBV B8/RAK x Pool 4 (stimulator 13-16); EBV B35/EPL x Pool 3 (stimulator 9-12); EBV B35/HPV x Pool 1 (stimulator 1-4). B) Example of individual HD4 showing alloreactivity of the polyclonal immune response against CMV. Plots show: CMV A2/NLV x Pool 1 (stimulator 1-4); CMV B7/RPH x Pool 4 (stimulator 13-16); CMV B7/TPR x Pool 3 (stimulator 9-12). All plots are gated on CD8⁺ lymphocytes.

Within polyclonal anti-viral immune responses, T cells with different viral epitope specificities were able to proliferate in response to allogeneic stimulation. This was observed for EBV and CMV responses, and also for both viruses within the same individual (Figure 1, Table 3). Interestingly, single allogeneic stimulators were able to induce multiple different virus-specific CD8⁺ T-cell responses in the same responder (Figure 2).

Table 3. Overview of all virus-specific T cells responding to allogeneic cells in MLR per healthy individual[ab]

Virus	HD1	HD2	HD3	HD4	HD5	HD6	HD7	HD8	HD9	HD10	HD11	HD12	HD13	HD14	HD15	HD16	HD17	HD18	HD19	HD20	HD21	HD22	HD23	HD24
CMV	A1/VTE	A2/NLV	A1/VTE	A2/NLV	B7/RPH	A2/NLV	A1/VTE	A1/VTE	B7/RPH	A1/VTE	A1/VTE	A1/VTE	A2/NLV	A1/VTE	A1/VTE	A2/NLV	A2/NLV		A2/VLE	B7/RPH			A2/NLV	B7/RPH
	A1/YSE	B8/ELR	A2/NLV	B7/RPH	B7/TPR	B7/RPH	A1/YSE	B7/RPH	B7/TPR	A1/YSE	A1/YSE	A1/YSE	B7/TPR	B8/ELR	A1/YSE	B7/RPH	B7/RPH			B7/TPR			B35/IPS	B7/TPR
	A2/NLV	B8/QIK	B8/ELR	B7/TPR		B7/TPR	B7/RPH	B7/TPR		B8/ELR	A2/NLV	B8/ELR		B8/ELK	B8/ELR	B7/TPR	B7/TPR							
		B35/IPS					B7/TPR			B8/ELK	B8/ELR	B8/QIK					B35/IPS							
							B8/ELK			B8/QIK	B8/QIK													
							B8/QIK			B35/IPS														
EBV	A2/GLC	B8/FLR	A2/GLC		A2/GLC	A2/GLC	B8/FLR	B7/RPP	B7/RPP	n.d.	n.d.	n.d.	A2/GLC	n.d.	n.d.	n.d.	A2/GLC	A2/GLC	B7/RPP	A2/GLC	A2/GLC	A2/GLC		A3/RLR
	B8/FLR	B8/RAK	B8/FLR		B7/RPP	A3/RLR	B8/RAK	B8/RAK	B35/EPL								B7/RPP	B7/RPP	B35/EPL	B7/RPP	B8/FLR	A3/RLR		B7/RPP
	B8/RAK	B35/EPL	B8/RAK			B7/RPP			B35/HPV								B35/EPL	B35/EPL	B35/HPV	B8/FLR	B8/RAK	B7/RPP		
		B35/HPV	B7/RPP														B35/EPL	B35/HPV		B8/RAK		B35/HPV		
		B35/YPL															B35/HPV	B35/YPL						
																	B35/YPL							

[a]Results are based on MLRs using the individual stimulators from the stimulator panel

[b]Red = proliferation; n.d. = not determined

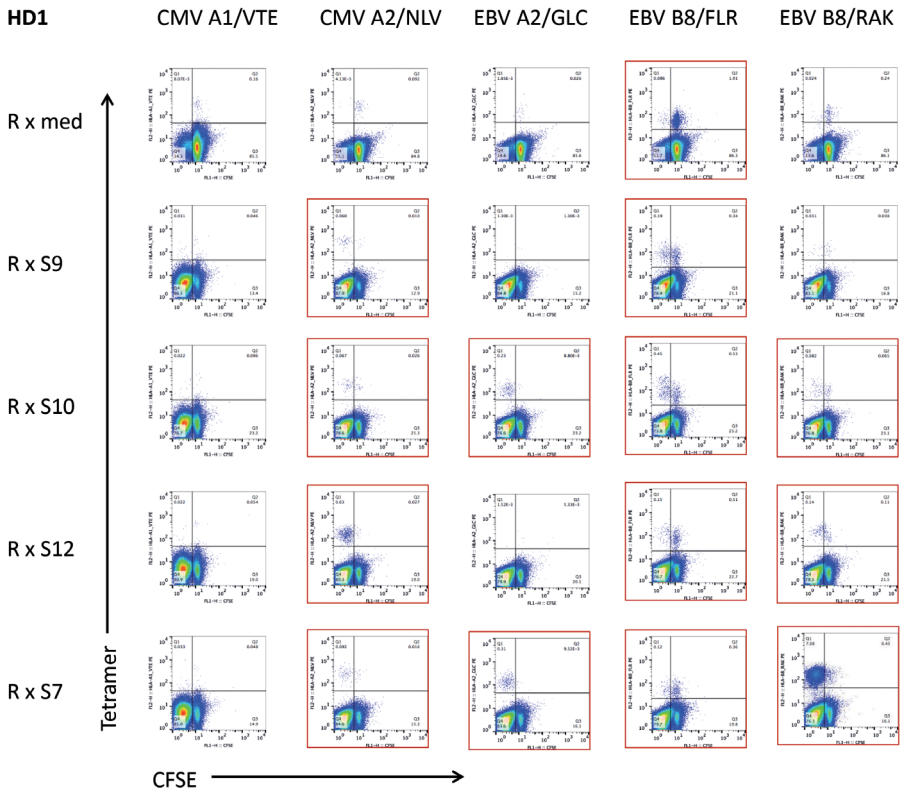


Figure 2. Single allogeneic stimulators induced multiple virus-specific CD8⁺ T-cell responses in MLR in the same responder (HD1). Although the EBV B8/FLR response should be interpreted with caution due to its proliferation background in media (% proliferated Tm-positive cells of total Tm-positive cells: 4.3%), its alloresponses were much more pronounced (% proliferated Tm-positive cells of total Tm-positive cells: respectively 35.8% (S9); 45.9% (S10); 22.7% (S12); and 25% (S7)). Plots are gated on CD8⁺ T cells. X-axis: CFSE. Y-axis: virus-specific tetramer.

The polyclonal CD8⁺ T-cell response directed against a virus contains multiple allo-HLA specificities

Virus-specific T cells with different viral specificities exerted different patterns of alloreactivity against the stimulator panel in MLR, indicating that they had different allo-HLA specificities as well. To confirm, virus-specific CD8⁺ memory T-cell clones were generated as a proof of principle to determine their allospecificity in IFNγ ELISA against a panel of EBV-immortalized B-cell lines (EBV-LCLs) (Supplemental Table 1). For example, responder HD23 showed cross-reactivity of CMV A2/NLV- and CMV B35/IPS-specific T cells. The CMV B35/IPS response was directed against

HLA-B*51:01 and HLA-B*58:01/B*57:01, a public cross-reactivity that was recently identified by our group (140). The CMV A2/NLV alloresponse showed cross-reactivity in response to multiple allo-HLA molecules: a CMV A2/NLV T-cell line (1A2) showed cross-reactivity against HLA-B*39:01, and a CMV A2/NLV T-cell clone (#1) against the combination of HLA-A2 and HLA-B50 (Table 4, Supplemental Figure 2). TCR Vb usage analysis confirmed that the CMV A2/NLV T-cell line and clone expressed multiple TCR clonotypes, whereas the CMV B35/IPS T-cell lines and clones expressed a public TCR (140). Findings were confirmed in additional MLRs (data not shown). Infection with CMV in this individual therefore enabled alloreactivity towards (a minimum of) six different allogeneic HLA molecules.

Table 4. Virus-specific T cells derived from the same individual and directed against the same virus show multiple allo-HLA cross-reactivities[a]

Viral specificity	Healthy Donor	T-cell clone / line	Reactivity against EBV-LCL	TCR Vβ usage	Allo-HLA cross-reactivity
CMV B35/IPS	HD23	Clone 7C8	7, 9, 10, 12	TRBV28	HLA-B*51:01 HLA-B*57:01 HLA-B*58:01
	HD23	Clone 8C1	9, 12[b]	n.d.*	HLA-B*58:01[b]
	HD23	Cell line 6A3	7, 9, 12	TRBV28 + TRBV12 + TRBV6-2	HLA-B*57:01 HLA-B*58:01
	HD23	Cell line 6A8	7, 9, 12	TRBV28 + TRBV20-1	HLA-B*57:01 HLA-B*58:01
CMV A2/NLV	HD23	Clone 1	23[c]	TRBV20-1	HLA-A*02 HLA-B*50:01
	HD23	Cell line 1A2	15	TRBV3-1 + TRBV18 + TRBV6 + TRBV20-1	HLA-B*39:01

[a]Reactivity against EBV-LCLs expressing syngeneic HLA-B*35:01 and HLA-A*02:01 was disregarded for analyses of CMV B35/IPS and CMV A2/NLV responses respectively, as it potentially reflects reactivity towards the cognate epitope

[b]Potential minor reactivity towards EBV-LCL 7 (HLA-B*57:01), however the response was too small to include in analysis

[c]All T-cell lines and clones were tested against EBV-LCL panel 1, except CMV A2/NLV Clone 1 (EBV-LCL panel 2)

*n.d. = not determined

DISCUSSION

As humans are exposed to a myriad of viruses throughout their life-time and TCR cross-reactivity is a common feature of T cells, it is not surprising that the majority of virus-specific T cells are able to cross-react to allo-HLA. Although our understanding of this cross-reactivity increases and even mechanisms underlying this cross-reactivity have been proposed (135, 141), the possible clinical relevance of these cross-reactive T cells remains under investigation (39-41, 142).

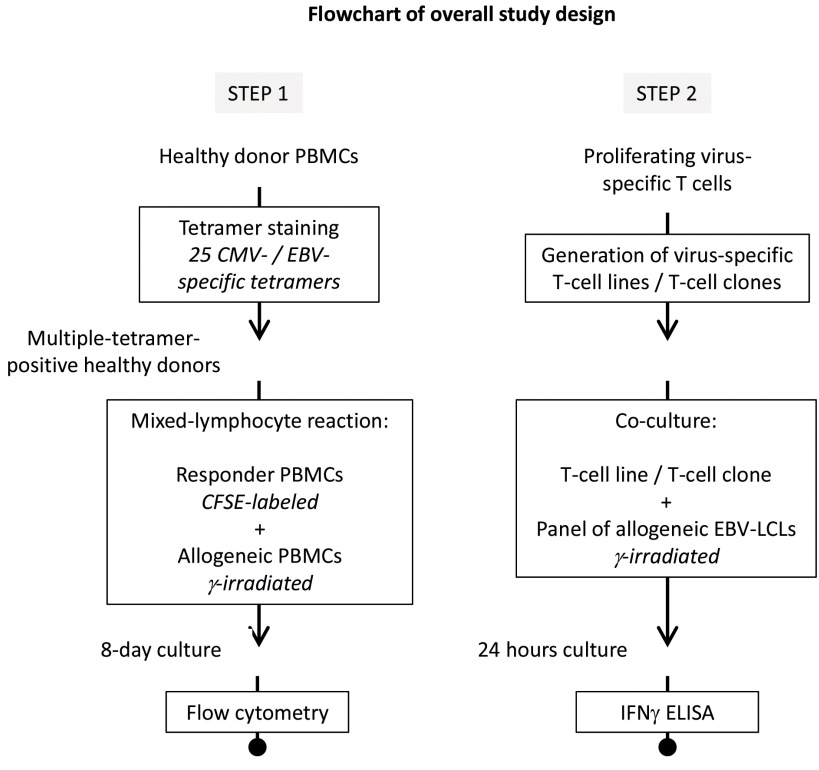
In this study, we aimed to determine the footprint of a single virus on the allorepertoire. We observed broad alloreactivity of virus-specific T cells on multiple levels: T cells with different viral epitope specificities, T cells with the same viral epitope specificities, and even T cells of the same clonotype were able to recognize multiple allogeneic HLA molecules. Polyclonal alloimmune responses of EBV and CMV T cells were identified in several individuals. This is particularly interesting given the fact that the experiments were restricted to known (dominant) viral epitopes for tetramer-staining. In total, 13 CMV- and 12 EBV-specific tetramers were available. It is thus remarkable that polyclonal alloresponses were found for both EBV and CMV, as the limited number of available tetramers inevitably leads to underestimation of the scope of the polyclonal alloresponse. Accordingly, a large population of tetramer-negative $CD8^+$ T cells responded to allostimulation (Figure 1, 2), possibly containing additional cross-reactive virus-specific T cells directed against unknown viral epitopes. In addition, alloreactivity screening was restricted to HLA-I alleles present in >5% of the Western population, and the allospecificity of polyclonal anti-virus responses will most likely be broader when taking into account less common HLA class I molecules as well.

Finally, we previously published that functional virus-specific T-cell responses can be induced by stimulation with allogeneic cells (143). We again observed that allostimulation was able to induce proliferation of virus-specific T cells, and in addition that a single allogeneic stimulator was able to stimulate T cells of multiple viral specificities (belonging to the same individual): further illustrating the impact of virus-specific immune responses on the allorepertoire.

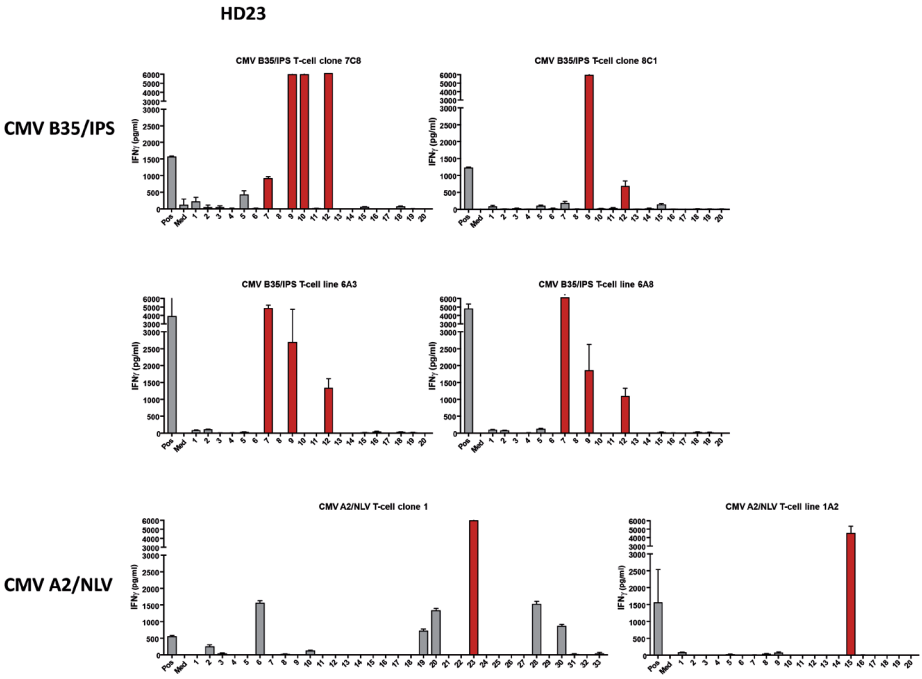
In conclusion, infection with a single virus can generate a diverse allorepertoire. Cross-reactive memory T-cells in the polyclonal anti-viral immune response can have broad alloreactive potential, as not only T cells with different viral epitope specificities, but also T cells sharing viral specificity and T cells of the same clonotype can be cross-reactive with multiple allo-HLA molecules. Thereby, the many viruses encountered throughout life could induce a

broad repertoire of (donor-specific) alloreactive memory T cells in transplant recipients already in place at the time of transplantation. This message is important to keep in mind, especially when seeking alternative immunosuppression strategies. Current standard-of-care immunosuppression covers suppression of the memory compartment, and it is still unclear what will happen to the alloresponse when the naïve compartment is selectively targeted instead. For example, based on the high prevalence of pre-existing allo-HLA cross-reactivity, one could argue that clinical rejection rates should be higher than is currently the case; potent immunosuppression is likely to play an important role here. In addition, the functional characteristics of the allo-HLA cross-reactive virus-specific T cells may not be sufficient to mount potent immune responses: for example due to low TCR avidity for the alloepitope (144). Yet, also low-avidity cross-reactive clonotypes could gain momentum when triggered upon viral infection or reactivation; and current standard-of-care anti-viral prophylaxis may also play an indirect role in preventing alloresponses (145, 146). Finally, continuous allostimulation, as is the case in a transplantation setting, may induce mechanisms of regulation or T-cell exhaustion (147). Answering these questions will make an invaluable contribution to unravel the clinical relevance of allo-HLA cross-reactive virus-specific memory T cells in transplantation.

SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Flowchart of the experimental procedure to determine alloreactivity of polyclonal T-cell immune responses.



Supplemental Figure 2. Allospecificity of CMV-specific CD8⁺ memory T-cell clones was determined in IFNγ ELISA against a panel of HLA-typed EBV-LCLs. All T-cell lines and clones were derived from HD23 and tested against EBV-LCL panel 1 or EBV-LCL panel 2 (CMV A2/NLV Clone 1) (Supplemental Table 1). Reactivity of CMV B35/IPS T-cell clones against EBV-LCLs expressing syngeneic HLA-B*35:01 (e.g. reactivity of CMV B35/IPS T-cell clone 7C8 versus EBV-LCL 5) and reactivity of CMV A2/NLV T-cell clones against EBV-LCLs expressing syngeneic HLA-A*02:01 (e.g. reactivity of CMV A2/NLV T-cell clone 1 versus EBV-LCL 30) were disregarded for analysis, as these potentially reflect reactivity towards the cognate epitope. X-axis: EBV-LCLs. Y-axis: IFNγ production in pg/ml. Positive control: EBV-LCL expressing syngeneic HLA + viral peptide (1000ng/ml). Red = reactivity against these EBV-LCLs was confirmed.

Supplemental Table 1. Panels of HLA-typed EBV-LCLs designed to cover the most commonly occurring HLA-I antigens in the Western population (>5%)[1]

HLA ALLELES REPRESENTED IN EBV-LCL PANEL 1				
HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A1	B7	Cw1	DR1	DQ1
A2	B8	Cw2	DR2	DQ2
A3	B13	Cw4	DR3	DQ4
A11	B14	Cw5	DR4	DQ5
A24	B18	Cw6	DR5	DQ7
A25	B35	Cw7	DR7	DQ8
A26	B38	Cw8	DR8	DQ9
A29	B39	Cw9	DR10	
A30	B41	Cw10	DR11	
A31	B42	C*12	DR12	
A32	B44	C*15	DR13	
A33	B49	C*16	DR14	
A68	B51	C*17	DR15	
	B52		DR17	
	B55		DR18	
	B57		DR103	
	B58			
	B60			
	B62			

HLA ALLELES REPRESENTED IN EBV-LCL PANEL 2				
HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A1	B7	Cw1	DR1	DQ1
A2	B8	Cw2	DR2	DQ2
A3	B13	Cw4	DR3	DQ4
A11	B14	Cw5	DR4	DQ5
A23	B15	Cw6	DR7	DQ6
A24	B18	Cw7	DR8	DQ7
A25	B27	Cw8	DR9	DQ8
A26	B35	Cw9	DR10	DQ9
A30	B37	Cw10	DR11	
A31	B38	C*12	DR12	
A32	B39	C*15	DR13	
A33	B40	C*16	DR14	
A66	B42	C*17	DR15	
A68	B44		DR16	
A80	B45		DR17	
	B46		DR18	
	B49			
	B50			
	B51			
	B52			
	B53			
	B55			
	B57			
	B58			
	B60			
	B62			
	B63			
	B70			

HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 1										
EBV-LCL	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ	
1	A*02:01:01	A*32:01:01	B*08:01:01	B*44:05:01	C*02:02:02	C*07:01/07:06/07:18	DR4	DR7	DQ2	DQ7
2	A*24:02	A*33:01:01	B*14:02:01		C*02:02/02:32	C*08:02/08:29	DR1		DQB1*05:01	
3	A*03:01/03:22	A*29:02/29:09	B*07:02/07:61/07:114	B*44:03/44:105	C*07:02:01	C*16:01:01	DR2			
4	A*11:01/11:43	A*33:03/33:51	B*18:01/18:17N	B*52:01:01	C*07:02:01	C*07:04:01	DR15	DRB1*14:10	DQB1*05:01	DQB1*05:03
5	A*11:01/11:33	A*30:01/30:54	B*13:02:01	B*35:01/35:42	C*04:01:01	C*06:02:01	DR13	DR14	DQ1	
6	A*01:01:01:01	A*26:01:01	B*08:01:01	B*49:01:01	C*07:01:01		DRB1*01	DRB1*03:04	DQB1*03:04	DQB1*05:04
7	A*11:01:01	A*31:01:02	B*15:01:01	B*57:01:01	C*03:03:01	C*06:02/06:55	DR7	DRB1*04:01	DQB1*03:01	DQB1*03:03
8	A*02:03:01	A*24:02	B*38:02:01	B*40:01:02	C*03:04:01	C*07:02:01	DR2	DR5		
9	A*29:02:01	A*31:01:02	B*18:01/18:17N	B*58:01:01	C*05:01	C*07:18/07:01	DR3	DR8	DQ2	
10	A*24:03:01		B*51:01:01		C*15:02:01		DRB1*11:04		DQB1*03:01	
11	A*26:01:01		B*38:01:01		C*12:03:01		DRB1*04:02		DQB1*03:02	
12	A*24:02:01	A*30:01:01	B*51:01:01	B*58:01:01	C*01:02:01	C*03:02:02	DR1	DR7	DQ1	DQ2
13	A*02:01:01	A*03:01:01	B*15:01:01:01		C*03:03:01		DR4	DR13	DQ1	DQ3
14	A*68:01:02	A*68:02:01	B*44:02/44:19N	B*55:01:01	C*03:03:01	C*07:04:01	DR14		DQ1	
15	A*24:02	A*31:01:02	B*39:01	B*55:01:01	C*03:03:01	C*12:03:01	DR13		DQ1	
16	A*30:01:01	A*68:02:01	B*42:01:01		C*17:01:01		DRB1*03:02		DQB1*04:02	
17	A*01:01:01:01		B*41:01		C*17:01:01		DRB1*11:01		DQB1*03:01	
18	A*02:01:01	A*11:01:01	B*35:01:01	B*44:03:01	C*04:01:01	C*16:01:01	DRB1*01:03			
19	A*02:01:01	A*25:01:01	B*18:01/18:17N/18:43	B*44:02/44:19N/44:55	C*05:01:01	C*12:03:01	DR1	DR4	DQ1	DQ7
20	A31	A*24:02	B7		Cw4	Cw7	DR12	DR15		

HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 2

EBV-LCL	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ	
1	A*24:02	A*33:01:01	B*14:02:01		C*02:02/02:32	C*08:02/08:29	DR1		DQB1*05:01	
2	A*11:01:01	A*31:01:02	B*15:01:01	B*57:01:01	C*03:03:01	C*06:02/06:55	DR7	DRB1*04:01	DQB1*03:01	DQB1*03:03
3	A*02:01		B*15:01	B*39:01	C*03:03/03:11/03:13	C*07:01	DRB1*13:03	DRB1*14:54/14:01/14:07	DQB1*03:01	DQB1*05:03
4	A2		B44(12)	B57(17)	Cw6		DR7	DR9		
5	A*68:01:02	A*68:02:01	B*44:02	B*55:01:01	C*03:03:01	C*07:04:01	DR14		DQ1	
6	A1	A*24:03(9)	B52(5)	B49(21)	Cw7		DR15(2)	DR11(5)	DQ6(1)	DQ7(3)
7	A*01:01:01:01		B*40:01:01		C*06:02:01:01		DRB1*13:01:01:02		DQB1*06:03:01	
8	A31	A*24:02	B7		Cw4	Cw7	DR12	DR15		
9	A1	A3	B37		Cw6		DR10		DQ5(1)	
10	A3	A24(9)	B39	B50			DRB1*03:01	DRB1*16:01	DQB1*02:01	DQB1*05:02
11	A*03:01	A*33:01	B*14:02	B*51:24	C*08:02	C*15:02	DRB1*04:01	DRB1*16:01	DQB1*03:01	DQB1*05:02
12	A*02:01/02:17/02:04		B*18:01/18:03/18:05		C*07:01/07:05/07:06+		DRB1*14:54/14:01/14:05		DQB1*05:03/05:05	
13	A*01	A*02:01	B*08:01	B*53:06	C*02:02	C*07:01/07:06	DRB1*03:01	DRB1*11:01	DQB1*02:01	DQB1*03:01
14	A*02:10	A*30:01	B*13:02	B*40:06	C*06:02	C*08:01	DRB1*07:01	DRB1*09:01	DQB1*02	DQB1*03:03
15	A*24:02	A*26:01/26:08/26:02	B*38:01	B*51:01/51:03/51:11N	C*05	C*12:03/12:06	DRB1*07	DRB1*13:22	DQB1*02:02	DQB1*06:03
16	A*02:01		B*08:01	B*39:06:02	C*07:02		DRB1*08:01	DRB1*09:01	DQB1*03:03	DQB1*04:02
17	A*66:02	A*30:02	B*18:01	B*58:01	C*07:01					
18	A*01:01	A*02:07	B*08:01/08:08N/08:18	B*27:04/27:68/27:69	C*07:01/07:06/07:18	C*12:02/12:17/12:22				
19	A*24:02	A*25:01	B*15:01/15:12/15:14	B*55	C*03:03/03:11/03:12		DRB1*08:06	DRB1*15:01	DQB1*05:01	DQB1*06:02
20	A*02:17:01		B*15:01:01:01		C*03:03:01		DRB1*14:02:01		DQB1*03:01:01	
21	A*01:01		B*35:02		C*04:01		DRB1*11:04:00		DQB1*03:01	
22	A*02:01:01:01		B*45:01:01		C*16:01:01		DRB1*13:01:01		DQB1*06:03:01	
23	A*02:08	A*01:01:01:01	B*50:01:01	B*08:01:01	C*07:01:01:01	C*06:02:01:02	DRB1*03:01:01:01	DRB1*07:01:01:01	DQB1*02:01/02:02/02:04	

HLA TYPINGS OF THE INDIVIDUAL STIMULATORS OF EBV-LCL PANEL 2 Continued

EBV-LCL	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ	
24	A*23:01		B*14:01		C*08:02		DRB1*04:01:01		DQB1*03:02:01	
25	A*24:02:01:01	A*26:01	B*35	B*38:01	C*04:01	C*12:03	DRB1*03:01	DRB1*08:06	DQB1*02:01	DQB1*06:02
26	A*30:01:01	A*68:02:01	B*42:01:01		C*17:01:01		DRB1*03:02		DQB1*04:02	
27	A2	A*80:01	B58	B70	Cw2	Cw6	DR17	DR11	DQ2	DQ7
28	A*02:04		B62(15)		Cw3		DRB1*14: 02/14:06		DQ8(3)	
29	A*66:01		B*38:01		C*12:03		DRB1*14:01		DQB1*05:03	
30	A*02:01:01	A*02:07	B*46:01:01		C*01:02:01		DRB1*09:01:02	DRB1*12:02:01	DQB1*03:01	DQB1*03:03
31	A*31:01/31: 02/31:06	A*32:01/32: 05/32:08	B*40:01/40: 02/40:11	B*40:02/40: 27/40:29	C*02:02/02: 04/02:08+	C*03:04/03: 05/03:08	DRB1*04:04/ 04:07/04:05	DRB1*04:04/04: 03/04:07+	DQB1*03:02/03: 05/03:07+	
32	A*01:01/01 :04N/01:09		B*15:17	B*57:01/57: 06/57:08	C*04:01/04 09N/04:05	C*07:01/07: 05/07:06	DRB1*04:05	DRB1*08:08	DQB1*02:01	DQB1*04:02
33	A*24:02		B*40:01		C*03:04		DRB1*09:01:02		DQB1*03:03:02	

[1] All T-cell lines and clones were tested against EBV-LCL panel 1 except CMV A2/NLV Clone 1 (EBV-LCL panel 2)

CHAPTER

5

STIMULATION OF HUMAN EBV- AND CMV-SPECIFIC CYTOLYTIC EFFECTOR FUNCTION USING ALLOGENEIC HLA MOLECULES

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J Immunol. 2012 Nov 15;189(10):4825-31

ABSTRACT

Viral infection is a major cause of morbidity and mortality, and there are few therapeutic options available to augment a virus-specific T-cell response. Although allo-HLA cross-reactivity from virus-specific memory T cells is common, it is unclear whether priming with specific allogeneic cells could conversely elicit a viral peptide/self-HLA restricted cytotoxic T-cell response in humans. First, we used the previously described allo-HLA-B*44:02 cross-reactivity of EBV peptide/HLA-B8 restricted T cells, to determine whether allogeneic HLA stimulation can elicit a cytolytic immune response against EBV. HLA-B8^{+} 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 d MLR. The allo-HLA stimulated responder cells were then evaluated for cytotoxicity using EBV peptide loaded autologous target cells and unloaded HLA-B8^{+} EBV LCL target cells. PBMCs from EBV-seropositive donors gained EBV-specific cytolytic effector function following specific allo-HLA stimulation. Finally, we also elicited cytolytic CMV-specific responses using specific allogeneic cell stimulation, to confirm that this technique can be used to elicit viral peptide/self-HLA restricted responses even from nonpublic TCR responses. Allogeneic cell stimulation used as a cell therapy may be a potential tool to augment an antiviral T-cell response in patients with EBV or CMV infection.

INTRODUCTION

Control of viral replication depends primarily on virus-specific memory T-lymphocyte activity (148, 149). In the normal course of viral infections, antiviral 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 (150), and the use of lymphocyte-targeted biologic therapies has recently been associated with viral reactivation that might not respond to antiviral antibiotics (151). For example, whereas allogeneic marrow depleted of T cells prevents acute and chronic forms of graft-versus-host disease (GVHD) post-transplant, the risk of infections, particularly with EBV and CMV, is increased (152). Furthermore, viral infection can cause severe morbidity and mortality, even in healthy individuals without specific immune defects.

New therapies are therefore required to increase the number and/or effector function of virus-specific T cells. Antiviral prophylaxis can be toxic and does not result in an increase in virus-specific T cells, nor does it achieve long-term eradication. Adoptive transfer of third-party cell lines may be associated with GVHD or failure due to allogeneic rejection (153), and is technically difficult (154). Peptide stimulation does not induce a polyclonal T-cell response and can fail to induce cytotoxic $CD8^+$ T-cell responses (155).

Furthermore, although Ag-specific T-cell responses are actively maintained, they are reversible and short lived in the absence of stimulating Ag (156-158). We have recently confirmed that alloreactivity from virus-specific T cells is common, and that the allo-HLA reactivity and virus specificity is mediated via the same TCR (43). Forty-five percent 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-$B8^+$ individual always selects for a dominant "public" Vb6S2 TCR (7), which cross-reacts against allo-HLA-B^*44:02 (11). We confirmed the previously described alloreactivity of this EBV EBNA3A–specific T cell (HLA-B8/FLRGRAYGL restricted) against allogeneic HLA-B^*44:02 (43, 121). Allo-HLA cross-reactivity was also shown for CMV, varicella-zoster virus (VZV) and influenza virus–specific T cells, which express nonpublic TCRs (43).

A high level of cross-reactivity against allo-HLA molecules is therefore an essential feature of the virus-specific memory TCR (7, 11, 24, 35, 43, 112, 121, 128, 159-161). This allo-HLA cross-reactivity by virus-specific T cells can be reproducibly detected in vitro. However, it is currently unknown whether stimulation with allogeneic-HLA molecules could conversely and specifically augment an HLA-restricted virus-specific T-cell response. The purpose of this study was to

assess whether allogeneic HLA challenge could be a useful tool to augment an HLA-restricted antiviral $CD8^+$ T-cell response, as determined by cytolytic functional assays. We used virus-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 Ags. Our proof-of-principle results demonstrate that allo-HLA stimulation may be a potential tool to augment cytolytic antiviral $CD8^+$ T cell effector responses in patients with viral infection. 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. PBMCs were isolated from heparinized blood by standard density gradient centrifugation and were subsequently cryopreserved until use. EBV-transformed lymphoblastoid cell lines (EBV-LCLs) were generated using standard procedures and were cultured in RPMI 1640 (Cambrex) with 10% FCS. The HLA type of all cells used in our experiments was determined molecularly by sequence-specific oligonucleotide and sequence-specific primer genotyping at the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, The Netherlands.

Proliferation assays for EBV EBNA3A–specific T-cell responses

For the proliferation assays, 1 x 10^6 CFSE-labeled PBMCs from an HLA-$B8^+$ HLA-B^*44^- EBV-seropositive healthy donor were co-cultured with 1 x 10^6 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 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/ml). Next, 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 proliferation assays for EBV EBNA3A-specific T-cell responses were repeated using 20 different HLA-$B8^+B44^-$ responder - HLA-$B8^-B^*44{:}02^+$ stimulator pairings, and eight different HLA-$B8^+B44^-$ responder - HLA-$B8^-B^*44{:}03^+$ stimulator pairings. The HLA typing of the selected responder-stimulator example is given below Figure 1A.

Proliferation assays for CMV-specific T-cell responses

To determine whether allo-HLA stimulation could elicit an antiviral response against any virus or specificity, we had to first determine a new method whereby specific allogeneic cells stimulating the proliferation of virus-specific T cells from any given individual could be identified easily. CFSE-labeled PBMCs (1 x 10^6) from CMV-seropositive healthy donors were first co-cultured with a pool of 1 x 10^6 total mismatched irradiated PBMCs (3000 rad) from four different healthy donors (0.25 x 10^6 cells of each individual stimulator), in a 24-well flat-bottom plate. Each responder was screened against four different pools of four PBMCs. The 16 total different allogeneic stimulator cells were selected to cover the most common occurring HLA molecules. Cells were incubated for 7-10 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/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 after stimulation with a screening pool of four different allogeneic PBMCs, then the same responder PBMCs were tested individually against the four stimulator PBMCs to determine which allogeneic cells 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 allogeneic 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 below). The HLA typing of the selected responder-stimulator examples is given below Figures 2B and 3.

Proliferation assay for combined stimulation of two virus specificities from one responder

T-cell alloresponses are polyclonal and polyclonal antiviral T-cell responses targeting different viral epitopes are required for effective antiviral immunity. To confirm that allo-HLA stimulation could induce a polyclonal antiviral T-cell response targeting different viral epitopes, we stimulated a single responder PBMC with a combination of two allogeneic PBMCs, expressing HLA molecules that were known to stimulate different CMV-specific T-cell responses, from within that individual responder. CFSE-labeled PBMCs (0.85 x 10^6) from a CMV-seropositive healthy donor responder were first co-cultured with a pool of 1 x 10^6 total mismatched irradiated PBMCs (3000 rad) from two different healthy donors S1 and S2 (0.5 x 10^6 cells of each individual stimulator), in a 24-well flat-bottom plate. S1 and S2 expressed allogeneic HLA molecules (HLA-A*02:05 and HLA-B*51:01, respectively) known to stimulate different CMV-

specific T-cell responses (pp65/A2 and pp65/B35 respectively) from the responder. A single stimulator cell expressing both HLA-A*02:05 and HLA-B*51:01 was not available. Cells were incubated for 7-10 d in RPMI 1640 culture medium with 15% human serum and IL-2 (60 IU/ml). Fluorescence-activated cell sorter analysis was performed after staining the cells with PE-labeled CMV pp65/A2 and CMV pp65/B35 specific tetrameric complexes to detect cell division.

Cytotoxicity assays

To confirm that allogeneic cell stimulation resulted in increased virus-specific cytolytic effector function from the stimulated PBMCs, and not just proliferation, 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 d MLR with allogeneic irradiated cells to stimulate a virus-specific memory T cell of interest (see 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 4h [51Cr] release assay. Cognate viral peptide or control viral peptide was directly added to the autologous target cells and incubated for 60 min, simultaneously with chromium incubation, and then washed three times. Supernatants were harvested for gamma counting: percent-specific lysis = (experimental release 2 spontaneous release) / (maximum release – spontaneous release) x 100%. Values for specific lysis are presented as the mean of triplicate wells with SD.

RESULTS

EBV-specific CD8$^+$ T cells proliferate following specific allo-HLA stimulation

To determine whether an allogeneic HLA challenge could specifically stimulate a virus-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 virus-specific T cells by allogeneic HLA molecules (Figure 1). EBV EBNA3A-specific T cells did not proliferate in response to stimulation with allogeneic HLA-B8$^+$HLA-B44$^-$ 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 (Supplemental Figure 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. These results confirm that virus-

specific CTL can directly recognize and proliferate in response to allogeneic HLA to which they are cross-reactive and have never been exposed.

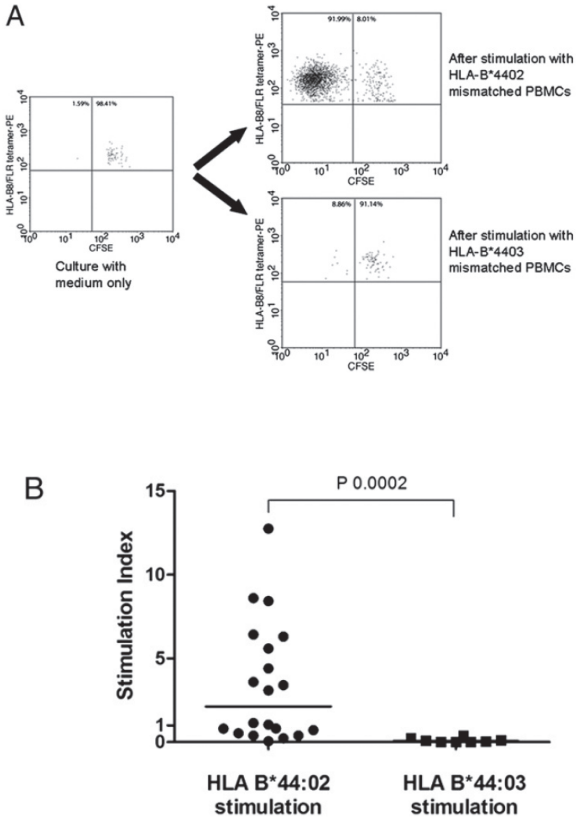


Figure 1. EBV-specific CD8⁺ memory T cells specifically proliferate after allogeneic cell stimulation. (A) EBV EBNA3A-specific T cells are specifically stimulated to proliferate after 7-10 d in vitro co-culture with HLA-B*44:02⁺, but not HLA-B*44:03⁺, mismatched irradiated PBMCs. Bystander activation was excluded. FACS plots gated on total HLA-B8/FLR-tetramer-complex-positive lymphocytes. Assay repeated multiple times with different responder-stimulator pairings, with similar results. A representative result is shown. Responder: HLA-A*02, A31; B*08, B39; DRB1*03, DR16. HLA-B*44:02⁺ stimulator: HLA-A*11, -; B*44:02, B51; DRB1*12, DR15. HLA-B*44:03⁺ stimulator: HLA-A*02, A68; B*44:03, B51; DRB1*08, DR13. (B) Proliferation of EBV EBNA3A-specific T cells is reproducible across different responder-stimulator pairings. Assay repeated with 20 different HLA-B8⁺B44⁻ responder - HLA-B8⁻B*44:02⁺ stimulator pairings, and 8 different HLA-B8⁺B44⁻ responder - HLA-B8⁻B*44:03⁺ stimulator pairings. Results are expressed as a relative proportion of EBV EBNA3A-specific T cells within the CD8⁺ T-cell compartment after HLA-B*44:02 or HLA-B*44:03 stimulation, as compared with the non-stimulated PBMCs. HLA-B*44:02 stimulation significantly increased the proportion of EBV EBNA3A-specific T cells, as compared with HLA-B*44:03 stimulation ($p = 0.0002$).

CMV-specific CD8$^+$ T cells proliferate following specific allo-HLA stimulation

To determine whether allo-HLA stimulation can elicit proliferation of CMV-specific T cells, we screened for responder CMV-specific T-cell proliferation using pools of PBMC stimulator cells from four different donors who were chosen to cover the most commonly occurring HLA molecules. Proliferation of CMV-specific CD8$^+$ memory T cells was detectable using pools of four different PBMC stimulators together (Supplemental Table I). 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 four different PBMCs (pool 4; Figure 2A, Supplemental Table I). The same responder was then tested individually against the stimulators present in the screening pool to identify the specific stimulator (Figure 2B).

Proliferation was associated with a specific increase in the proportion of CMV pp65-specific T cells within the CD8$^+$ T-cell compartment (Figure 3). 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 (Supplemental Table I). Furthermore, this stimulation is demonstrable without the need to generate virus-specific T-cell clones from the responder, even when the virus-specific T cell of interest does not express a public TCR, thereby suggesting that allogeneic cells stimulating viral-peptide/HLA-restricted T cells from any given responder are readily identifiable in the routine laboratory.

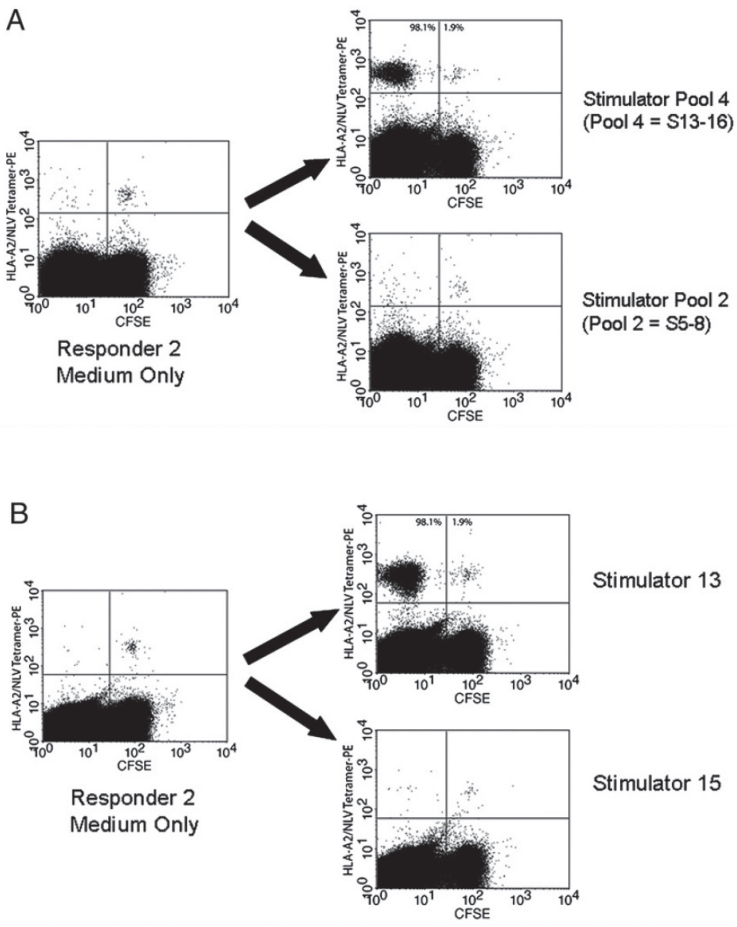


Figure 2. Screening for allo-HLA cross-reactivity of virus-specific memory T cells. A representative example is shown. (A) CMV pp65-specific $CD8^+$ memory T cells (A2/NLV restricted) from Responder 2 (R2) proliferate following stimulation with a pool of four PBMCs (pool 4 containing stimulators 13-16), but not other pools of four different stimulator PBMCs (pool 2 shown). (B) R2 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 three stimulators present in pool 4 (S15 shown), thereby confirming that the CMV pp65-specific T cells from R2 were specifically stimulated by only S13 allogeneic cells.

HLA typing of responder 2: HLA-A*02, A11; B*35, B40; DRB1*11, DR15.

Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.

Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.

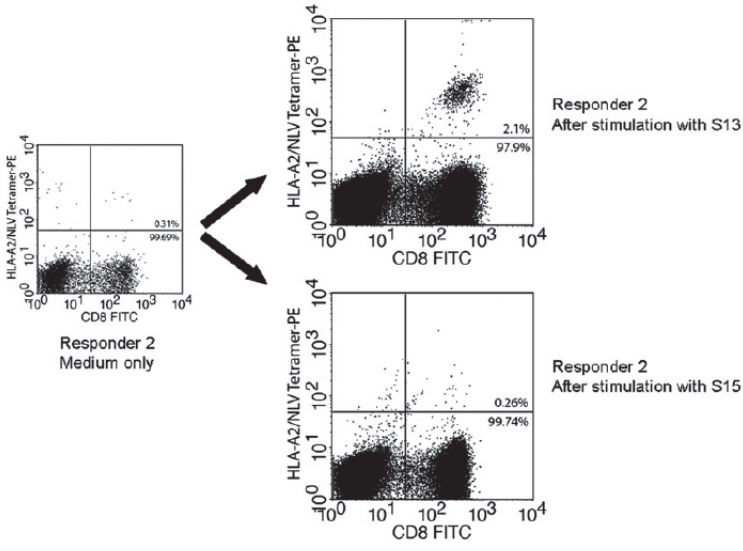


Figure 3. The proportion of CMV-specific CD8⁺ T cells is specifically increased following allogeneic cell stimulation. A representative example is shown. CMV pp65-specific CD8⁺ T cells accounted for 2.1% of total CD8⁺ T cells from responder 2 (R2), following 8 d co-culture with stimulator 13 (S13). The 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, A11; B*35, B40; DRB1*11, DR15.
Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.

Allo-HLA stimulation is associated with an increase in the total number, not just proportion, of virus-specific memory T cells

To confirm that specific allo-HLA stimulation was associated with an increase in the total number of virus-specific T cells, not just an increased proportion of virus-specific T cells, we extrapolated the total number of CMV-specific T cells based on the total number of harvested lymphocytes and the proportion of tetramer-positive T cells before and after stimulation. In two separate experiments after stimulation with S13, the number of CMV-specific T cells increased from 320 to 35,200 and from 2280 to 33,110, respectively (Table I). These data are consistent with specific in vitro allo-HLA stimulation being associated with a 10- to 100-fold increase in the total number of virus-specific T cells, as compared with the number of virus-specific T cells after stimulation with an allogeneic control cell (Table 1).

Table 1. Specific allo-HLA stimulation is associated with an increase in the total number, not just proportion, of virus-specific memory T cells

Responder	Stimulator	Total No. of Lymphocytes before Stimulation	Total No. of Lymphocytes after Stimulation	% of Tetramer-Positive Cells within Total Lymphocyte Population after Stimulation	Estimated Total No. of CMV-Specific T Cells after Stimulation	Fold Increase in Total No. of CMV-Specific T Cells after Allo-HLA Stimulation[a]
R2	Medium only	2×10^6	2.9×10^6	0.015	435	1.36
	S13	1×10^6	3.2×10^6	1.1	35,200	110
	S15	1×10^6	3.2×10^6	0.01	320	1
R2	Medium only	2×10^6	0.5×10^6	0.04	200	0.09
	S13	3×10^6	7.7×10^6	0.43	33,110	14.5
	S15	3×10^6	5.7×10^6	0.04	2280	1

[a]*Relative to control stimulation with allogeneic S15*

Allo-HLA stimulation may stimulate polyclonal antiviral T-cell responses

Polyclonal T-cell responses targeting different viral epitopes are required for effective antiviral immunity. We therefore stimulated a single responder PBMC with a combination of two allogeneic PBMCs, expressing HLA molecules that were already known to stimulate different CMV-specific T-cell responses, from within that one individual responder. CMV A2/pp65- and CMV B35/pp65-specific T cells from the same responder both proliferated in response to stimulation with two different HLA molecules, present on the surface of two different allogeneic PBMCs, within the same assay. Negative controls gave appropriate results, suggesting that allo-HLA stimulation may be capable of stimulating multiple different virus-specific T cells from within the one responder (Figure 4).

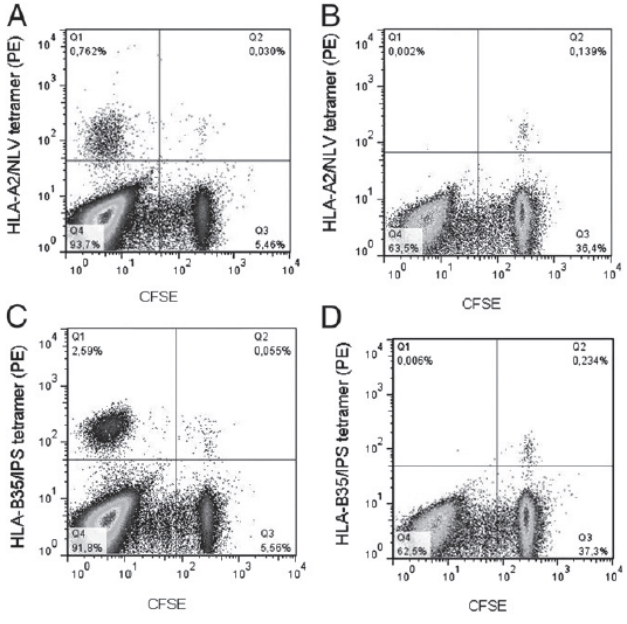


Figure 4. Allo-HLA stimulation may stimulate polyclonal antiviral T-cell responses. (A) CMV A2/pp65- and (C) CMV B35/pp65-specific T cells from the same responder both proliferated in response to stimulation with two different HLA molecules, present on the surface of two different allogeneic stimulator PBMCs, in the same assay. Negative controls gave appropriate results (B, D). Thereby suggesting that allo-HLA stimulation may be capable of stimulating multiple different virus-specific T cells, targeting different viral epitopes, from within the one responder.

HLA typing responder PBMCs: HLA-A*02:01, A11; B*35, B40; DR*11, DR15.
Stimulator 1: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 2: HLA-A*02, A36; B*51, B72; DRB1*03, DR10.

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 virus-specific T cells following allogeneic stimulation is associated with a gain of cytolytic effector function against the original viral peptide/self-HLA restricted target Ag. We therefore performed a cytolytic assay using responder HLA-B8⁺ 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 d of stimulation with HLA-B*44:02-mismatched irradiated PBMCs, primed responder cells from an HLA-B8⁺ EBV-seropositive healthy donor showed increased cytolytic

effector function against both HLA-B8$^+$ 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 with the same PBMCs co-cultured with either HLA-B*44:03-mismatched PBMCs or cultured 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 (Supplemental Figure 1). 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). Confirming that allogeneic HLA challenge can indeed increase the (in vitro) cytolytic effector function of human CMV- and EBV-specific CD8$^+$ T cells against their original cognate viral Ag. We argue that these proof-of-principle results may have important implications for treatment of viral infections, if confirmed in vivo.

DISCUSSION

This study demonstrates that human virus-specific memory T cells gain cognate viral-Ag-specific cytolytic effector function following stimulation with allogeneic HLA molecules against which they are cross-reactive. Stimulation of peripheral blood from a non-sensitized HLA-B8$^+$ EBV-seropositive healthy donor with HLA-B*44:02-mismatched irradiated PBMCs increases (in vitro) cytolytic effector function against EBV. Furthermore, we show that this technique can be used to elicit cytolytic effector function against any potential viral Ag, as shown for CMV. These results provide proof-of-principle evidence that stimulation with specific allogeneic HLA molecules could be useful for treatment of viral infections.

The importance of our findings is 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.

EBV infection in an HLA-B8$^+$HLA-B44$^-$ individual selects for a public BV6S2 TCR, which cross-reacts against allogeneic HLA-B*44:02 (7). Although not all virus-specific immune responses give rise to a public TCR, the allo-HLA cross-reactivity of virus-specific T cells from a given individual can be detected easily in vitro using techniques we have described here and elsewhere (43, 121).

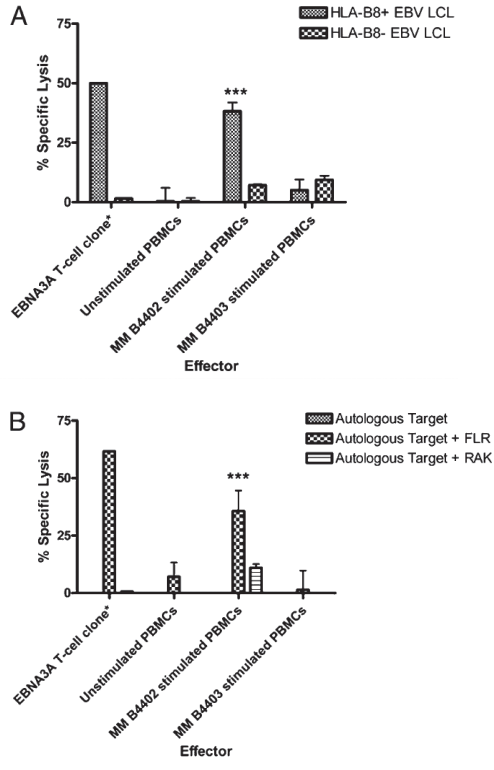


Figure 5. EBV-specific cytolytic effector function of allo-HLA primed cells using EBV-LCL target cells and viral peptide-loaded autologous target cells. (A) PBMCs from a HLA-B8$^+$ EBV-seropositive donor gain EBV-specific cytolytic effector function following allogeneic HLA-B*44:02$^+$ cell stimulation. Unstimulated HLA-B8$^+$ PBMCs and HLA-B*44:03-stimulated HLA-B8$^+$ PBMCs do not demonstrate cytolytic effector function against HLA-B8$^+$ EBV-LCLs. E:T ratio 50:1; targets 2000 and EBV EBNA3A-specific T cells accounted for 20.8% of effector cell population after HLA-B*44:02 stimulation. Positive control EBNA3A T-cell clone is described previously (121), 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, A*02; B*08:01, -; DRB1*03, -.

HLA-B8$^+$ EBV-LCL: HLA-A*01, -; B*08, -; DRB1*03, -.

HLA-B8$^-$ EBV-LCL: HLA-A*03, -; B*07, -; DRB1*15, -.

***p < 0.0001 versus HLA-B8$^-$ EBV-LCL.

(B) PBMCs from an HLA-B8$^+$ EBV-seropositive donor gain HLA-B8/FLR-restricted cytolytic effector function following allogeneic HLA-B*44:02 stimulation. Unstimulated HLA-B8$^+$ PBMCs and HLA-B*44:03-stimulated HLA-B8$^+$ PBMCs do not demonstrate cytolytic effector function against FLR-peptide-loaded autologous cells. E:T ratio 50:1; targets 2000 and EBV EBNA3A-specific T cells accounted for 20.8% of effector cell population after HLA-B*44:02 stimulation. Positive control EBNA3A T-cell clone is described previously (15), 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, A*02; B*08:01, -; DRB1*03, -.

***p = 0.0094 versus RAK-peptide-loaded autologous cells.

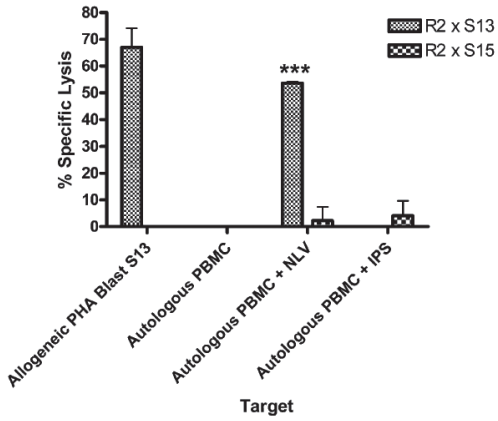


Figure 6. CMV-specific cytolytic effector function of allo-HLA primed cells. PBMCs from a CMV-seropositive HLA-A2$^+$ donor (responder 2; R2) gain HLA-A2/NLV-restricted cytolytic effector function following heterozygote allogeneic cell stimulation with stimulator 13 cells (R2 x S13). S15-stimulated PBMCs do not demonstrate cytolytic effector function against NLV-peptide-loaded autologous cells (R2 x S15). A strong secondary response against stimulator 13 (S13) is demonstrated from R2 responder cells primed with S13 (positive control), but not S15. E:T ratio 100:1; targets 2000 and CMV A2/NLV-specific T cells accounted for 2.1% of effector cell population after stimulation with S13.

HLA typing of responder 2: HLA-A*02, A11; B*35, B40; DRB1*11, DR15.
Stimulator 13: HLA-A*02:01, A*02:05; B*18, B50; DRB1*11, DR13.
Stimulator 15: HLA-A*23, A29; B*15, B53; DRB1*11, DR13.
***$p < 0.0001$ versus IPS-loaded autologous cells.

Indeed, successful stimulation of cytolytic effector function against CMV Ag reveals that this technique could potentially be useful to elicit T-cell cytolytic effector function against any virus or specificity. Furthermore, stimulation of two different CMV-specific T-cell responses from one individual suggests that allo-HLA stimulation may also be capable of stimulating a polyclonal antiviral T-cell response targeting different viral epitopes, which does not occur with single peptide stimulation. Techniques described here should 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 Ags. We used irradiated isolated PBMCs for stimulation of the virus-specific memory T cells, thereby excluding any contributions by plasma, platelets, or erythrocytes. Therefore, we suggest that allogeneic cell therapy should be investigated using only isolated leukocytes as stimulators.

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

The results presented in this study suggest that specific allogeneic cell therapy could prime or maintain virus-specific memory. The proportion and total number of virus-specific T cells in the $CD8^+$ compartment increased significantly following specific allo-HLA stimulation. CFSE dilution and counting experiments confirmed that the increase in the proportion of virus-specific memory T cells was secondary to proliferation, and not just better survival of memory T cells in culture, with the number of virus-specific memory T cells increasing between 10- and 100-fold. Data from preliminary clinical studies suggest that CMV-specific $CD8^+$ T-cell levels greater than 1×10^7 / L of peripheral blood may correlate with protection (162); therefore, the total number of virus-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 (156, 158). Transfer of memory T cells to naive mice, in the presence or absence of priming Ag, reveals that maintenance of T-cell memory is short lived in the absence of TCR-mediated signaling (156). Furthermore, recently activated memory T cells can bypass the requirement for CD28/CD80/CD86 costimulation, as compared with resting memory T cells that are still dependent on CD28 triggering for their activation (163). Although at baseline in our EBV-specific cytolytic assay 1.5% of $CD8^+$ T cells in the peripheral blood of the individual were EBV EBNA3A-specific T cells, no cytolysis of FLR-peptide-loaded autologous cells could be detected prior to allo-HLA-B^*44:02 stimulation, suggesting specific 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 virus-specific T cells via triggering TCR signaling or abrogating costimulation requirements, regardless 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 (164-166), most of which interfere with the various steps necessary for MHC class I restricted Ag presentation. For example, CMV evades MHC class I Ag presentation by reducing the stability of class I heavy chains (167) and also by dislocating MHC class I heavy chains from the endoplasmic reticulum (168). The coordinated function of murine CMV genes can completely inhibit CTL lysis (169). Among others, the EBV EBNA1 protein contains an element

that interferes with its proteasomal proteolysis, and the HSV ICP47 protein inhibits the TAP complex (170, 171). HIV is highly efficient at evading immune responses through mechanisms such as modulation of MHC class II presentation (172) and downregulation of MHC class I molecules (150, 172-174). Many other viral immune evasion strategies are also described (175-179).

Allogeneic cell therapy may be capable of bypassing all these viral strategies of immune evasion as the virus-specific memory T cells are directly stimulated via molecular mimicry (44). The allo-HLA molecule against which the virus-specific T cell is cross-reactive 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 et al. (180) demonstrated that preemptive CMV-specific $CD8^+$ T-cell immunotherapy, guided by viral DNA load, prevented lethal disease and reduced the risk of virus recurrence. 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.

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. Whereas 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 also cause sensitization of a recipient to future transplantations. It is also unclear whether such treatment would stimulate a de novo virus-specific response from naive T cells (181-183). Nonetheless, results demonstrated in this study suggest allo-HLA stimulation may have potential as an alternative to adoptive transfer or pharmacological therapy to treat viral infections.

The high frequency of allo-HLA cross-reactivity by virus-specific T cells is increasingly being recognized. We provide (in vitro) evidence that allogeneic cell therapy may be useful to conversely stimulate a beneficial antiviral 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.

ACKNOWLEDGMENTS

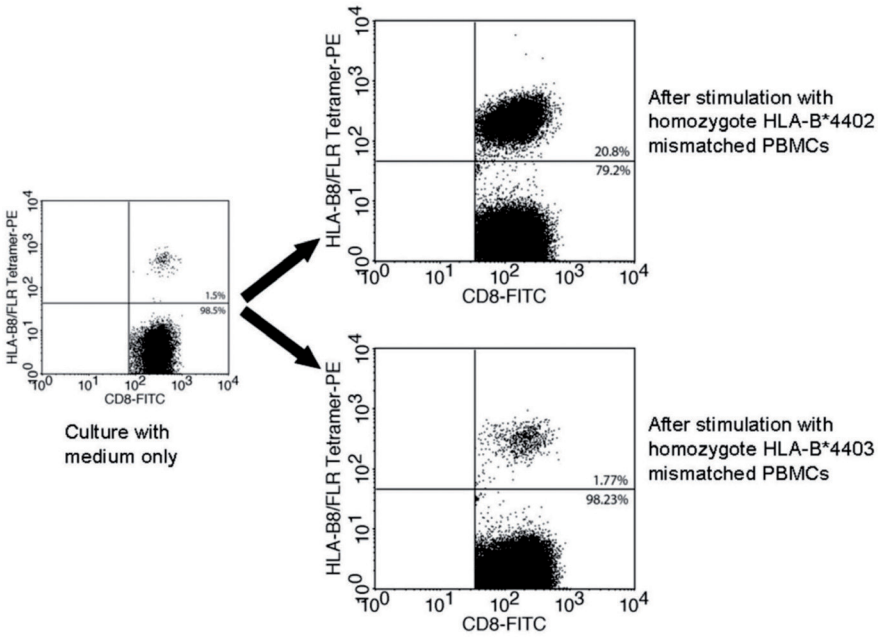
We thank Prof. Frank Christiansen for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

Supplemental Table 1. Screening for allo-HLA cross-reactivity 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 cross-reactivity 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 virus-specific T-cell response were identifiable for most responders and specificities. + Specific proliferation detected. - No proliferation detected. PBMCs from responder 2 were used in the assays described in Figures 2, 3 and 6.



Supplemental Figure 1. 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, A*02; B*08, -; DRB1*03, -.

HLA-B*44:02⁺ stimulator: HLA-A*02, A68; B*44:02, -; DRB1*07, DR14.

HLA-B*44:03⁺ stimulator: HLA-A*02, A32; B*44:03, -; DRB1*01, DRB1*08.

CHAPTER

6

ALLO-HLA CROSS-REACTIVITIES OF CYTOMEGALOVIRUS-, INFLUENZA- AND VARICELLA ZOSTER VIRUS-SPECIFIC MEMORY T CELLS ARE SHARED BY DIFFERENT INDIVIDUALS

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Am J Transplant. 2017 Aug;17(8):2033-2044

ABSTRACT

Virus-specific T cells can recognize allogeneic HLA (allo-HLA) through TCR cross-reactivity. The allospecificity often differs by individual (*private* cross-reactivity), but can also be shared by multiple individuals (*public* cross-reactivity); however, only a few examples of the latter have been described. Because these could facilitate alloreactivity prediction in transplantation, we aimed to identify novel public cross-reactivities of human virus-specific $CD8^+$ T cells directed against allo-HLA by assessing their reactivity in mixed lymphocyte reactions. Further characterization was done by studying TCR usage with primer-based DNA sequencing, cytokine production with ELISA, and cytotoxicity with ^{51}chromium-release assays. We identified three novel public allo-HLA cross-reactivities of human virus-specific $CD8^+$ T cells. CMV B35/IPS $CD8^+$ T cells cross-reacted with HLA-B51 and/or HLA-B58/B57 (23% of tetramer-positive individuals), FLU A2/GIL $CD8^+$ T cells with HLA-B38 (90% of tetramer-positive individuals) and VZV A2/ALW $CD8^+$ T cells with HLA-B55 (two unrelated individuals). Cross-reactivity was tested against different cell types including endothelial and epithelial cells. All cross-reactive T cells expressed a memory phenotype, emphasizing the importance for transplantation. We conclude that public allo-HLA cross-reactivity of virus-specific memory T cells is not uncommon and may create novel opportunities for alloreactivity prediction and risk estimation in transplantation.

INTRODUCTION

Alloreactive T cells are a major cause of graft loss after solid organ transplantation and acute graft-versus-host disease (aGVHD) after hematopoietic stem cell transplantation (HSCT) (114, 184). In particular alloreactive T cells expressing a memory phenotype are a potential threat, since their activation threshold is substantially lower compared with their naïve counterpart, while their effector function is enhanced (161, 185). Interestingly, alloreactive memory T cells are present in all individuals, even without prior exposure to alloantigen. This can be explained by heterologous immunity of virus-specific T cells: the inherent capacity of T-cell receptors (TCRs) to cross-react with multiple antigens. Heterologous immunity thereby arms virus-specific T cells with a TCR that can recognize a range of related and unrelated viral peptides, creating a survival benefit to the host. This scenario describes the recognition of different peptides presented by a self-HLA. TCR cross-reactivity, however, can also be directed against allogeneic HLA (allo-HLA). As a consequence, memory T cells that are primed by viral infections may contribute to allograft rejection. Alarmingly, such TCR cross-reactivity of virus-specific memory T cells against allogeneic HLA is indeed common (43).

The TCR repertoire differs by individual due to both intrinsic (thymic selection) and extrinsic (allergens / viral infections) factors. Although most TCRs appear to be unique to individuals (*private* TCRs), dominant TCR sequences have also been found in multiple individuals (*public* TCRs). Public TCRs are selected for optimal recognition of immunodominant viral epitopes (186), and cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) in particular are known for inducing public TCR responses (6, 7, 187). Inherent to their structural differences, the allospecificity of cross-reactive private TCRs (even with the same viral specificity) is directed toward different alloepitopes (private cross-reactivity) (188), and alloreactivity by private TCR cross-reactivity is therefore impossible to predict. In contrast, cross-reactivity of public TCRs is directed against the same alloepitopes in multiple individuals and thus could facilitate the prediction of alloreactivity by memory T cells. A classic example of such public cross-reactivity is found in HLA-B8^{+} individuals: when infected with the EBV virus, they select a public TCR that, in addition to the viral epitope (HLA-B8/FLR), recognizes allogeneic HLA-B*44:02 (11). Such cross-reactive virus-specific T cells were also identified in HLA-B8^{+}B44^{-} lung-transplant recipients transplanted with an HLA-B*44:02 graft (39, 40).

To date, public cross-reactivity of virus-specific T cells is considered uncommon because it requires strict TCR preservation in unrelated individuals; however, reports of other TCR cross-reactivities with similar allospecificity in unrelated individuals give reason to challenge this

conception (189-192). Furthermore, Nguyen et al. recently identified cross-reactivity of CMV A2/NLV-specific T cells with nearly identical TCR usage against HLA-B27 in two unrelated individuals (86).

To the best of our knowledge, these indications of public cross-reactivity of virus-specific T cells are the only ones described so far. Identification of additional public cross-reactivities of virus-specific T cells and knowledge concerning their prevalence and functional characteristics could enable monitoring and facilitate risk estimation in a transplantation setting. Therefore, we aimed to identify novel public cross-reactivities of virus-specific $CD8^+$ memory T cells directed against allogeneic HLA antigens.

MATERIALS AND METHODS

Collection of responder and target cells

Responder and target peripheral blood mononuclear cells (PBMCs) were derived from healthy donors with informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated from whole blood by standard density gradient centrifugation (Ficoll-Isopaque separation) and cryopreserved until usage.

EBV-transformed lymphoblastoid cell lines (EBV-LCLs) were generated according to standard protocol. Supernatant of the EBV-producing marmoset cell line B95.8 was added to bulk PBMCs, incubated for 1.5 h at 37°C, and cultured in RPMI 1640 Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine.

Phytohemagglutinin (PHA) blasts were generated from PBMCs by culturing in RPMI 1640 medium (Gibco) supplemented with penicillin/streptomycin, glutamine, 15% human serum (HS), and PHA (4mg/mL; Murex Biotech Ltd).

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium (Gibco) supplemented with 10% FCS, sodium pyruvate (Gibco), penicillin/streptomycin (Gibco) and 0.01% β-mercaptoethanol (0.05M; Sigma Aldrich, St. Louis, MO). HUVECs were used at passages 1-4.

Proximal tubular epithelial cells (PTECs) were cultured in DMEM ham F12 medium (Lonza) supplemented with 26µg/mL Hydrocortisone (Sigma Aldrich), 1% 1x insulin-transferrin-sodium selenite media supplement (ITS; Sigma Aldrich), 10µg/mL human Epidermal Growth Factor (hEGF; Sigma Aldrich), 0.08 pg/mL 3,3',5-Triiodo-L-thyronine sodium salt (Sigma Aldrich), and penicillin/streptomycin (Gibco). PTECs were used at P1-P4.

HLA typing was achieved by sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) genotyping at the European Federation of Immunogenetics (EFI)- accredited national reference laboratory for histocompatibility testing at the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands.

Proliferation assays

Proliferation of virus-specific $CD8^+$ T cells was determined at bulk level by mixed lymphocyte reactions (MLRs). Responder PBMCs were labeled with carboxyfluorescein succinimidyl ester (5mM; Molecular Probes) and incubated for 8 days with 1 x 10^6 irradiated stimulator PBMCs (3000 Rad, responder:stimulator (R:S) ratio 1:1) in RPMI 1640 Medium (Gibco) supplemented with penicillin/streptomycin, glutamine, 15% HS and IL-2 (10 U/mL). Analysis was done by flow cytometry (FACS Calibur; BD Biosciences) using viral tetramers conjugated with phycoerythrin (PE) and CD8 antibody conjugated with allophycocyanin (APC) (Protein facility of the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands).

In addition, proliferation of $CD8^+$ T-cell clones was determined by thymidine (^3H) incorporation. $CD8^+$ T-cell clones (0.5 x 10^6 cells; responders) were stimulated with irradiated PBMCs and EBV-LCLs (0.5 x 10^6 cells; stimulators) and incorporated with ^3H-thymidine (20 µCi) at day 4. After 24 h, cells were harvested using a Tomtec cell harvester (Tomtec, Hamden, CT) and read by liquid scintillation spectrophotometry on a 1450 LSC MicroBeta TriLux microplate scintillation and luminescence counter (Perkin Elmer, Waltham, MA). The stimulation index (SI) was calculated as: mean counts per minute (CPM) of experimental wells / mean CPM unstimulated (medium-only) wells.

Generation of virus-specific $CD8^+$ T-cell clones

Virus-specific $CD8^+$ memory T-cell clones were generated by fluorescence-activated cell sorting (FACS; FACSAria; BD Biosciences), as described previously (118). PBMCs of healthy donors were stained with PE-labeled viral tetramers CMV pp65(123-131) HLA-B*35:01/IPSINVHHY (CMV B35/IPS), influenza (FLU) IMP(58-66) HLA-A*02:01/GILGFVFTL (FLU A2/GIL), and varicella zoster virus (VZV) IE62(593-601) HLA-A*02:01/ALWALPHAA (VZV A2/ALW) (Protein facility of the Leiden University Medical Center) and fluorescein isothiocyanate (FITC)-labeled mAb against CD4, CD19, CD45-RA, CD14, CD40, CD16 and CD56 (BD Pharmingen, San Diego, CA). The FL1 channel was used as a dump channel to avoid direct CD8 mAb staining, as simultaneous CD8/MHC staining triggers TCR internalization. TCR usage was determined by primer-based DNA sequencing using primers against the Vβ and Vα alleles (119).

For optimal conditioning, $CD8^+$ T-cell clones were cultured with irradiated PBMCs (4000 rad) from anonymous buffy coats (Sanquin, Leiden, the Netherlands) 8 days prior to functional testing.

Cytokine production assays

Interferon γ (IFNγ) production of $CD8^+$ T-cell clones was measured by ELISA according to the manufacturer's protocol (U-CyTech ELISA kit; U-CyTech, Utrecht, the Netherlands). Overall, 5×10^3 $CD8^+$ T cells were stimulated with 5×10^4 EBV-LCLs for 24 h at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% FCS (Lonza), 5% HS, and IL-2 (10 U/mL) in triplicate wells.

Cytotoxicity assays

Cytotoxicity was determined by ^{51}chromium (^{51}Cr)-release assay, as previously described (118, 121). In short, $CD8^+$ T-cell clones were stimulated with ^{51}Cr-labeled PHA blasts, EBV-LCLs, PTECs and HUVECs for 4 h at 37°C in IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% FCS (Lonza), 5% HS, and IL-2 (10 U/mL). ^{51}Cr release was measured on a γ-counter (PerkinElmer 2470 Wizard^2, PerkinElmer) and specific lysis was determined by the following calculation: (experimental ^{51}Cr release - spontaneous ^{51}Cr release) / (maximum ^{51}Cr release - spontaneous ^{51}Cr release) x 100. Spontaneous ^{51}Cr release of the target cells was determined in medium alone, and maximum ^{51}Cr release was determined by adding Triton. Values for specific ^{51}Cr lysis represent the mean ± standard deviation of triplicate wells.

RESULTS

Virus-specific CD8$^+$ T cells from different individuals proliferate in response to the same allo-HLA antigens

A cohort comprising 30 healthy individuals was screened for allo-HLA reactivity of virus-specific CD8$^+$ T cells in MLRs against a panel of stimulators expressing the most frequent HLA class I antigens (>5%) in the Western population. In multiple responders, CD8$^+$ T cells with the same viral specificity showed a similar proliferative pattern against the panel (Table 1), and additional MLRs with different stimulators confirmed corresponding allo-HLA specificity. First, CD8$^+$ T cells directed against self-HLA-B35 presenting CMV-derived peptide IPSINVHHY (pp65 123-131) proliferated in response to HLA-B51$^+$ and HLA-B58$^+$ allogeneic cells in 23% (3 of 13) of CMV B35/IPS tetramer-positive individuals. Second, CD8$^+$ T cells directed against self-HLA-A2 presenting FLU-derived peptide GILGFVFTL (MP 58-66), proliferated in response to HLA-B38$^+$ allogeneic targets in 90% (18 of 20) of FLU A2/GIL tetramer-positive individuals. Third, CD8$^+$ T cells directed against self-HLA-A2 presenting VZV-derived peptide ALWALPHAA (IE62 593-601) proliferated in response to HLA-B55$^+$ allogeneic targets in two unrelated individuals. The percentage of VZV A2/ALW tetramer-positive individuals showing this cross-reactivity could not be determined because the low precursor frequency of VZV A2/ALW-specific T cells in peripheral blood hampers their accurate detection by tetramer staining. Representative FACS plots are shown in Figure 1A, and heat maps of all MLRs are shown in Data S1.

Furthermore, CMV B35/IPS, FLU A2/GIL, and VZV A2/ALW CD8$^+$ T cells from individuals that expressed the cross-reactive HLA antigen themselves did not proliferate in response to the latter, indicating self-tolerance. Interestingly, CMV B35/IPS CD8$^+$ T cells from an HLA-B35$^+$B51$^+$ heterozygous individual did not proliferate against syngeneic HLA-B51 but did against allogeneic HLA-B58 (Figure 1B).

Table 1. Cohort of healthy individuals

	Relevant self-HLA	Tetramer-positive	Cross-reactive
CMV B35/IPS	16	13	3
FLU A2/GIL	22	20	18
VZV A2/ALW	22	n.a.	1[1]

CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; n.a. = not applicable

[1]This cross-reactivity has been identified in two additional individuals outside the cohort

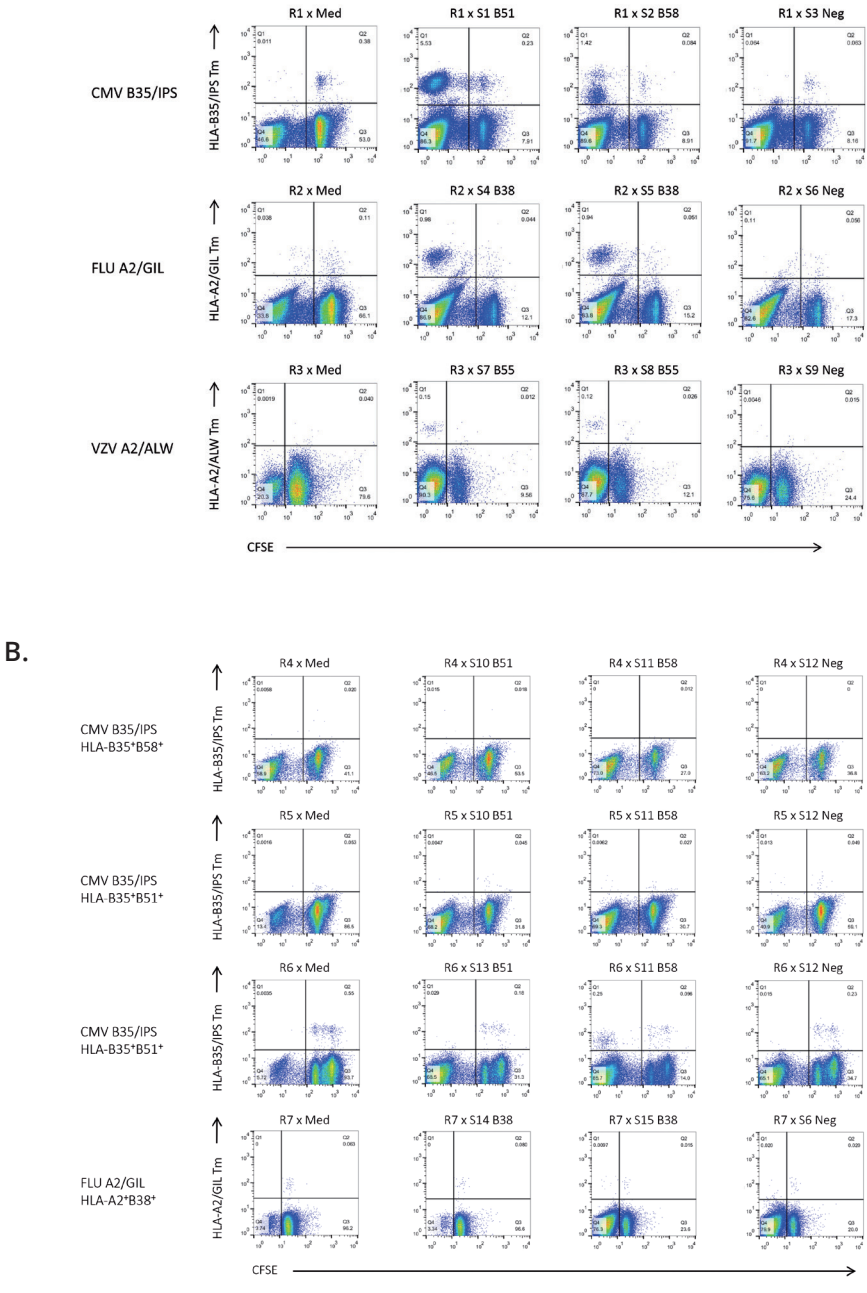

Figure 1. Proliferation of virus-specific CD8⁺ T cells directed at dominant viral epitopes in response to allogeneic HLA. (A) Proliferation of CMV B35/IPS, FLU A2/GIL, and VZV A2/ALW CD8⁺ T cells against respectively allogeneic HLA-B51 and -B58, HLA-B38 and HLA-B55 in mixed lymphocyte reaction. Shown plots are representative examples of positive responders that showed CMV B35/IPS T-cell proliferation (3

of 13 responders), FLU A2/GIL T-cell proliferation (18 of 20 responders) and VZV A2/ALW T-cell proliferation (2 of 2 responders). (B) Proliferation of CMV B35/IPS and FLU A2/GIL CD8$^+$ T cells of individuals expressing the cross-reactive HLA antigen in response to allogeneic cells expressing the antigen. In total, 4 HLA-B35$^+$B51$^+$, 1 HLA-B35$^+$B58$^+$ and 4 HLA-A2$^+$B38$^+$ responders were tested against 5 HLA-B51$^+$, 5 HLA-B58$^+$ and 6 HLA-B38$^+$ stimulators respectively. Representative examples are shown. All plots were gated on PBMCs and CD8$^+$ antibody staining. Full HLA typing of the responders and stimulators is included in Data S2. CFSE, carboxyfluorescein succinimidyl ester; CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Shared allo-HLA cross-reactivity is assigned to public TCR usage of virus-specific CD8$^+$ memory T cells

CMV B35/IPS-, FLU A2/GIL-, and VZV A2/ALW-specific CD8$^+$ memory T-cell clones were generated from the individuals that showed proliferation in response to identical allo-HLA antigens, and highly conserved TCR Vα and Vβ usage was observed. Remarkably, the TCR usage of CMV B35/IPS- and FLU A2/GIL-specific T-cell clones derived from individuals also expressing the cross-reactive HLA antigen highly resembled the TCR usage of allo-HLA cross-reactive T-cell clones. Only minor amino acid differences within the CDR3α (FLU A2/GIL) and CDR3β (CMV B35/IPS) regions were able to abrogate the occurrence of cross-reactivity (Table 2, Data S2).

Stimulation with allogeneic PBMCs confirmed that T-cell clones expressing public TCRs were able to proliferate in response to the cross-reactive alloantigen. Allorecognition of HLA-B51, in contrast to HLA-B58, induced robust proliferation of cross-reactive CMV B35/IPS T cells, similar to stronger compared with cognate antigen (B35 + IPS). Allorecognition of HLA-B38 also induced comparable proliferation of cross-reactive FLU A2/GIL T cells as cognate antigen (A2 + GIL), whereas HLA-B55 allorecognition induced less pronounced proliferation of VZV A2/ALW T cells compared to cognate antigen (A2 + ALW). T-cell clones derived from individuals that also expressed the cross-reactive HLA antigen (CMV B35/IPS 8A2, FLU A2/GIL 4A2) did not proliferate in response to syngeneic cross-reactive HLA. Of interest, HLA-B38 allorecognition of FLU A2/GIL T cells was hampered by differences in Va usage (FLU A2/GIL 3F8), and even in CDR3a usage alone (FLU A2/GIL 4A2) (Figure 2).

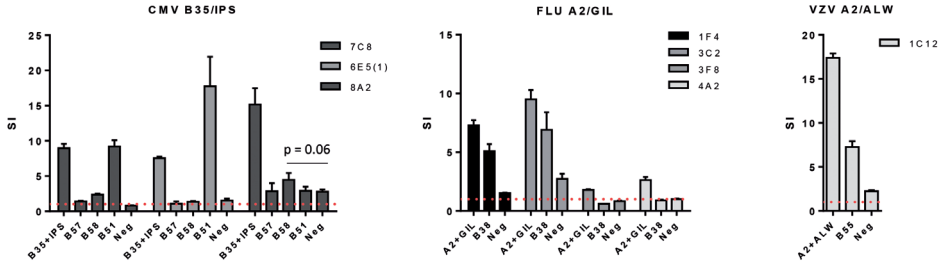

Figure 2. Allo-HLA cross-reactive cytomegalovirus-, influenza-, and varicella zoster virus-specific CD8⁺ memory T-cell clones are able to proliferate in response to allogeneic PBMCs. Clones derived from individuals expressing the cross-reactive HLA antigen (CMV B35/IPS 8A2, FLU A2/GIL 4A2) did not. SI = stimulation index. All experiments were performed in triplicate, and error bars represent the standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; SI, stimulation index; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Virus-specific CD8⁺ memory T-cell clones with public cross-reactive TCRs produce IFNγ on stimulation with allogeneic targets

IFNγ production of the T-cell clones was determined by ELISA. CMV B35/IPS-, FLU A2/GIL-, and VZV A2/ALW-specific CD8⁺ memory T-cell clones bearing public TCRs produced IFNγ in response to EBV-LCLs expressing self-HLA and viral peptide and EBV-LCLs expressing cross-reactive allo-HLA antigens. Interestingly, minor HLA-B57 cross-reactivity was observed for HLA-B58 cross-reactive CMV B35/IPS T cells (Figure 3A and 3B), whereas no significant proliferation against HLA-B57 was observed in MLR at the bulk or clonal level (data not shown; Figure 2). Cross-reactive FLU A2/GIL T-cell clones produced IFNγ mainly on HLA-B*38:01 allorecognition, whereas HLA-B*38:02 allorecognition resulted in only limited IFNγ production. FLU A2/GIL T-cell clones with the same Vb but different Va usage did not cross-react against HLA-B38. Cross-reactive VZV A2/ALW T-cell clone 1C12 produced IFNγ on HLA-B55⁺ allorecognition, comparable to VZV A2/ALW T-cell clone 12, as published previously (193). Furthermore, despite highly similar TCR usage, virus-specific T cells isolated from individuals carrying cross-reactive HLA antigens did not produce IFNγ when stimulated with syngeneic HLA. Interestingly, CMV B35/IPS T-cell clone 8A2 (derived from the heterozygous HLA-B35⁺B51⁺ individual that showed minor proliferation in response to HLA-B58) produced high levels of IFNγ on recognition of HLA-B58⁺ EBV-LCLs, and limited levels on recognition of HLA-B57⁺ EBV-LCLs (Figure 3B). Heat maps of the IFNγ ELISAs are included in Data S1, and the allospecificity of all T-cell clones is indicated in Table 2.

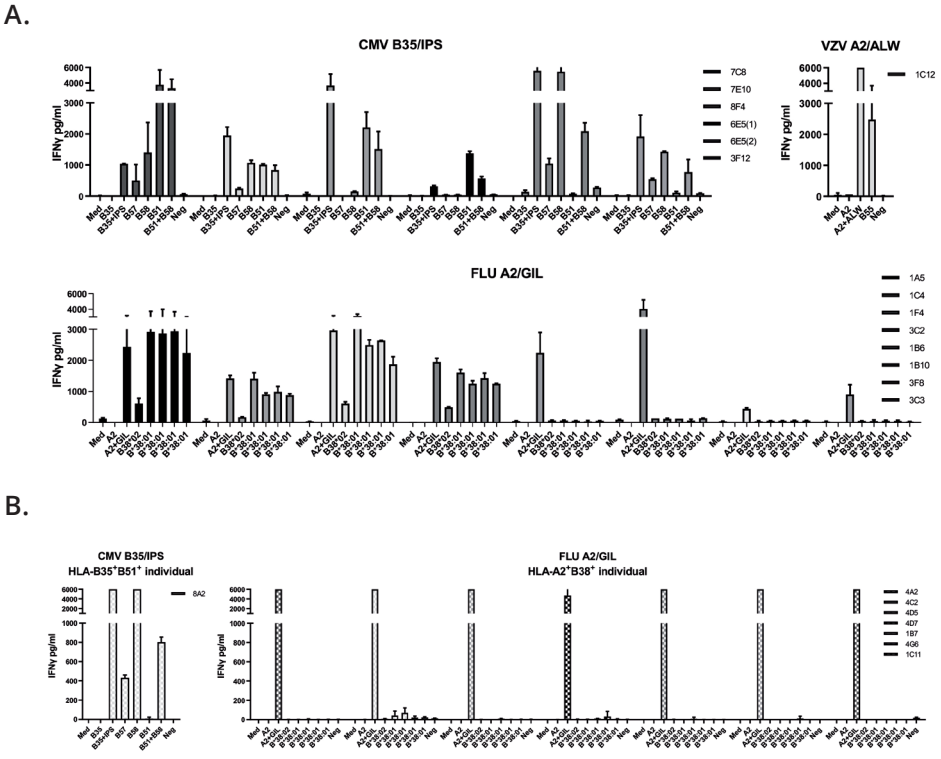

Figure 3. Allo-HLA cross-reactive virus-specific CD8⁺ memory T cells from different individuals produce IFNγ in response to allogeneic EBV-LCLs. (A) Cross-reactive CMV B35/IPS T-cell clones produced IFNγ upon HLA-B51 and HLA-B58 allorecognition. In addition, HLA-B58 cross-reactive CMV B35/IPS T cells consistently produced limited amounts of IFNγ upon HLA-B57 allorecognition. Cross-reactive FLU A2/GIL T-cell clones produced IFNγ upon HLA-B*38:01, and to a limited extent, HLA-B*38:02 allorecognition. Finally, HLA-B55 allorecognition induced IFNγ production of cross-reactive VZV A2/ALW T-cell clone 1C12. (B) Virus-specific CD8⁺ memory T-cell clones derived from individuals expressing the cross-reactive HLA antigen do not produce IFNγ against EBV-LCLs expressing syngeneic HLA. All experiments were performed in triplicate at E:T ratio 1:10, and error bars represent the standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; IFNγ, interferon γ; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

Public allo-HLA cross-reactive CMV B35/IPS-, FLU A2/GIL-specific CD8$^+$ memory T cells are cytotoxic, unlike public allo-HLA cross-reactive VZV A2/ALW-specific CD8$^+$ memory T cells

^{51}Cr-release assays showed efficient lysis of HLA-B51$^+$ EBV-LCLs and PHA blasts by HLA-B51 cross-reactive CMV B35/IPS T cells, whereas HLA-B58/B57 cross-reactive CMV B35/IPS T cells (including T-cell clone 8A2 derived from the heterozygous HLA-B35$^+$B51$^+$ individual) were unable to lyse HLA-B58$^+$ and HLA-B57$^+$ EBV-LCLs and PHA blasts**.** Interestingly, the same applied for T cells that recognized all three allo-HLA antigens. Furthermore, FLU A2/GIL T-cell clones efficiently lysed HLA-B*38:01$^+$ EBV-LCLs and PHA blasts, whereas HLA-B*38:02$^+$ EBV-LCL lysis was considerably lower. None of the FLU A2/GIL T-cell clones derived from the HLA-A2$^+$B38$^+$ individual were cytotoxic toward HLA-B38 targets. VZV A2/ALW T-cell clones showed only minor cytotoxicity towards HLA-B55$^+$ EBV-LCLs and PHA blasts, below the generally applied cut-off level of 10% (194) (Figure 4A).

Finally, additional ^{51}Cr-release assays were performed with PTECs and HUVECs as targets to determine possible tissue specificity of the alloimmune response. HLA-B51 cross-reactive CMV B35/IPS T cells were able to lyse HLA-B51$^+$ PTECs and HUVECs with efficiency comparable to PHA-blasts and EBV-LCLs. Similar to HLA-B58$^+$ EBV-LCLs and PHA blasts, HLA-B58/B57 cross-reactive CMV B35/IPS T cells were incapable of lysing HLA-B58$^+$ and HLA-B57$^+$ PTECs. HLA-B58$^+$ HUVECS were not available for testing, but no cytotoxicity was observed against HLA-B57$^+$ HUVECs. Furthermore, HLA-B38 cross-reactive FLU A2/GIL T cells efficiently lysed HLA-B38$^+$ PHA-blasts, EBV-LCLs, HUVECs and PTECs, although the latter to a lesser extent. VZV A2/ALW cross-reactive T cells showed consistent limited cytotoxicity towards HLA-B55$^+$ PHA blasts, EBV-LCLs, PTECs, and HUVECs. Finally, FLU A2/GIL T-cell clones generated from the HLA-A2$^+$B38$^+$ individual again showed no recognition of HLA-B38$^+$ targets (Figure 4B). Cytotoxicity results of the T-cell clones that were able to lyse target cells expressing the cross-reactive HLA antigen are summarized in Table 3.

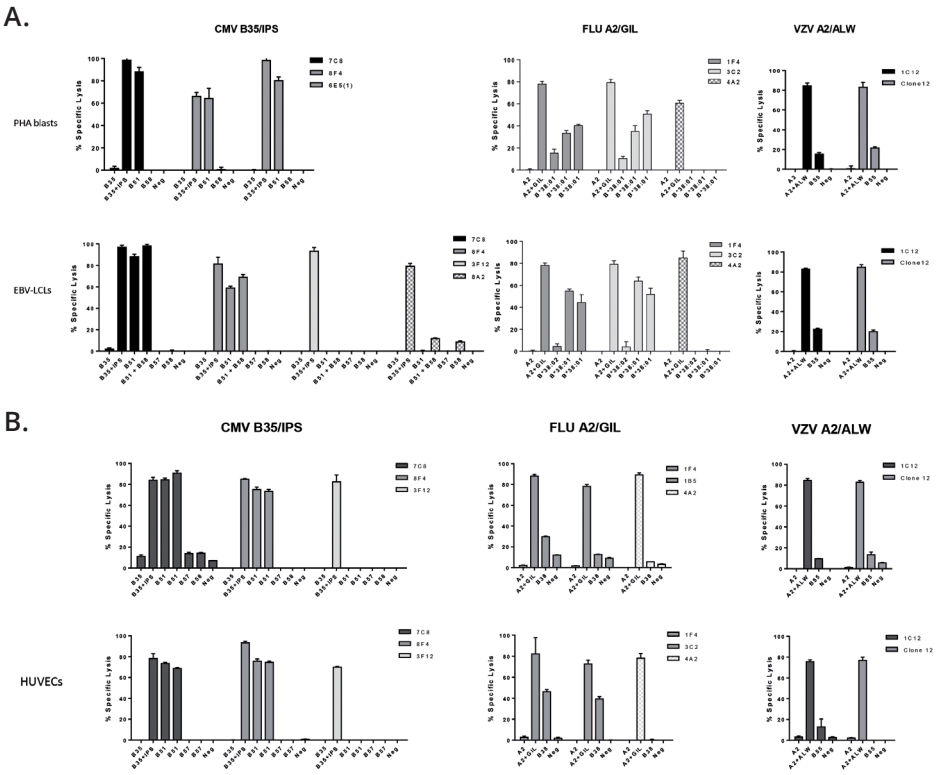

Figure 4. Allo-HLA cross-reactive virus-specific CD8⁺ memory T cells from different individuals are cytotoxic to allogeneic targets. Cytotoxicity was observed to (A) PHA blasts and EBV-LCLs, and (B) PTECs and HUVECs expressing the cross-reactive allo-HLA molecules. Non-cross-reactive T cells (including T cells from individuals expressing the cross-reactive HLA) were not cytotoxic to allogeneic targets. Plots are representative examples, all experiments were performed in triplicate and at different effector:target (E:T) ratios (30:1; 10:1; 1:1; 0.1:1); E:T ratio 30:1 is shown. Error bars represent standard deviation. CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; HUVEC, human umbilical vein endothelial cell; PHA, phytohemagglutinin; PTEC, proximal tubular epithelial cell; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA.

DISCUSSION

Because public cross-reactivity hinges on strict TCR preservation in multiple individuals, it is allegedly uncommon. Nevertheless, using a small cohort and a restricted set of viral tetramers, we were able to identify three novel public allo-HLA cross-reactivities of virus-specific memory T cells. All TCRs of HLA-B51- and HLA-B58/57-cross-reactive CMV B35/IPS T cells shared strong public features (187) and only minor amino acid differences within their CDR3 loops differentiated between HLA-B51 and HLA-B58/B57 allorecognition. TCR clonotypes recognizing HLA-B51, HLA-B58/B57, and the combination were detected alongside each other in different individuals. FLU A2/GIL cross-reactivity against HLA-B38 was observed in almost all (18 of 20) tetramer-positive individuals, and TCR usage was identical to an abundant public FLU A2/GIL TCR clonotype described in literature (12, 195). Although public TCR usage has not been described for VZV A2/ALW-specific T cells, $CD8^+$ T-cell responses against the VZV A2/ALW epitope are shared by the majority of HLA-$A2^+$ individuals (196) and HLA-B55 cross-reactivity of VZV A2/ALW-specific T cells with corresponding TCR Vβ usage has in fact been identified previously in yet another individual (43). The nearly identical TCR usage in three unrelated individuals again firmly points toward the involvement of a public TCR.

FLU A2/GIL and CMV B35/IPS T cells sorted from HLA-$A2^+B38^+$ and HLA-$B35^+B51^+$ individuals, respectively, did not recognize HLA-B38 and HLA-B51 alloantigens, which is likely the result of thymic TCR selection to avoid autoimmunity. This confirms the observation in HLA-$B8^+B^*44{:}02^+$ heterozygous individuals, which select oligoclonal EBV B8/FLR TCRs that are completely disparate from the public HLA-B^*44:02-cross-reactive TCR (197, 198). Furthermore, even individuals heterozygous for HLA-B^*08:01 and HLA-B^*44:03 (which highly resembles HLA-B^*44:02 but is not functionally cross-reactive) select EBV B8/FLR TCRs that are highly distinct from the public HLA-B^*44:02-cross-reactive TCR (199). In contrast, we did not find such cautious TCR selection. First, unlike HLA-B^*44:03 skewing of the EBV B8/FLR TCR repertoire, self-expression of HLA-B51 did not abrogate HLA-B58 cross-reactivity of CMV B35/IPS cross-reactive T cells in a heterozygous HLA-$B35^+B51^+$ individual. Moreover, FLU A2/GIL and CMV B35/IPS TCRs from HLA-$A2^+B38^+$ and HLA-$B35^+B51^+$ individuals, respectively, were nearly identical to the public cross-reactive TCRs, and only small amino acid insertions within both CDR3 regions (CMV B35/IPS) and even within the CDR3α region alone (FLU A2/GIL) were able to abrogate cross-reactivity. Plausibly, this exceptionally conserved TCR usage illustrates the superiority of these TCR clonotypes in generating antiviral immune responses.

The mechanisms by which the newly identified public TCRs cross-react to allo-HLA remain unknown, however, because HLA-B35 is structurally highly similar to HLA-B51 and HLA-B58/B57, and HLA-B35 and HLA-B51 present a similar peptide repertoire (200, 201), the CMV-B35/IPS cross-reactivity against HLA-B51 and HLA-B58/B57 may be explained by structural mimicry. Structural mimicry between an allo- and viral epitope is the most described mechanism of TCR cross-reactivity and underlies EBV B8/FLR cross-reactivity against HLA-B*44:02 as well as HLA-B*35:01 (44, 135). Both FLU A2/GIL and VZV A2/ALW public cross-reactivities were directed against allo-HLA antigens with only little similarity to the cognate HLA antigen. Although this does not exclude structural mimicry (after all HLA-B*08:01 and HLA-B*44:02 show substantial polymorphism and divergent peptide expression), the finding that FLU A2/GIL cross-reactivity appears to depend on CDR3a usage, whereas TCR docking to the cognate viral epitope depends primarily on binding of the Vβ chain (9, 202), could indicate a different mechanism for allorecognition and points toward an altered TCR docking mode, but this should be investigated further.

The strong functional difference in HLA-B51 versus HLA-B58/B57 allorecognition by CMV B35/IPS T-cell clones is remarkable. A possible explanation may be different TCR affinities for these alloantigens. The small amino acid differences in the α_1 domain between HLA-B51 and HLA-B58/B57 for example, may affect TCR binding (203), especially because these involve the "generic" MHC class I restriction elements on positions 65, 66, and 69 (190). These restriction elements are conserved for HLA-B35 and HLA-B51. In addition, variations in peptide presentation between HLA-B51 and HLA-B58/B57 could affect TCR avidity and lead to suboptimal or alternative TCR signaling and T-cell activation in response to HLA-B58/B57 (204, 205). However, for conclusive statements on the mechanisms behind TCR cross-reactivity, the (allo)peptides presented in the cross-reactive HLA should be identified and crystal structures need to be assembled, which was beyond the scope of this research.

We recently identified FLU A2/GIL cross-reactivity to donor HLA-B38 in an HLA-A2$^+$ renal transplant patient (38), emphasizing that (public) cross-reactivities can indeed present in a clinical setting. As one of the key endeavors in transplantation research is to predict alloreactivity, public cross-reactivity of virus-specific TCRs could provide a useful tool. However, potential predictive value will differ by public cross-reactivity.

The prevalence of the public cross-reactive TCR clonotypes, and thus the percentage of patients that harbor these TCRs, greatly affects prediction. The here-described FLU A2/GIL public cross-reactivity against HLA-B38 is highly abundant, with 90% of FLU A2/GIL tetramer-

positive individuals expressing the public HLA-B38 cross-reactive TCR. In fact, this public TCR is considered the most abundant human TCR clonotype (9). This high prevalence is facilitated by the facts that HLA-A2 is the most common HLA allele worldwide, that infection with the influenza virus occurs in virtually all individuals, and that the $CD8^+$ T-cell response to influenza is dominated by T cells that recognize the HLA-A2/GIL epitope. The CMV B35/IPS HLA-B51 and HLA-B58/B57 cross-reactive TCR clonotypes were found in 23% of CMV B35/IPS tetramer-positive individuals. Although HLA-B35 is less predominant than HLA-A2, CMV affects approximately 40-100% of the world population and is one of the most prevailing infections after transplantation. The prevalence of the HLA-B55 cross-reactive VZV A2/ALW TCR cross-reactivity remains to be established, because the low precursor frequency of VZV A2/ALW-$tetramer^+$ T cells in the peripheral blood hampers its accurate detection. Yet, VZV also affects nearly all individuals, the majority of $HLA\text{-}A2^+$ individuals show $CD8^+$ T-cell responses against the VZV A2/ALW epitope (196), and the identification of highly identical TCRs in different unrelated individuals suggests it is indeed common.

Furthermore, functionality differs by cross-reactivity. CMV B35/IPS cross-reactivity against HLA-B51 and HLA-B58/B57 were both characterized by proliferation and IFNγ production, but only HLA-B51 cross-reactivity induced cytotoxicity. FLU A2/GIL T cells were able to proliferate, produce IFNγ, and exert cytotoxicity toward $HLA\text{-}B38^+$ targets, whereas VZV A2/ALW-specific T cells expressed limited to no cytotoxicity but were able to proliferate and produce IFNγ upon allorecognition. Nevertheless, it is still unclear which functional characteristics of cross-reactive T cells in vitro affect clinical outcome: where cytotoxic responses may harm the allograft directly, cytokine production could harm the allograft indirectly by inducing an inflammatory environment (113). In addition, one should keep in mind that functionality may differ in response to different cell types (tissue specificity), as shown for the public EBV-B8/FLR cross-reactivity to allo-$HLA\text{-}B^*44{:}02$ (87). When the cross-reactive allopeptide has a restricted tissue distribution, (public) TCR cross-reactivity may be restricted to specific organs, whereas if it is expressed in multiple cell types, it could have broader implications for organ transplantation. In our experiments, there were no indications for tissue-specificity of CMV B35/IPS cross-reactivity against HLA-B51 or VZV A2/ALW cross-reactivity against HLA-B55. HLA-B38 cross-reactive FLU A2/GIL T cells, however, appeared less potent in lysing PTECs compared with HUVECs, EBV-LCLs, and PHA blasts, suggesting there may be a certain degree of tissue specificity.

Furthermore, precursor frequencies should be determined because they could affect clinical outcome. Unlike low precursor frequencies, high precursor frequencies of alloreactive memory T cells are associated with impaired tolerance induction and contribution to graft rejection

and graft-versus-host disease (61, 84, 109, 206, 207); therefore, cross-reactive T-cell precursor frequencies strongly determine alloreactivity predictive value and should be determined per individual. Of note, precursor frequencies of virus-specific T cells have been shown to be subject to some fundamental principles that apply to most individuals and that could aid in initial risk estimation. FLU-specific memory T cells, for example, compose a small proportion of the T-cell repertoire, plausibly due to clearance of the virus after infection. CMV-specific memory T cells, in contrast, make up a substantial part of the $CD8^+$ T-cell repertoire (208), likely because CMV is (like EBV) a herpes virus that latently persists after infection. In addition, they have been shown to dominate T-cell repopulation following lymphodepletion (209) and are even suggested to promote solid organ rejection (42). Despite VZV also being a persistent virus, the precursor frequency of VZV A2/ALW-specific memory T cells is low: perhaps due to VZV "hiding" from the immune system in nerve cells. Nevertheless, the precursor frequency of HLA-B55 cross-reactive VZV A2/ALW T cells could increase as a result of routine vaccination against VZV in transplantation recipients. Indeed, one of the HLA-B55 cross-reactive VZV A2/ALW T-cell clones studied in this paper (Clone 12) was derived from a transplant recipient that was vaccinated against VZV (193). In addition, adoptive transfer of enriched virus-specific T cells may introduce cross-reactive virus-specific T cells at high precursor frequencies.

In conclusion, we were able to identify three novel public cross-reactivities of virus-specific memory T cells in a small cohort of individuals, while subjected to a restricted number of known dominant viral epitopes and tetramers. It is therefore expected that this is only the tip of the iceberg - and public cross-reactivity of virus-specific T cells is much more common than anticipated. Increasing the knowledge concerning public cross-reactivities could benefit the prediction of anti-donor reactivity, enable monitoring of potentially harmful alloresponses, and ultimately support clinical decision making.

Table 2. TCR usage of virus-specific CD8⁺ memory T cells in different individuals

	Donor	Clone	TRAV	TRAJ	CDR3α	TRBV	TRBJ	TRBD	CDR3β	Allo-HLA cross-reactivity
CMV B35/IPS	*#1*	7C8	TRAV17*01	TRAJ33*01	C A T E T S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S L S T S P N E K L F F	B51+B58
		6E5(1)	TRAV17*01	TRAJ33*01	C A V E D S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S D G T G P N E K L F F	B51
		6A4	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P A G G P N E K L F F	B58
	#2	3F12	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5D12	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5F8	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
		5G11	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S G T A P N E K L F F	B58
	#3	6C8	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		6B11	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		6E5(2)	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S S Q T G P N E K L F F	B58
		8F4	TRAV17*01	TRAJ33*01	C A T E D S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S A G T G P N E K L F F	B51
		8F11	TRAV17*01	TRAJ53*01	C A T E S S N Y K L T F	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S P G T A P N E K L F F	B58
		7E10	TRAV17*01	TRAJ33*01	C A T E H S N Y Q L I W	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S S L S T A P N E K L F F	B51+B58
	#4	8A2	TRAV17*01	TRAJ33*01	**C A T E G S N Y Q L I W**	TRBV28*01	TRBJ1-4*01	TRBD1*01	C A S T T S G G P N E K L F F	B58
FLU A2/GIL	*#1*	1A5	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		1B6	TRAV29DV5*01	TRAJ38*01	n.d.	TRBV19*01/02/03	TRBJ2-7*01	TRBD2*02	C A S S I R S T G E L F F	-
		1B10	TRAV29DV5*01	TRAJ38*01	n.d.	TRBV19*01/02/03	TRBJ2-7*01	TRBD2*02	C A S S I R S T G E L F F	-
		1C4	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38

Table 2. Continued

	Donor	Clone	TRAV	TRAJ	CDR3α	TRBV	TRBJ	TRBD	CDR3β	Allo-HLA cross-reactivity
		1F4	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
	#3	1B5	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		1B7	TRAV8-6*01	TRAJ42*01	C A V S G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	C A S S L R S S Y E Q Y F	-
		1C11	TRAV17*01	TRAJ42*01	C A T D E G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-2*01	TRBD1*01	C A S S M R S T G E L F F	-
	#5	3C2	TRAV27*01	TRAJ42*01	**C A G G G S Q G N L I F**	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	B38
		3C3	TRAV38-1*01/04	TRAJ52*01	C A F M I G A G G T S Y G K L T F	TRBV19*01/02/03	TRBJ1-2*01	TRBD1*01	C A S S I G A Y G Y T F	-
		3F8	TRAV41*01	TRAJ42*01	C A V D A G G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
	#6	4A2	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4C2	TRAV27*03	TRAJ42*01	C A G A G G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-2*01	TRBD1*01	C A S S G R A T G E L F F	-
		4D5	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4D7	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
		4G6	TRAV27*01	TRAJ42*01	C A G A G D G G S Q G N L I F	TRBV19*01/02/03	TRBJ2-7*01	TRBD1*01	**C A S S I R S S Y E Q Y F**	-
VZV A2/ALW	#7	1C12	TRAV12-2*01	TRAJ40*01	C A A F T S G T Y K Y I F	TRBV27	TRBJ1-1*01	TRBD1*01	C A S S S D R G Y E A F F	B55
	#8	Clone 12	TRAV12-2*01	TRAJ40*01	C A V F T S G T Y K Y I F	TRBV27	TRBJ2-7*01	n.d.	C A S S S D R G Y E Q Y F	B55

Sequences indicated in bold: identical CDR3 regions (indicated per viral specificity)

Table 3. Cytotoxic potential of virus-specific memory CD8+ T-cell clones with public TCR usage able to lyse allogeneic cells expressing cross-reactive HLA antigens[a,b]

	Donor	Clone	Allo-HLA	PHA-blast	EBV-LCL	PTEC	HUVEC
CMV B35/IPS	1	7C8	B51	92 (±2.1)[1]	83 (±17.7)[1]	104	98 (±9.2)[1]
		6E5(1)	B51	49	44 (±7.1)[1]	n.d.	76
	3	8F4	B51	97	73 (±15.4)[1]	87	91 (±15.6)[1]
FLU A2/GIL	1	1A5	B38	43	53	n.d.	58
		1F4	B38	47	63	34	56 (±0.7)[1]
		1C4	B38	48	65 (±2.1)[1]	67	n.d.
	3	1B5	B38	5	33	16	n.d.
	4	3C2	B38	54	63 (±14.1)[1]	n.d.	54
VZV A2/ALW	5	Clone 12	B55	24(±2.9)[1]	21 (±9.1)[1]	16	0
	6	1C12	B55	14(±6.1)[1]	11 (±0.7)[1]	11	13 (±8.7)[1]

CMV B35/IPS, cytomegalovirus pp65(123-131) HLA-B*35:01/IPSINVHHY; EBV-LCL, Epstein–Barr virus transformed lymphoblastoid cell line; FLU A2/GIL, influenza IMP(58-66) HLA-A*02:01/GILGFVFTL; HUVEC, human umbilical vein endothelial cell; PHA, phytohemagglutinin;
PTEC, proximal tubular epithelial cell; VZV A2/ALW, varicella zoster virus IE62(593-601) HLA-A*02:01/ALWALPHAA. n.d. = not determined.

[a]Numbers represent the allo-HLA response as a percentage of the anti-virus response

[b]All experiments were performed in triplicate and at different effector: target (E:T) ratios (30:1, 10:1, 1:1, 0.1:1) of which E:T ratio 30:1 is shown

[1]Means of two independent experiments with standard deviation

SUPPLEMENTARY MATERIAL

Data S1

Data S1 can be found at: https://tinyurl.com/yyx8ygj4

Data S2.

Figure 1	HLA typing
R1 (= Donor #1 Table 2)	A*02:01 A*11:01 **B*35:01** B*40:01 C*03:04 C*04:01 DRB1*15:01 DRB1*11:01 DQB1*06:02 DQB1*03:01
R2 (= Donor #3 Table 2)	**A*02:01** A*24:02/24:09N/24:11N+ B*08:01/08:05/08:08N+ B*35:01/35:07/35:11+ C*03:04/03:05/03:08+ C*04:01/04:09N/04:05+ DRB1*03:01 DRB1*13:02 DRB3*01:01 DRB3*03:01 DQB1*02:01 DQB1*06:04 DQA1*01:02 DQA1*05:01/05:03 DPB1*04:01 DPB1*10:01
R3 (= Donor #7 Table 2)	**A2** A3 B51(5) B7 Cw2 Cw7 DR15 DR13 DQ6 DQ1
R4	A2 A24(9) **B35 B58(17)** Cw3 DR2 DR13 DR6 DR51 DR52 DQ6 DQ1
R5	A*03:01 A*31:01 **B*35:01 B*51:0**1 C*04:01/04:09N/04:05 C*15 DRB1*01:01 DRB1*09 DRB4*01:03 DQB1*03:03 DQB1*05:01 DPB1*04:02
R6 (= Donor #4 Table 2)	A*03:01/03:03N/03:04+ A*24:02/24:05/24:09N+ **B*35:01/35:07/35:11+ B*51:01/51:03/51:09+** C*07:02/07:03/07:10+ C*14:02/14:07N DRB1*04:07 DRB1*15:01 DRB4*01:03 DRB5*01:01, DQB1*03:01 DQB1*06:02
R7 (= Donor #6 Table 2)	**A*02:01** A*26:01 **B*38:01** B*55:01 C*01:02 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03
S1	A2 A3 **B51(5)** B7 Cw2 DR15(2)(51) DR11(5)(52) DQ6(1) DQ7(3)
S2	A11 A31(19) **B58(17)** B18 Cw7 DR11(5)(52) DR8 DQ7(3) DQ4(3)
S3	A3 A31(19) B60(40) Cw10 Cw3 DR4(53) DR11(5)(52) DQ7 DQ3
S4	A1 A24(9) B8 **B38(16)** DR4 DR14(6) DQ5(1) DQ8(3)
S5	A*24:02 A*26:01 **B*38:01** B*35:03 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03 DQB1*06:03
S6	A*03:01 A*26:01 B*07:02 B*40:01 C*03:04 C*07:02 DRB1*15 DRB1*04:04 DQB1*06:02 DQB1*03:02
S7	A*03 A*24 B*15:01/15:33/15:34+ **B*55:01/55:02/55:05+** C*03 DRB1*04 DRB1*13:01/13:02/13:06+ DRB3*03 DRB4 DQB1*03:02/03:07/03:08 DQB1*06:04/06:08/06:17
S8	A24(9) A30(19) B13 **B55(22)** Cw9 Cw3 Cw6 DR11 DR5 DR13 DR6 DR52 DQ6 DQ1 DQ7 DQ3
S9	A24(9) A11 B35 B60 (40) Cw10 Cw3 Cw4 DR15 DR2 DR4 DR51 DR53 DQ6 DQ1 DQ7 DQ3
S10	A24(9) A68(28) **B51(5)** B45(12) DR13 DR6 DR10 DR52 DQ5 DQ6 DQ1
S11	A*0301 A*2402 B*0702 **B*58:01** Cw*0701 DRB1*01 DRB1*1301
S12	A*01:01 A*02:01 B*08:01 C*07:01/07:06/07:07 DRB1*03:01/03:04/03:05+ DRB3*01:01 DQB1*02:01/02:02/02:04 DQA1*05:01/05:03 DPB1*01:01 DPB1*04:02
S13	A*02:01 A*03:01 B*07:02 **B*51:01** C*02:02 C*07:02 DRB1*11:01 DRB1*15:01 DRB3*02:02 DRB5*01:01
S14	A*02:01 A*26:01 **B*38:01** B*55:01 C*01:02 C*12:03 DRB1*13:01 DRB1*14:54 DQB1*05:03
S15	A2 **B38(16)** B72(70) Cw2 DR3 DR13(6) DQ1

Data S2. Continued

Table 2	HLA typing
Donor 1	**A*02:01** A*11:01 **B*35:01** B*40:01 C*03:04 C*04:01 DRB1*15:01 DRB1*11:01 DQB1*06:02 DQB1*03:01
Donor 2	A1 A11 B8 **B35** Bw6 Cw4 Cw7 DRB1*0103 DRB1*0301 DQ5 DQ2
Donor 3	**A*0201** A*2402 B*0801 **B*3501** Cw*0304 Cw*0401 DRB1*0301 DRB1*1302
Donor 4	A3 A24(9) **B51(5) B35** C*0702 C*14:02 DRB1*04:07 DRB1*15:01 DRB4*01:03 DRB5*01:01 DQB1*03:01 DQB1*06:02
Donor 5	**A*02** A*03 B*07 B*35 C*04 C*07 DRB1*01 DRB1*08 DQB1*04:02 DQB1*05:01
Donor 6	**A*02:01** A*03:01 B*37:01 **B*38:01** C*06:02 C*12:03 DRB1*03:01 DRB1*13:01 DQB1*02:01 DQB1*06:03
Donor 7	**A2** A3 B51(5) B7 Cw2 Cw7 DR15 DR13 DQ6 DQ1
Donor 8	**A*02:01** B*13:02 B*40:01 C*03:04 C*06:02 DRB1*07:01 DRB4 DQB1*02:01

CHAPTER

7

THE AVIDITY OF CROSS-REACTIVE VIRUS-SPECIFIC T CELLS FOR THEIR VIRAL AND ALLOGENEIC EPITOPES IS VARIABLE AND DEPENDS ON EPITOPE EXPRESSION

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Hum Immunol. 2018 Jan;79(1):39-50

ABSTRACT

Virus-specific T cells can recognize allogeneic HLA (allo-HLA) through cross-reactivity of their T-cell receptor (TCR). In a transplantation setting, such allo-HLA cross-reactivity may contribute to harmful immune responses towards the allograft, provided that the cross-reactive T cells get sufficiently activated upon recognition of the allo-HLA. An important determinant of T-cell activation is TCR avidity, which to date, has remained largely unexplored for allo-HLA-cross-reactive virus-specific T cells. For this purpose, cold target inhibition assays were performed using allo-HLA-cross-reactive virus-specific memory $CD8^+$ T-cell clones as responders, and syngeneic cells loaded with viral peptide and allogeneic cells as hot (radioactively-labeled) and cold (non-radioactively-labeled) targets. CD8 dependency of the T-cell responses was assessed using interferon γ (IFNγ) enzyme-linked immunosorbent assay (ELISA) in the presence and absence of CD8-blocking antibodies. At high viral-peptide loading concentrations, T-cell clones consistently demonstrated lower avidity for allogeneic versus viral epitopes, but at suboptimal concentrations the opposite was observed. In line, anti-viral reactivity was CD8 independent at high, but not at suboptimal viral-peptide-loading concentrations. The avidity of allo-HLA-cross-reactive virus-specific memory $CD8^+$ T cells is therefore highly dependent on epitope expression, and as a consequence, can be both higher and lower for allogeneic versus viral targets under different (patho)physiological conditions.

INTRODUCTION

In humans, the estimated T-cell receptor (TCR) repertoire after positive and negative selection in the thymus covers around 10^8 unique TCR clonotypes (210). If these would only reflect single specificities, the TCR repertoire would be far too restricted to cope with the broad array of mutating pathogens encountered throughout life. T cells therefore have the intrinsic ability to cross-react to multiple viral epitopes, a phenomenon known as heterologous immunity. In recent years, it became clear that virus-specific T cells not only have the ability to cross-react to multiple viral peptides, but also to allogeneic HLA (allo-HLA) molecules. Such cross-reactivity is very common and occurs within all individuals (43). Heterologous immune responses of virus-specific T cells that are directed against allo-HLA molecules could pose a threat to both hematopoietic and solid organ transplantation. Indeed, animal models have shown that virus-specific T cells can actively hamper tolerance induction and mediate allograft rejection (35). Recently, we reported the first ex vivo analysis of virus-specific T cells possessing cross-reactivity to donor peptide:HLA antigens in renal allograft recipients. In 13 of 25 transplant recipients, cross-reactivity to donor antigen was demonstrated within T-cell populations specific for viral epitopes (142). Remarkably, the presence of donor cross-reactive T cells in the circulation of these transplant patients was not associated with inferior outcomes relative to patients who lacked these cells. However, to determine the clinical consequences of virus-specific T cells with cross-reactivity to allo-HLA, further and more extensive (prospective) clinical studies are necessary.

The potential of allo-HLA cross-reactive T cells to harm an allograft depends on their effector function, which in turn hinges on TCR avidity: the cumulative strength of all non-covalent binding interactions between a T cell and its target cell. Indeed, high avidity donor-reactive cytotoxic T cells have been associated with acute rejection of cardiac allografts (211, 212). Avidity is largely defined by the interaction between a TCR and its peptide-MHC (pMHC) ligand (TCR affinity), but additional cell surface molecule interactions (e.g. TCR dimerization), co-receptor binding and cell adhesion molecules also play an important role. Indeed, TCR affinity and avidity are unmistakably correlated with TCR signal strength and T-cell activation (213-215).

Variation in T-cell ligation has been shown to induce differences in downstream signaling pathways, mainly by altered phosphorylation downstream of the TCR (216, 217). Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) plays a crucial role in determining "T-cell fate" by promoting different TCR signaling pathways (218). TCR ligation of a T cell with high avidity for its ligand is more likely to induce a full-blown T-cell response compared to

TCR ligation of a T cell with low avidity for its ligand, resulting in more pronounced or even qualitatively different effector functions (219, 220). Accordingly, TCRs generally bind with higher affinity to agonistic peptides compared to partial agonists or antagonists (221). As a result of thymic selection, the peripheral TCR repertoire expresses low to moderate avidity for self-HLA, thereby avoiding T-cell activation and autoimmunity. Since allo-HLA is not expressed in the thymus, allo-HLA cross-reactivity is not restricted by positive and negative thymic selection. Therefore, TCR avidity for allo-HLA could in theory have a much broader spectrum compared to self-HLA.

TCR affinity and avidity can be addressed by several techniques: for example, competitive tetramer-staining can be used to estimate the hierarchy of TCR avidity for nominal and allogeneic epitopes (44, 222) while techniques such as surface plasmon resonance (SPR) (223), Förster resonance energy transfer (FRET) (224) and the mechanical micropipette adhesion frequency assay (225) can address technical kinetics such as half-life and association / dissociation rates of the TCR-pMHC complex. Yet, a downside of these assays is that they require comprehensive knowledge of the recognized epitope(s), which in case of allo-HLA cross-reactive T cells not only requires identification of the allo-HLA, but also the allopeptide. The latter is laborious and time-consuming and has only been done for two human TCRs with the same viral specificity (44, 192). As a consequence, much is still unknown about TCR affinity and avidity of virus-specific cross-reactive T cells. Here, we aimed to characterize the relative avidity of human cross-reactive virus-specific $CD8^+$ memory T cells for nominal and allogeneic epitopes by using different techniques.

MATERIALS AND METHODS

Collection of responder and target cells

Peripheral blood mononuclear cells (PBMCs) of healthy individuals were obtained after informed consent in accordance with the Declaration of Helsinki. Phytohaemagglutinin (PHA) blasts were generated by incubating PBMCs for 7 days in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with penicillin/streptomycin, glutamine, 15% human serum (HS), and PHA (4 µg/mL; Murex Biotech Ltd, Dartford, UK).

Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCLs) were generated by incubating PBMCs for 1.5 hours at 37°C with supernatant of the EBV-producing marmoset cell line B95.8, and subsequently cultured in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with pen/strep, glutamine and 10% fetal calf serum (FCS).

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium (Gibco) supplemented with 10% FCS, sodium pyruvate (Gibco), penicillin/streptomycin (Gibco) and β-mercaptoethanol (0.05M; Sigma Aldrich, St. Louis, MO), and were used at passages P1-4.

HLA typing was performed by sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) genotyping at the European Federation of Immunogenetics (EFI)-accredited national reference laboratory for histocompatibility testing at the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands.

Generation of virus-specific CD8$^+$ T-cell lines and clones

Virus-specific CD8$^+$ memory T-cell lines and clones were generated by fluorescence-activated cell sorting (FACS Aria; BD) as previously described (118). PBMCs were stained with phycoerythrin (PE)-labeled viral tetramers CMV pp65(123-131) HLA-B*35:01/IPSINVHHY (CMV B35/IPS), FLU MP(58–66) HLA-A*02:01/GILGFVFTL (FLU A2/GIL), VZV IE62(593-601) A*02:01/ALWALPHAA (VZV A2/ALW), and EBV EBNA3A(325-333) B*08:01/FLRGRAYGL (EBV B8/FLR) (Protein facility of the Leiden University Medical Center, Department of Immunohematology and Blood Transfusion, the Netherlands) and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAb) against CD4, CD19, CD45-RA, CD14, CD40, CD16 and CD56 (BD Pharmingen, San Diego, CA). The FITC channel (FL1) was used as a dump channel.

Cold-target inhibition

Cold target inhibition assays were performed to define TCR avidity for syngeneic HLA + viral peptide versus allo-HLA + endogenous (allo)peptide. Hereto, the ^{51}Chromium release assay (CRA) (226) was altered by including not only hot (radioactively-labeled) but also cold (non-radioactively-labeled) targets in different cold / hot target ratios (1:1; 2.5:1; 10:1; 20:1). EBV-LCLs and HUVECs were used as target cells. Both syngeneic HLA + viral peptide and allogeneic HLA + (allo)peptide target cells were employed as hot and cold targets, and different peptide-loading concentrations were used (ranging between 0.01ng/ml to 1000ng/ml). All conditions (disregarding validation) were performed in triplicate at effector:target (E:T) ratio 1:1.

CD8-blocking assays

CD8 blocking was assessed in both IFNγ ELISA and ^{51}Chromium release assay. Virus-specific CD8$^+$ T-cell clones (5 x 10^3) were incubated with or without CD8-blocking antibody FK18 (7.7μg/ml; 1 hour 37°C) as described previously (227). For IFNγ ELISA, T-cell clones were co-cultured for 24 hours with EBV-LCLs (5 x 10^4) expressing either self-HLA, self-HLA + viral peptide, or allo-HLA molecules (triplicate wells; 24 hours at 37°C). Culture medium consisted of IMDM (Lonza) supplemented with penicillin/streptomycin, glutamine, 5% FCS (Lonza), 5% HS, and

IL-2 (10 U/mL). After 24 hours, IFNγ production was assessed in a standard enzyme-linked immunosorbent assay (ELISA; U-CyTech, Utrecht, The Netherlands) according to protocol. Different peptide-loading concentrations were used (ranging between 0.01ng/ml to 1000ng/ml). For ^{51}Chromium release assay, the experiments were performed with EBV-LCLs and PHA blasts as previously described (226). All conditions were performed in triplicate at effector:target (E:T) ratio 1:10 (ELISA) and 1:1 (CRA) respectively.

RESULTS

Lysis of allogeneic cells by cross-reactive virus-specific T cells can be inhibited by competition with syngeneic cells expressing the viral epitope

To assess the difference in TCR avidity of cross-reactive T cells for viral and allogeneic epitopes, cold target inhibition assays were performed (Figure 1). T cells expressing the following (public) cross-reactivities were used: CMV B35/IPS T cells cross-reacting to allo-HLA-B*51:01/B*57:01/B*58:01; FLU A2/GIL T cells cross-reacting to allo-HLA-B*38:01; VZV A2/ALW T cells cross-reacting to HLA-B*55:01; and EBV B8/FLR T cells cross-reacting to allo-HLA-B*44:02 (and the allopeptide EEYLQAFTY) (44, 140) (Figure 2).

First, the conditions of the assay were evaluated. The optimal effector:target (E:T) ratio was determined to prevent misinterpretation due to excess of responder and/or target cells. Hereto, the same target cells were used as hot and cold targets (both viral and allogeneic origin). Employing Epstein-Barr Virus-immortalized lymphoblastoid cell lines (EBV-LCLs) as target cells, only E:T ratio 1:1 met the expected inhibition corresponding to the cold:hot (C:H) target ratios. This optimal ratio was observed for all viral specificities and confirmed with human umbilical vein endothelial cells (HUVECs) as alternative targets (Figure 3A). Furthermore, steric hindrance was determined using non-cross-reactive virus-specific T-cells and a-specific cold target inhibition at increasing C:H target ratios. At E:T ratio 1:1, steric hindrance was observed from C:H target ratios of 10:1 onwards (Figure 3B).

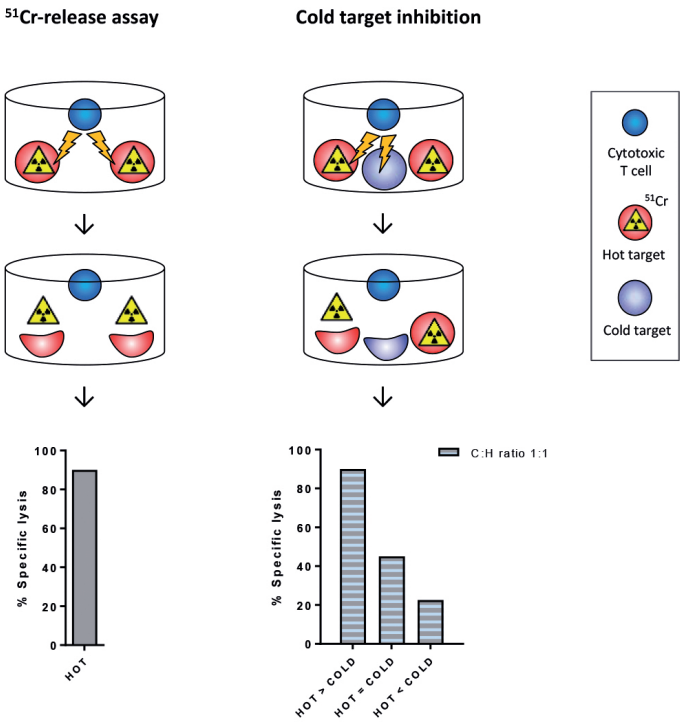


Figure 1. Principle of the cold target inhibition assay. The cold target inhibition assay (right pane) is based on the ^{51}Chromium-release assay (left pane) that measures lysis of radioactively-labeled target cells ("hot targets") by cytotoxic T cells ("responders"), however in addition introduces unlabeled target cells ("cold targets") to the system. This addition stimulates competition between hot and cold target lysis based on TCR avidity. A reduction in released ^{51}Chromium illustrates inhibition of hot target cell lysis by cold target cells. When TCR avidity is higher for the hot target, no or limited inhibition of hot target cell lysis is observed at cold:hot target (C:H) ratio 1:1 (left bar, right graph), when TCR avidity is equal for both hot and cold targets a two-fold reduction is observed at C:H ratio 1:1 (middle bar, right graph), and when TCR avidity is higher for the cold target strong inhibition of hot target cell lysis is observed at C:H ratio 1:1 (right bar, right graph). The C:H ratio in combination with the degree of inhibition indicates whether TCR avidity is higher for the hot or the cold target.

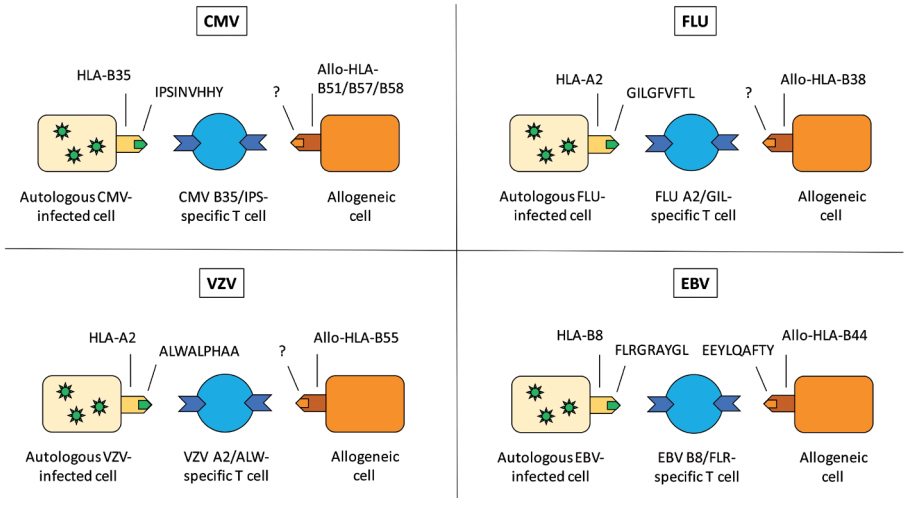


Figure 2. Schematic overview of the virus-specific TCR cross-reactivities used in this study. The cross-reactive allopeptides of the CMV, FLU and VZV cross-reactivities are still to be determined (indicated by "?").

Next, the assay was performed with different allo-HLA cross-reactive virus-specific memory CD8$^+$ T-cell clones (effectors) versus allogeneic EBV-LCLs and HUVECs (targets). Viral-peptide-loaded autologous cold targets were able to inhibit lysis of allogeneic hot targets, whereas allogeneic cold targets were not able to inhibit lysis of viral-peptide-loaded autologous hot targets. This was consistently observed regardless of viral specificity and target cell type. Moreover, it also held when both viral peptide and allopeptide were employed in the assay: EBV B8/FLR-specific T cells preferentially lysed HLA-B*08:01 EBV-LCLs loaded with viral peptide (FLRGRAYGL) over HLA-B*44:02 EBV-LCLs loaded with equal concentrations of the cross-reactive allopeptide (EEYLQAFTY) (Figure 4).

The alloresponse of cross-reactive virus-specific memory CD8$^+$ T cells is co-receptor dependent

In general, high-affinity TCRs do not require CD8 co-receptor binding for activation, whereas low-affinity TCRs do (228). For all viral specificities, CD8 co-receptor blocking on the T-cell surface hampered IFNγ production against allogeneic targets, but not against the viral targets (Figure 5A). CD8 co-receptor binding also hampered cytotoxicity towards allogeneic cells, but not towards syngeneic cells expressing the viral peptide (Figure 5B).

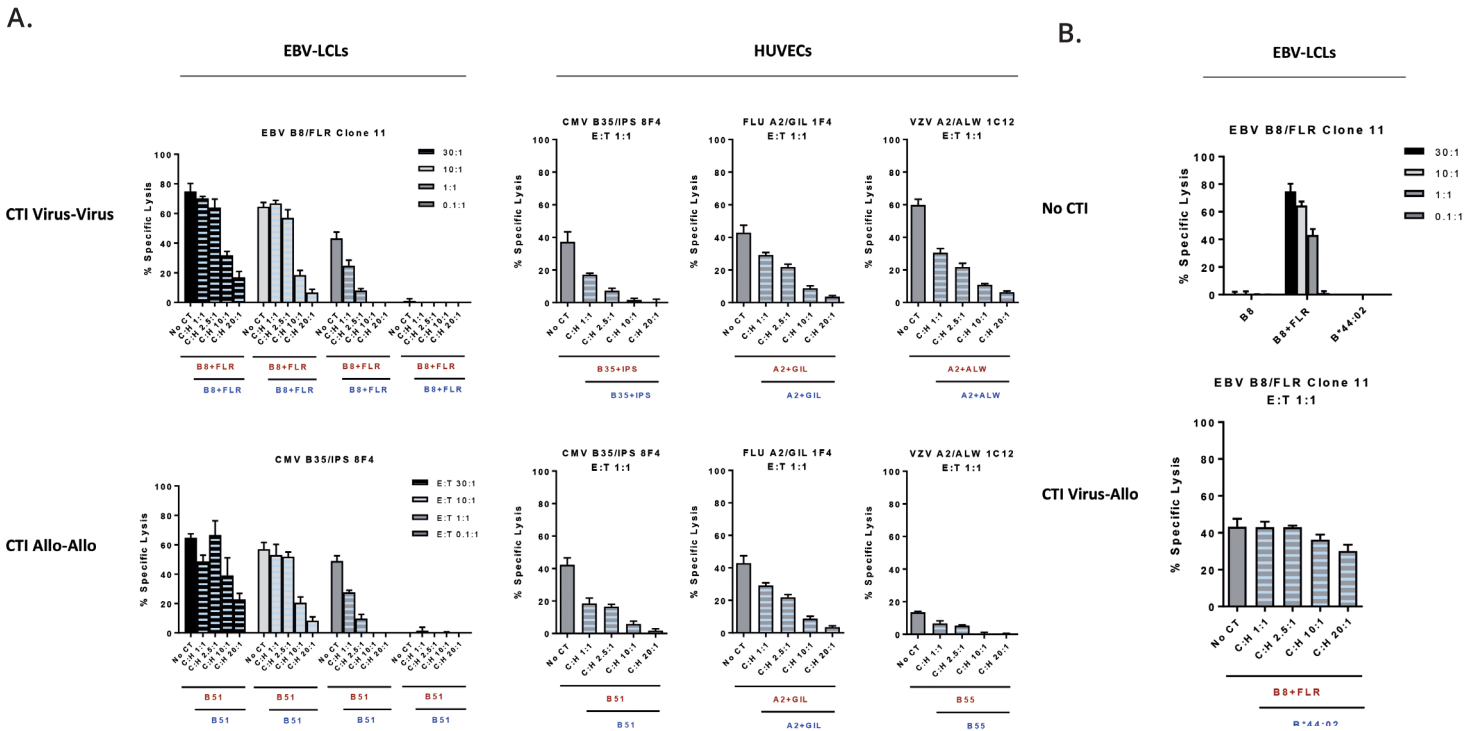
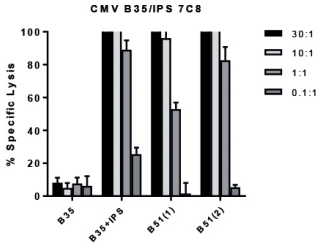
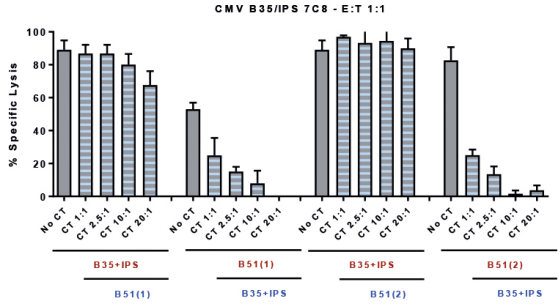
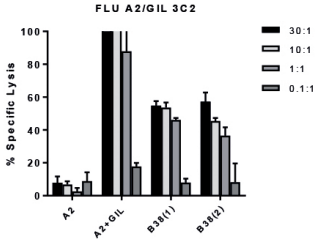


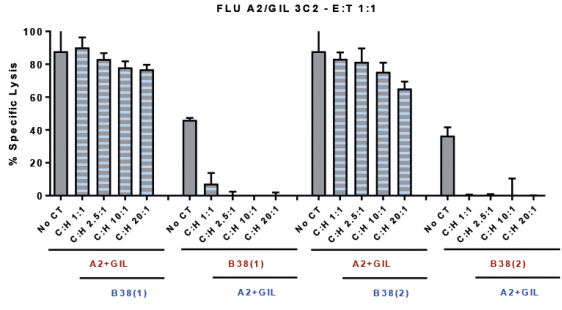
Figure 3. CTI assay evaluation. A) The optimal E:T ratio was determined by CTI using the same EBV-LCLs as hot and cold targets while varying E:T and C:H ratios. Upper left panel: CTI with HLA-B*08:01+ EBV-LCLs + viral peptide (FLR) as hot and cold targets. Lower left panel: CTI with allogeneic HLA-B*51:01+ EBV-LCLs as hot and cold targets. Regardless of TCR specificity E:T ratio 1:1 showed accurate inhibition for both viral- and alloepitope-expressing EBV-LCLs, and HUVECs confirmed these findings (right panels). B) To determine steric hindrance, non-cross-reactive target EBV-LCLs were used as cold targets to a-specifically hamper the anti-viral response. Red: hot targets. Blue: cold targets. Error bars represent the standard deviation (S.D.) of triplicate wells. Peptide concentrations: 1000 ng/ml.

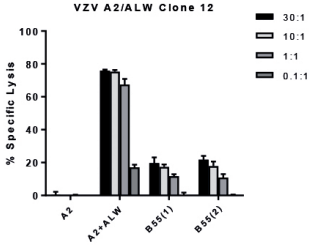
EBV-LCLs

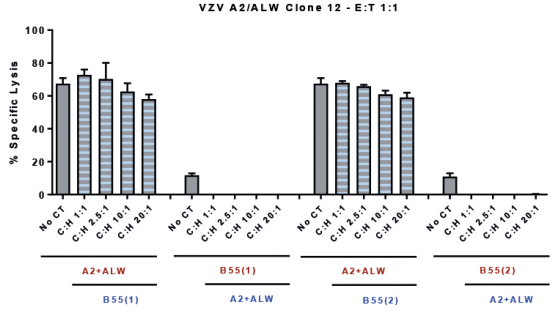

CMV B35/IPS 7C8
% Specific Lysis
30:1
10:1
1:1
0.1:1
B35
B35+IPS
B51(1)
B51(2)

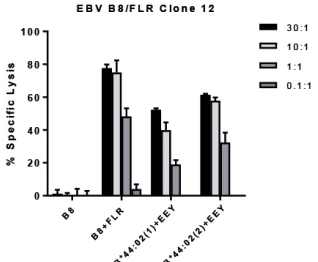

CMV B35/IPS 7C8 - E:T 1:1
% Specific Lysis
No CT
CT 1:1
CT 2.5:1
CT 10:1
CT 20:1
B35+IPS
B51(1)
B51(2)

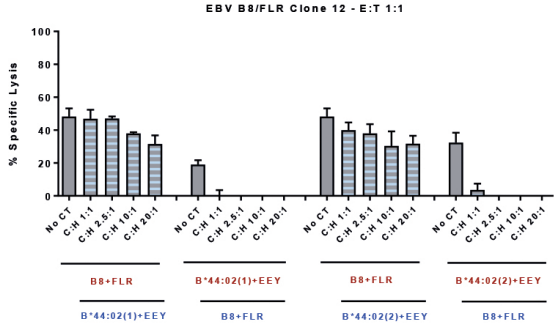

FLU A2/GIL 3C2
% Specific Lysis
30:1
10:1
1:1
0.1:1
A2
A2+GIL
B38(1)
B38(2)


FLU A2/GIL 3C2 - E:T 1:1
% Specific Lysis
No CT
C:H 1:1
C:H 2.5:1
C:H 10:1
C:H 20:1
A2+GIL
B38(1)
B38(2)


VZV A2/ALW Clone 12
% Specific Lysis
30:1
10:1
1:1
0.1:1
A2
A2+ALW
B55(1)
B55(2)


VZV A2/ALW Clone 12 - E:T 1:1
% Specific Lysis
No CT
C:H 1:1
C:H 2.5:1
C:H 10:1
C:H 20:1
A2+ALW
B55(1)
B55(2)


EBV B8/FLR Clone 12
% Specific Lysis
30:1
10:1
1:1
0.1:1
B8
B8+FLR
B*44:02(1)+EEY
B*44:02(2)+EEY


EBV B8/FLR Clone 12 - E:T 1:1
% Specific Lysis
No CT
C:H 1:1
C:H 2.5:1
C:H 10:1
C:H 20:1
B8+FLR
B*44:02(1)+EEY
B*44:02(2)+EEY

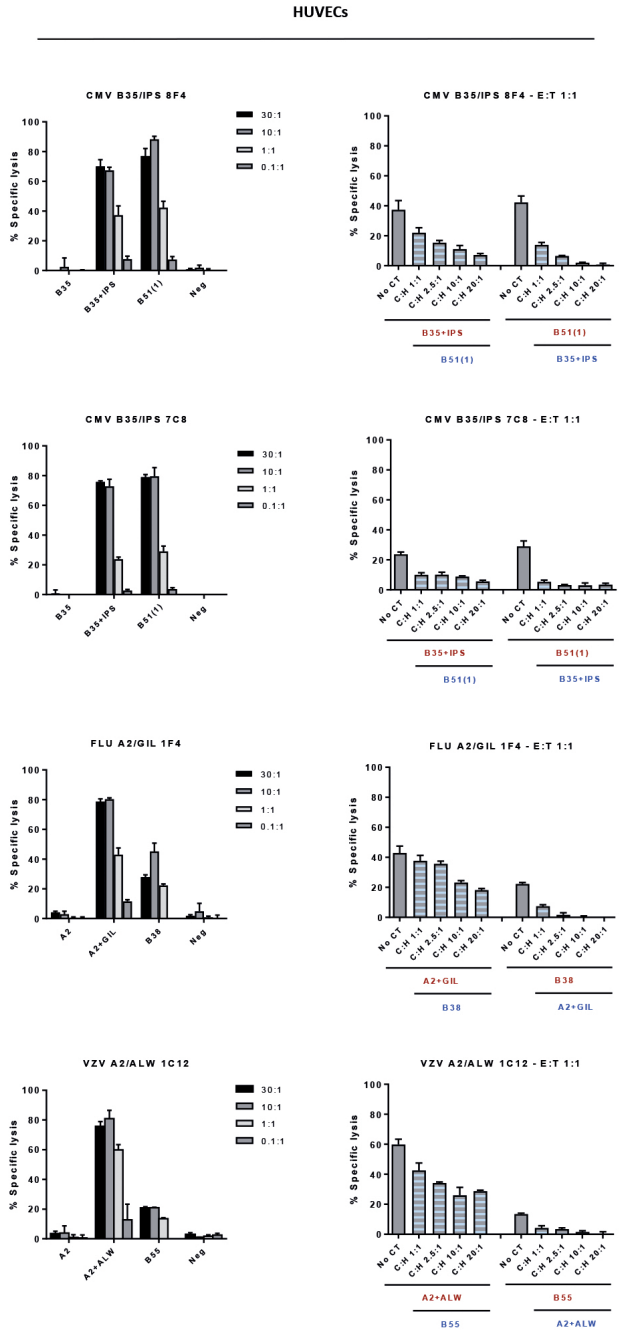


Figure 4. Cold target inhibition assays consistently showed higher TCR avidity for the viral epitope compared to the alloepitope, regardless of viral specificity of the T-cell clones and the target cell type (EBV-LCL or HUVEC). Left panels: CRAs with different E:T ratios (30:1; 10:1; 1:1; 0.1:1). Right panels: CTIs with E:T ratio 1:1 and different C:H ratios (1:1; 2.5:1; 10:1; 20:1). Red: hot targets. Blue: cold targets. Error bars represent the standard deviation (S.D.) of triplicate wells. Peptide concentrations: 1000 ng/ml.

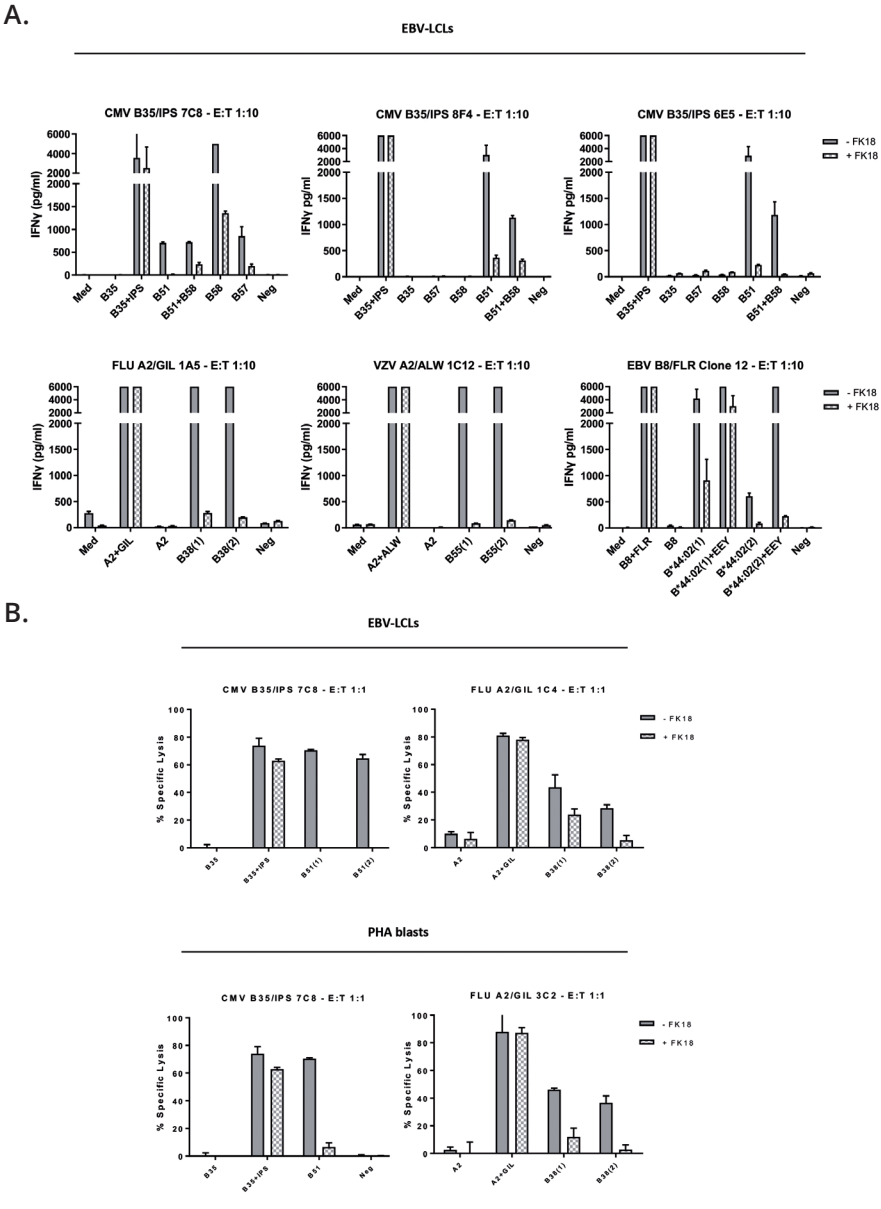
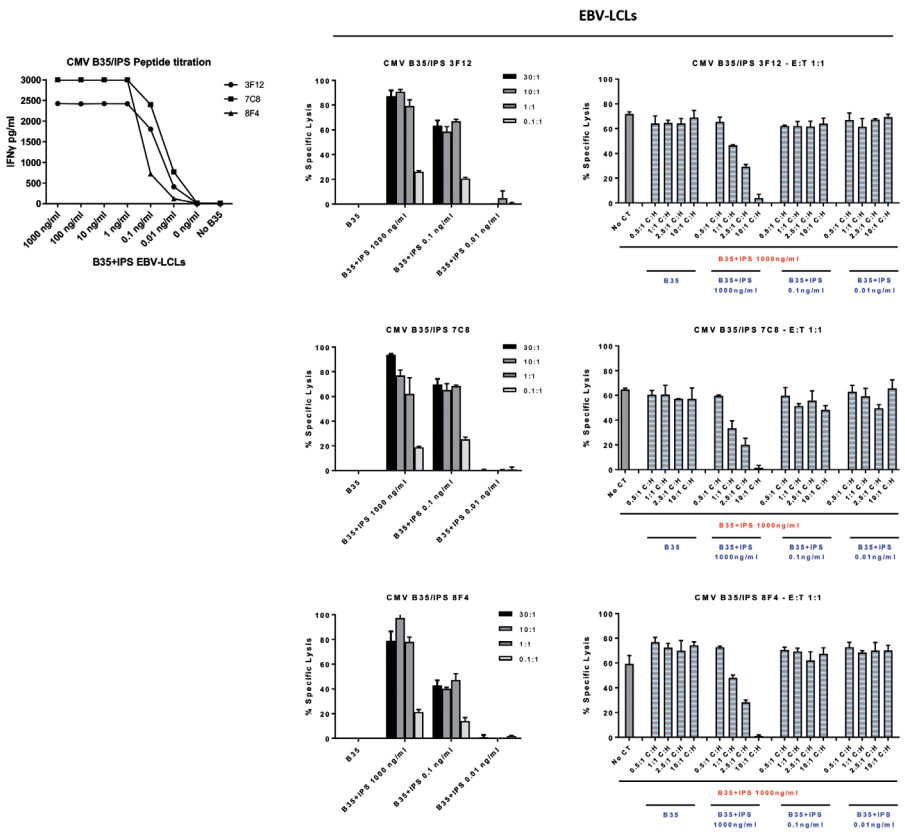


Figure 5. The effect of CD8 co-receptor binding on viral versus alloreactivity. A) CD8 blocking by the CD8-blocking antibody FK18 hampered IFNγ production in response to the alloepitope, but not the viral epitope. The HLA-B*44:02 alloresponse of EBV B8/FLR cross-reactive T cells was hampered by CD8 blocking even with addition of the allopeptide (EEY). B) CD8 blocking by the CD8-blocking antibody FK18 hampers cytotoxicity in response to the alloepitope, but not the viral epitope. Both EBV-LCLs (upper panels) and PHA blasts (lower panels) confirmed the findings. Error bars represent the standard deviation (S.D.) of triplicate wells. Peptide concentrations: 1000 ng/ml.

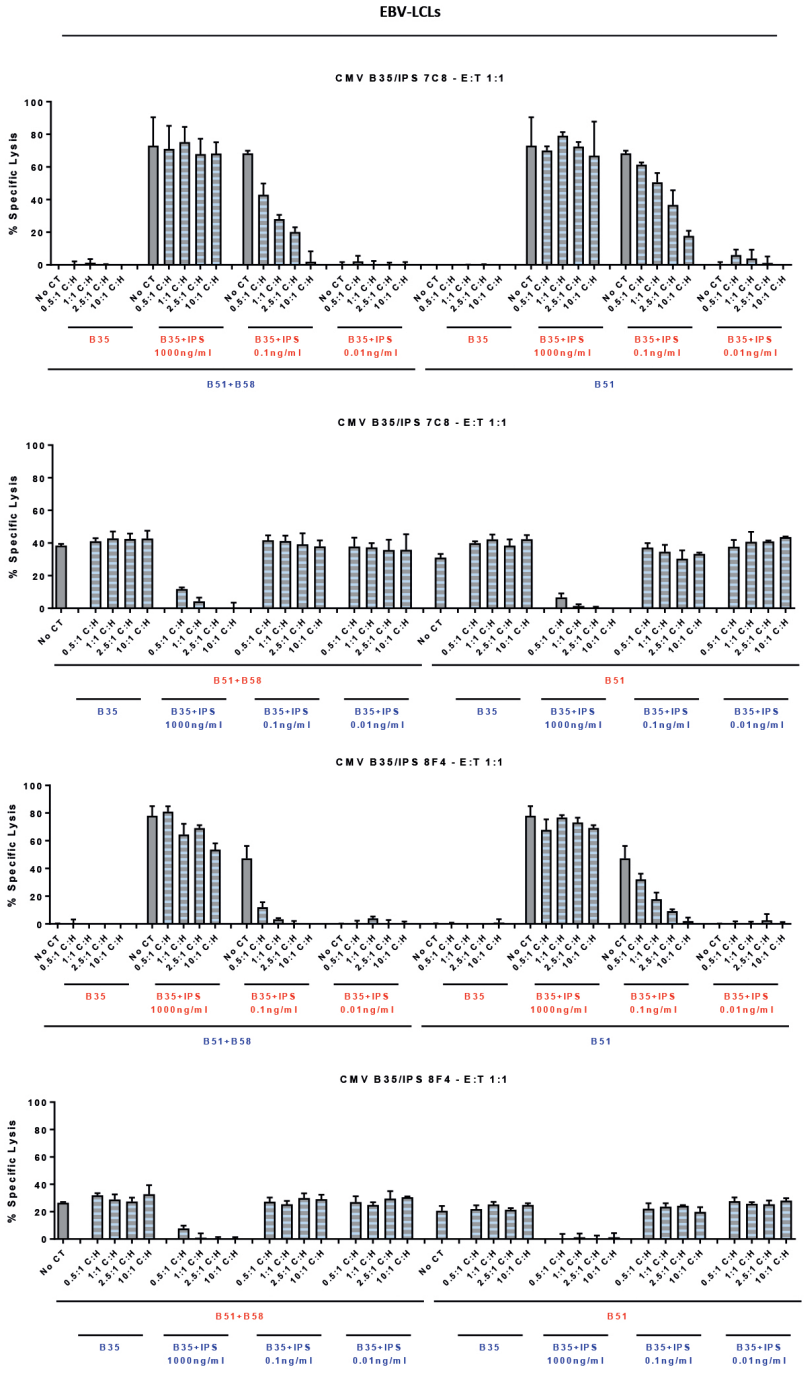
Variation in peptide expression affects TCR avidity for viral and alloepitopes

Considering that TCR avidity is directly related to epitope expression (214), the expression of the specific HLA-peptide complex is a crucial determinant of TCR avidity. As proof of principle, cold target inhibitions were performed using the same target cells expressing syngeneic HLA loaded with different concentrations of viral peptide. Indeed, T-cell reactivity and TCR avidity were positively correlated with viral peptide expression (Figure 6A). Thereupon, cold target inhibition assays with allogeneic targets were conducted using different viral-peptide-loading concentrations. At high levels of viral-peptide loading, TCR avidity was consistently higher for the viral epitope compared to the alloepitope, as observed in Figure 4. Yet, at suboptimal viral-peptide-loading levels TCR avidity appeared to be stronger for the alloepitope (Figure 6B). CD8-blocking experiments with peptide titration confirmed these findings: in contrast to optimal concentrations, suboptimal viral-peptide-loading concentrations required CD8 for IFNγ production and cytotoxicity (Figure 6C).

A.



B.


EBV-LCLs
CMV B35/IPS 7C8 - E:T 1:1
% Specific Lysis
No CT
0.5:1 C:H
1:1 C:H
2.5:1 C:H
10:1 C:H
B35
B35+IPS 1000ng/ml
B35+IPS 0.1ng/ml
B35+IPS 0.01ng/ml
B51+B58
B51
CMV B35/IPS 7C8 - E:T 1:1
CMV B35/IPS 8F4 - E:T 1:1
CMV B35/IPS 8F4 - E:T 1:1

C.

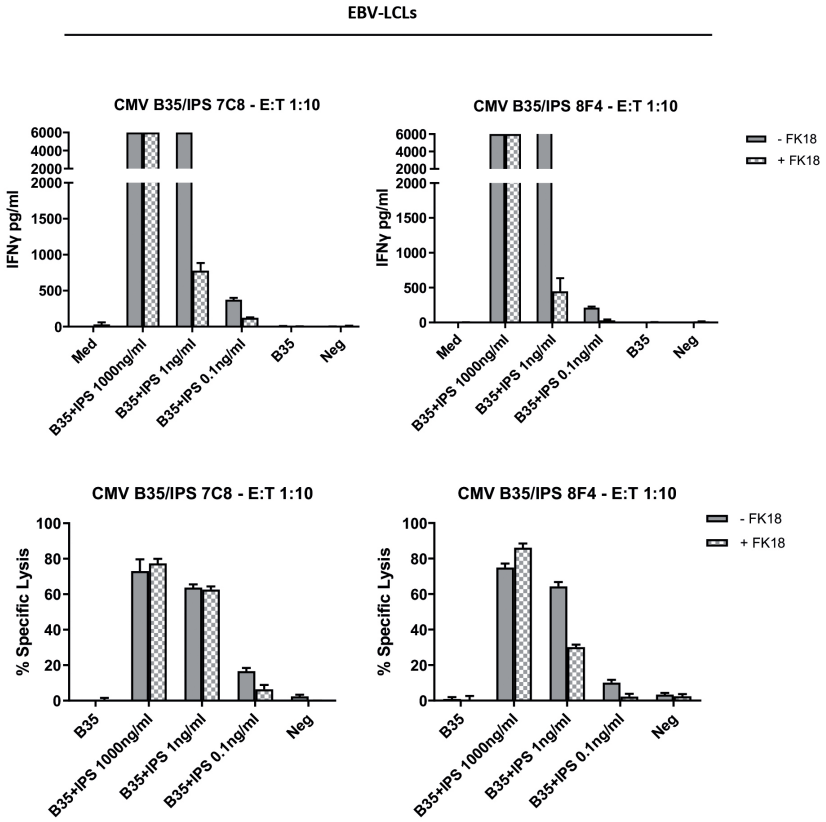


Figure 6. Cold target inhibition depends on epitope expression. A) Optimal and suboptimal viral peptide loading concentrations were determined in IFNγ ELISAs (left panel) and cytotoxicity for optimal and suboptimal viral peptide loading concentrations in chromium-release assays (middle panels). Cold target inhibitions showed that target cells with suboptimal epitope expression were unable to hamper lysis of target cells with optimal epitope expression (right panels). B) TCR avidity is higher for the viral epitope compared to alloepitope, but only at optimal viral epitope expression. C) Suboptimal levels of viral peptide expression require CD8 co-receptor binding for IFNγ production (upper panels) and cytotoxicity (lower panels). Error bars represent the standard deviation (S.D.) of triplicate wells.

DISCUSSION

The aim of the present study was to assess the differential avidity of cross-reactive virus-specific T cells for viral peptides presented by self-HLA molecules versus the cross-reactive allogeneic HLA molecule. The TCR cross-reactivities used in this study represent examples of the vast amount of allo-HLA cross-reactive virus-specific T cells that can be present in the human body (43). We selected for public cross-reactivities, and therefore the results of this study apply to multiple individuals (44, 229).

Cold target inhibition assays showed that at high levels of viral-peptide loading, TCR avidity of all cross-reactive virus-specific T-cell clones was consistently higher for the viral epitope compared to the allogeneic epitope. However, whether the high concentrations of viral peptide used in these in vitro studies are representative for the in vivo situation remains to be established. Interestingly, at suboptimal levels of viral peptide loading, TCR avidity shifted its preference to allogeneic epitopes instead. Indeed, peptide-MHC (pMHC) density is known to be a strong regulator of T-cell activation (214, 230), and whereas high peptide-MHC levels are able to compensate for low-affinity TCRs in the induction of T-cell proliferation (231), T-cell activation does not occur when epitope expression is insufficient (232). Important to keep in mind is that the physiological expression of viral and allopeptides in vivo remains elusive and is difficult to investigate, even when the hurdle of identifying the allopeptide is overcome. Furthermore, viral peptide expression differs during latency and active infection, and TCR avidity could thereby transiently change upon episodes of viral (re)activation. Likewise, depending on the origin of the allopeptide, alloepitope expression may also alter over time with TCR avidity following its lead. EBV- and CMV-peptide expression have been investigated on EBV-LCLs under latent infection for a selected number of HLA molecules, but these did not include the HLA molecules used as targets in the present study (233). However, in our experiments latent viral peptide expression of EBV-LCLs was too low to induce any EBV B8/FLR T-cell responses in vitro, and we were unable to detect cytokine production and/or lysis without the external addition of viral FLR peptide as far as up to 100 ng/ml (data not shown).

In addition, we compared TCR avidity under optimal levels of both viral and allopeptide expression. Hereto, the known human cross-reactive allopeptide EEYLQAFTY (EEY) was used. Target loading with optimal levels of both the allopeptide and the viral peptide continued to show that TCR avidity was higher for the HLA-B*08:01/FLR viral epitope compared to the HLA-B*44:02/EEY alloepitope (Figure 4). These results appear to agree with literature, as tetramer

competition and SPR studies have shown that TCR affinity is 15 times higher for the HLA-B*08:01/FLR viral epitope compared to the HLA-B*44:02/EEY alloepitope (44).

To further substantiate the findings, the avidity of the clones was also assessed by blocking the CD8 co-receptor on the T-cell surface, as CD8 co-receptor binding to MHC is an important determinant of TCR avidity. It enhances antigen sensitivity, is involved in early T-cell activation by stabilizing the TCR-pMHC complex and by mediating phosphorylation of the intracellular domain of the TCR/CD3z complex, and is furthermore involved in the formation of TCR-pMHC microdomain structures at the cell surface (234-236). Accordingly, TCR affinity is negatively correlated to CD8 co-receptor dependency: low TCR affinity requires CD8 co-receptor signaling for T-cell activation whereas high TCR affinity generally does not (237). Interestingly, regulation of CD8 expression has been shown to alter TCR specificity (236) and even to facilitate T-cell cross-reactivity (238). In agreement with the cold target inhibition assays, CD8 blocking did not hamper anti-virus reactivity when viral peptide expression was optimal, but did when it was suboptimal, whereas the alloresponse was consistently hampered by CD8 blocking also under optimal allopeptide (EEY) loading conditions (Figure 5).

The present experiments show that cold target inhibition and CD8-blocking assays are useful tools to estimate allo-HLA TCR avidity of cross-reactive virus-specific T cells for which the allopeptide is unknown. Even when the allopeptide has been identified, TCR avidity assays are a useful addition to TCR affinity assays, as they take into account all non-covalent binding interactions and cluster formations at the cell surface membrane instead of TCR/pMHC interactions alone. While TCR affinity remains static, TCR avidity can change over time: for example, activation-induced membrane changes can enhance TCR avidity upon T-cell activation (239), an event that would go unnoticed in assays that measure TCR affinity alone. Furthermore, the assays employ live cells, which permits a natural state of antigen presentation. This includes spatio-temporal interactions between the TCR and its ligand and natural variation in HLA and (allo)peptide expression under physiological circumstances. For example, epitope expression can differ per cell type as a result of tissue-specificity, which can be addressed by employing different tissue cell types. In addition, epitope expression can be affected by inflammation: under non-inflammatory conditions HLA expression is reasonably constant and epitope expression is primarily determined by the level of (allo)peptide presentation, yet it is enhanced under inflammatory conditions (e.g. during acute rejection), which could boost TCR avidity for the (allo)epitope. By incubating the target cells with cytokines, the impact of inflammation on HLA expression can roughly be simulated, which will be subject of future studies.

Of note, TCR affinity assays that use soluble pMHC and tetramers can underestimate TCR affinity compared to membrane-bound assays (204, 224, 240), indicating that the cell membrane is important even for TCR-pMHC affinity studies. TCR affinity and avidity studies can complement each other and generate insights into TCR-ligand interactions beyond their own boundaries. In addition, the cold target inhibition and CD8-blocking assays allow for in vitro T-cell activation and determination of functional characteristics such as cytokine production and cytotoxicity.

In conclusion, without prior knowledge of the allopeptide we were able to compare the avidity of allo-HLA cross-reactive virus-specific T cells for both their viral and alloepitopes. Our observations show that the relative TCR avidity for these targets is variable depending on epitope expression, and that depending on the circumstances, the avidity of allo-HLA cross-reactive virus-specific T cells may be higher for its alloepitope compared to its viral epitopes - an issue that has been long debated. The clinical consequences of these in vitro observations are still to be established.

CHAPTER

8

SUMMARY & GENERAL DISCUSSION

Viral infections are an important health issue for transplant recipients. In healthy individuals, viral infections can be controlled by virus-specific T cells, but in immunosuppressed transplant recipients the response to viral infection is less efficient. As recently outlined by D'Orsogna et al, infectious pathogens can affect alloreactivity at all levels of the immune system (241). Therefore, common viruses that do not pose a significant health risk to non-immunosuppressed individuals, can become major threats to the health of transplant recipients and can severely affect transplantation outcome (242). Especially cytomegalovirus (CMV), a common virus with a prevalence of around 70% in the general population, is of great concern in kidney transplantation (243-246).

Current immunosuppressive drugs are not specific: they not only hamper anti-donor immune responses but also anti-viral immune responses. Ideally, these drugs should specifically target the immune response to the donor and only to a minor extent the anti-viral immune responses. For this purpose, the differences between these immune responses need to be investigated. One way to make this distinction is by looking at their composition: in theory, the anti-viral immune repertoire largely consists of memory cells that are primed by previous viral infections, whereas the donor-specific immune repertoire consists of naïve cells that are yet to be primed upon transplantation. Consequently, this implies that immunosuppressive drugs directed at the naïve T-cell compartment (such as co-stimulatory inhibitors) prevent the priming of naïve donor-reactive T cells, while they render anti-virus T cells with a memory phenotype unaffected. Thereby the host is protected from anti-donor immunity and life-threatening viral infections.

However, recent clinical trials in which calcineurin inhibitor-based immunosuppression against the memory repertoire was tapered or withdrawn, repeatedly reported impaired tolerance and acute graft rejection (75, 139, 247-249), which may (partly) be explained by heterologous immunity. Some of the virus-specific memory T cells that are so urgently needed for viral control also respond (cross-react) to donor HLA in addition to their cognate viral epitope – a "mistake" that occurs frequently (43). As a consequence, these virus-specific memory T cells can elicit potentially harmful immune responses against the donor allograft. Indeed, studies in mice have shown that cross-reactive virus-specific memory T cells can induce solid organ rejection (35, 250), but the impact of cross-reactive virus-specific memory T cells on allograft survival in humans remains a matter of debate. Clinical studies do not, as yet, show a significant impact on transplantation outcome (38, 40, 41). The number of studies and cross-reactivity models studied are however too limited to draw any generalizable conclusions. Besides, if the cross-reactive T cells are hampered by immunosuppression, they may become a serious issue in the future when alternatives are sought to replace or taper immunosuppression. Therefore,

it remains important to strive towards understanding the mechanisms and clinical impact of virus-specific TCR cross-reactivity. The role of T-cell alloreactivity, and in particular of cross-reactive virus-specific T cells, in transplantation outcome is discussed in **Chapter 2.**

There are several ways in which TCR cross-reactivity of virus-specific T cells can be detected in an experimental setting, and their potencies and flaws are discussed in **Chapter 3** (226). Although these methods do enable accurate detection, it is necessary to keep their limitations in mind. Importantly, one should be aware that current methods based on cross-reactivity detection with viral tetramers rely heavily on known public viral epitopes: and therefore only address the tip of the iceberg regarding the scope of all possible cross-reactivities mediated by virus-specific T cells.

Regardless of this limitation, we were able to show that infection with a single virus can induce many different allo-HLA cross-reactivities **(Chapter 4)**. For example, a large percentage of CMV-specific memory $CD8^+$ T cells are cross-reactive with allogeneic HLA, and we identified a cross-reactivity of CMV A2/NLV-specific T cells specifically recognizing the complex of allo-HLA-A*02:05 presenting a peptide derived from allo-HLA-B*50:01. Interestingly, a recent paper describes how CMV peptides often mimic human peptides and thereby induce alloreactivity (251), and therefore it is not unlikely that HLA-derived peptides could be among those as well.

Furthermore, we found identical allo-HLA cross-reactivity of virus-specific T cells in multiple individuals (public cross-reactivity), as described in **Chapter 6** (140). Partly because dominant TCR usage was considered uncommon, public cross-reactivity was believed to be extremely rare, despite the fact that a public allo-HLA response of EBV-directed T cells had already been documented. Our studies show that public alloresponses are more common than anticipated, which could potentially hold promise for the prediction of alloreactivity in transplant recipients and risk estimation for specific donor-recipient combinations. However, with the exception of the unlikely event of having a surplus of equally suitable donors, we stress that increased knowledge concerning public cross-reactivities should not lead to the discouragement of donor-recipient combinations. In the current climate of organ scarcity, it is not recommended to complicate donor selection or further restrict organ allocation. Rather, the identification of public cross-reactivities could provide a tool to monitor patients at risk for generating memory-like alloreactive T-cell responses, and ultimately to support clinical decision making. Such knowledge may for instance affect the composition of immunosuppressive regimens and the decision whether or not to taper immunosuppression (252). In addition, insights into public cross-reactivity could be used to selectively tailor the composition of pre-transplant vaccines

and adoptive anti-viral T-cell grafts (253). Yet, there is still a long way to go to determine whether public cross-reactivities could have clinical implications.

First of all, the functional characteristics of the cross-reactive virus-specific T cells should be thoroughly analysed. The newly identified public cross-reactive T cells were able to produce cytokines, show cytotoxicity towards alloantigen-expressing target cells derived from different tissues, and reach similar cytokine and cytotoxicity levels in response to alloantigen as to viral antigen in vitro. If they retain these capabilities in vivo, these T cells could contribute to the generation of an inflammatory environment in or around the allograft or even directly kill donor cells.

Important to keep in mind is that a functional alloreactive T-cell response depends on T-cell activation, which is determined by the interaction of the TCR with the alloepitope. Indeed, TCR affinity and avidity for the alloepitope play a crucial role in determining whether a T cell becomes activated upon allorecognition (214, 231). In **Chapter 7**, we have shown that TCR avidity depends on the expression of the (viral and allo-) peptide (144). In other words: the onset of alloreactivity (and whether it is persistent or transient) could depend on alloepitope expression. Defining parameters that affect this expression could provide novel insights into if and when potentially harmful alloreactive responses may emerge in a transplantation setting. For example, if the cross-reactive allopeptide is involved in specific cell processes (for instance damage repair or cell division) cross-reactive alloresponses may be transient, and if its expression is affected by genetic polymorphisms the immunogenicity of allografts could vary between donors. Preliminary experiments in which public cross-reactive CMV B35/IPS T-cell clones were stimulated by cells of first-degree related individuals indeed suggest a genetically determined stimulation capacity. Additional experiments are needed to make conclusive statements (FIGURE 1, unpublished data).

Moreover, we showed that, depending on epitope expression, TCR avidity for the alloepitope could surpass TCR avidity for the viral epitope - indicating that the alloresponse could have similar immune potential as the anti-virus response.

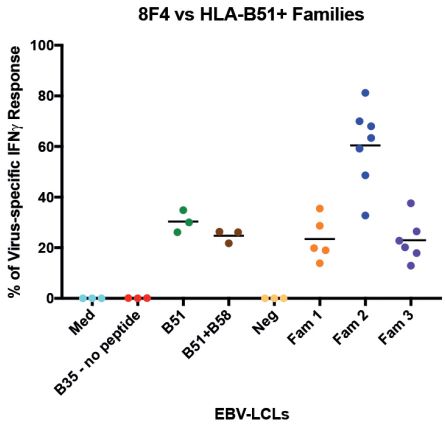


Figure 1. Family members may share immunogenicity. CMV B35/IPS T-cell clone 8F4, cross-reactive against allo-HLA-B*51:01, showed increased IFNγ production in ELISA towards HLA-B*51:01+ members of Family 2 (n = 7) compared to HLA-B*51:01+ members of Family 1 (n = 5) and Family 3 (n = 6). Depicted is the percentage of the virus-specific response (EBV-LCL B35 + IPS peptide) and experiments were performed in triplicate.

Two major determinants of TCR avidity are the interaction between the TCR and pMHC molecule (TCR affinity) and CD8 co-receptor binding to the side of the MHC molecule. TCR affinity is directly correlated with T-cell activation, however, most T-cell responses are mediated by T cells expressing intermediate TCR affinity to prevent overstimulation leading to rapid T-cell senescence and that could potentially pose a threat to the host (214). Unfortunately, TCR affinity for an alloepitope can only be accurately determined when the allopeptide is known, using methods as surface plasmon resonance (SPR) (223). When TCR affinity is sufficiently strong, CD8 co-receptor binding is not needed to elicit an immune response (237), but when TCR affinity is low, CD8 co-receptor binding is needed to strengthen the TCR-pMHC interaction. It thereby compensates for the low TCR affinity to ensure full T-cell activation (234, 254). Interestingly, CD8 co-receptor binding can also play an important role in allorecognition, and differences in CD8 expression on a cross-reactive T cell have been shown to alter its fine-specificity (255). Of note, such shaping would only occur for alloreactive T cells that are CD8 dependent. Although alloreactive T cells are generally considered to be CD8 dependent, recently alloreactive HCV-specific T cells have been documented that do not require CD8 binding for allorecognition (256).

CD8 dependency varies between HLA class I molecules, depending on mutations in the a3 domain (primarily due to a negatively charged loop at residues 223-229) (257). Decreased CD8 binding capacity can hamper the recognition of certain allo-HLA antigens and shape

the fine specificity of the allorepertoire. All self-HLA molecules (HLA-B*35:01; HLA-A*02:01) and cross-reactive allo-HLA molecules (HLA-B*51:01, HLA-B*58:01, HLA-B*57:01; HLA-B*38:01; HLA-B*55:01) tested in this thesis were identical in amino acid composition in their CD8 binding region of the a3 domain (DQTQDTE), indicating that the binding potential of the CD8 co-receptor was comparable between the different HLA molecules. We evaluated CD8 co-receptor dependency in functional assays by CD8 blocking and observed that CD8 co-receptor dependency was generally stronger for allorecognition compared to viral recognition **(Chapter 7)**. Although one should keep in mind that other interactions at the cell surface, such as those mediated by adhesive molecules, contribute to TCR avidity as well, a potential explanation for the difference in CD8 co-receptor dependency may be lower TCR affinity for the alloepitope versus the viral epitope (especially since the CD8 binding potential was similar for all HLA molecules). For example, the FLU A2/GIL TCR is known to recognize its cognate antigen in an CD8-independent manner, plausibly due to its specific pMHC docking mode that results in sufficient TCR affinity that diminishes the need for CD8 co-receptor signaling (9, 258). Our TCR sequencing data suggest that the CDR3a region, in contrast to cognate docking, is vital for HLA-B*38:01 allorecognition, hence a potential explanation for the observed CD8 dependency for allorecognition could be an alternative TCR docking mode. It is however impossible to make any conclusive statements without generating crystal structures of this interaction, which was beyond the scope of this thesis.

Interestingly, TCR affinity of the EBV B8/FLR cross-reactivity model is lower for the alloepitope versus the viral epitope (44). However, it remains to be investigated whether this can be generalized for all virus-specific T cell cross-reactivities. Perhaps more importantly, one should keep in mind that partial T-cell activation as a result of low affinity T cell - antigen interaction could be detrimental as well (259). Interestingly, a recent study in mice shows that low-affinity priming with pathogen antigen induces a different differentiation program in cross-reactive T cells compared to high-affinity priming; yet these low-affinity primed T cells are equally potent in inducing graft rejection upon exposure to high-affinity alloantigen (260). This holds promise for the priming of HIV-cross-reactive allospecific T cells as suggested by Almeida et al (261): in **Chapter 5**, we showed that virus-specific T cells can indeed be induced by allostimulation (143), and if priming is independent of TCR affinity, this would suggest that TCR affinity for allo-HLA is no limiting factor for generating a potent HIV-reactive T-cell repertoire.

Finally, in the quest to determine the clinical relevance of (public) cross-reactivities, the most important element that is missing so far is the identification of the peptide presented by the cross-reactive HLA allo-antigen. If the origin of the peptide is known, this can provide

information on tissue-specificity, as well as on the (patho)physiological expression of the alloepitope. In addition, TCR affinity can be assessed using SPR and functional assays can be optimized: for example, knowing the allopeptide would allow for TCR avidity determination using the same levels of viral and alloepitope expression. Furthermore, the allopeptide can unravel the structural mechanisms behind the TCR cross-reactivities using crystallography. With regard to monitoring, tetramers of the allo-HLA and allopeptide could be used to determine the frequency of T cells recognizing this complex in the blood of patients, pre-transplant and at follow-up. When alloreactivity mediated by these cross-reactivities plays an important role in graft rejection, knowing the allopeptide may ultimately even hold therapeutic potential with regard to the administration of altered peptide ligands (205). Unfortunately, the identification of the allopeptide is a long and complicated process with no universal experimental strategy. The only allopeptide that has been described so far is the EEY allopeptide, and its identification took several years. It was finally indirectly identified using the baculovirus vector system in insect cells that happened to yield a peptide mimic in the human genome (44, 262).

To benefit other cross-reactivity models as well, a more generalizable approach to identify allopeptides is therefore needed. A promising strategy for allopeptide identification combines functional T-cell assays with chromatography, mass spectrometry, biochemistry and crystallography, and is currently under development in the groups of Prof. Anthony Purcell and Prof. Jamie Rossjohn at Monash University (Melbourne, Australia) by Dr. Nicole Mifsud and Dr. Stephanie Gras, respectively. Using peptide elutions of the allogeneic target cells and HPLC fractionation of the resulting peptidome, functional screening of those HPLC fractions can reveal one (or more) positive fractions containing the allopeptide(s). Having joined their efforts for a year, we gathered preliminary results showing that a positive fraction for the CMV B35/IPS TCR cross-reactivity against HLA-B*51:01 can indeed be identified in CD69 assays (FIGURE 2, unpublished data).

Subsequently, the peptides present in the positive HPLC fraction are identified by mass spectrometry. A single HPLC fraction still contains hundreds to thousands of peptides - but comparing the positive and negative HPLC fractions can already rule out peptides that are unlikely to induce cross-reactivity. Furthermore, attempts are made to re-fractionate the positive fraction into smaller fractions, despite the requirement of extensive numbers of allogeneic cells for elution (>1 x 10^{10}). Another promising path aims to identify a peptide motif that the cross-reactive TCR favors for recognition, for comparison with the peptides in the positive HPLC fraction.

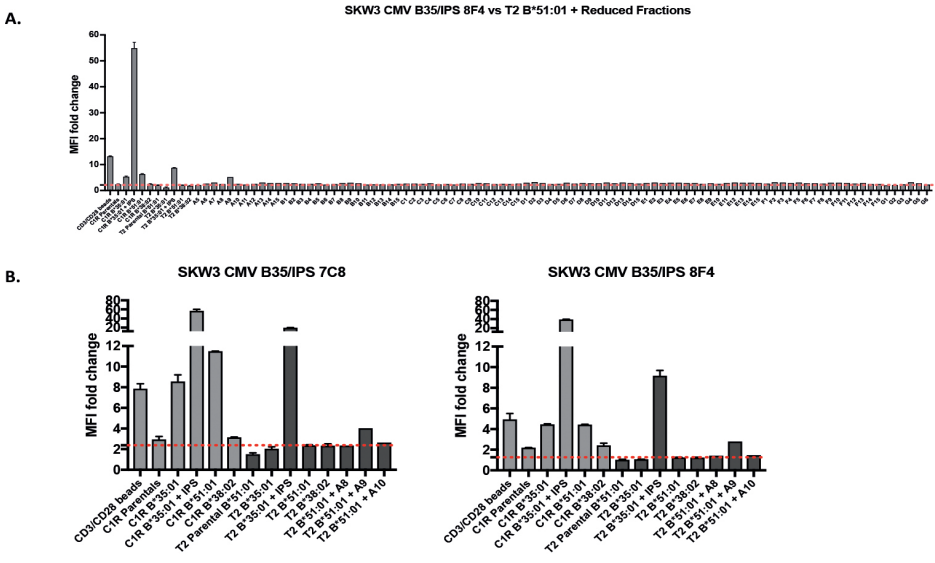


Figure 2. HPLC fractionation can be used to identify fractions containing allopeptides. A) A positive HPLC fraction (A9) was identified in CD69 assay for the CMV B35/IPS cross-reactivity model against HLA-B*51:01. B) Verification in additional CD69 assays showed both cross-reactive TCRs (7C8 and 8F4) were able to recognize peptides in the A9 faction. X-axis: Responder cells: retrovirally transduced SKW3s expressing the cross-reactive CMV B35/IPS TCRs; Stimulator cells: C1Rs and T2s retrovirally transduced with allo-HLA-B*51:01, of which three conditions HLA-B*51:01+ T2s loaded with HPLC fractions. Y-axis: MFI fold change of CD69 expression compared to media alone values.

The peptide motif can be achieved in an unbiased way using a combinatorial peptide ligand library (CPL), which covers the entire peptide spectrum divided into subgroups based on a single shared amino acid at a fixed position. Screening those subgroups in functional assays (the MIP-1b ELISA proves to be most sensitive) reveals an amino acid pattern, i.e. motif, that the TCR is most responsive to (238, 263, 264). Using this knowledge, peptides that are unlikely to cross-react based on the peptide motif can be discarded from the positive HPLC fraction. However, a downside of CPL is the requirement of cross-reactive T-cell clones, which are not always available, and it has only ever been used to identify cognate peptides rather than allopeptides. Currently, experiments are performed at Monash University to evaluate whether the above-mentioned approaches are suitable to identify allopeptides for different cross-reactivity models, including the public CMV B35/IPS and FLU A2/GIL cross-reactivity models described in this thesis. If successful, this would yield a generalizable strategy that can be implemented for the allopeptide identification of all (public and private) virus-specific TCR cross-reactivities, and their clinical relevance for transplantation can finally be resolved.

CHAPTER

9

Nederlandse samenvatting

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Curriculum vitae

Publicaties

Abbreviations

Dankwoord

Nederlandse samenvatting

Virusinfecties vormen een groot risico voor transplantatiepatiënten. Met name het cytomegalovirus (CMV) heeft een significante impact op transplantaatoverleving. Wat minder mensen weten, is dat de immuunrespons *tegen* dit soort virussen ook nadelig kan zijn voor de patiënt. Dit komt doordat de afweercellen die getraind zijn om een virus te herkennen, een "vergissing" kunnen maken en het donororgaan aan kunnen vallen. Er is echter nog veel onduidelijk over de rol van deze cellen in transplantaatafstoting. Kunnen deze cellen ook daadwerkelijk een sterke immuunrespons tegen een donororgaan opwekken? Welke factoren spelen hierbij een rol? En kunnen we deze immuunresponsen voorspellen? In dit proefschrift probeer ik antwoord te geven op deze vragen.

Virus-specifieke T cellen

Het immuunsysteem bestaat uit vele verschillende soorten afweercellen, die elk hun eigen kenmerkende eigenschappen hebben en hun eigen rol vervullen. In dit proefschrift is specifiek gekeken naar T cellen die gespecialiseerd zijn in de afweer tegen virussen ("virus-specifieke" T cellen) en tevens direct een virus-geïnfecteerde cel kunnen aanvallen en doden.

T cellen herkennen specifieke eiwitcomplexen op het celoppervlak, namelijk de combinatie van een humaan leukocytenantigeen (HLA) en het peptide dat daarin wordt gepresenteerd. Deze combinatie wordt ook wel het peptide-HLA (pHLA) complex genoemd. Het peptide dat in het HLA gepresenteerd wordt is een stukje eiwit afkomstig van binnenin de cel en reflecteert als het ware wat zich daar afspeelt. Als dit peptide afwijkt van de norm, zoals tijdens een virusinfectie of de ontwikkeling van een maligniteit, kan een T cel dit herkennen en wordt hij geactiveerd om een afweerreactie op gang te zetten. Nadat de afwijkende cellen zijn opgeruimd, blijven in het lichaam "geheugen" T cellen achter die, mocht het ongewenste materiaal zich nogmaals aandienen, extra snel en krachtig een aanval kunnen inzetten zodat de gastheer niet (opnieuw) ziek wordt.

Heterologe immuniteit

Een T cel herkent een pHLA complex door middel van T-cel receptoren op zijn celoppervlak, die zeer specifiek alleen bepaalde peptide-HLA combinaties herkennen. Dit wordt ook wel "T-cel specificiteit" genoemd. Wie een oud leerboek openslaat, zal daarin lezen dat een T-cel receptor slechts één specificiteit heeft. Vandaag de dag weten we echter dat dit gegeven achterhaald is. Een T-cel receptor kan wel degelijk meerdere specificiteiten hebben - hetgeen zelfs een groot voordeel heeft, omdat zulke "multispecificiteit" gepaard gaat met een gunstig effect op

de overleving van de soort. Immers, als een afweerreactie tegen virus X tevens bescherming biedt tegen virus Y, dan geeft dat de gastheer een evolutionair voordeel als hij later in aanraking komt met virus Y. Dit fenomeen wordt ook wel "heterologe immuniteit" genoemd. Bij heterologe immuniteit tussen virussen herkent een T-cel receptor dus verschillende pHLA complexen; waarin lichaamseigen HLA moleculen verschillende peptides presenteren afkomstig van verschillende virussen. Het herkennen van verschillende pHLA complexen door dezelfde T-cel receptor wordt ook wel "kruisreactiviteit" genoemd.

Heterologe immuniteit in transplantatie

Interessant genoeg komt heterologe immuniteit niet alleen voor bij antivirale immuunresponsen, maar ook bij immuunresponsen tegen vreemd ("allogeen") HLA - bijvoorbeeld met HLA van een donor in een transplantatiesetting ("alloreactiviteit"). In dat geval is het mogelijk dat dezelfde T-cel receptor niet alleen een pHLA complex met een ander peptide herkent, maar tevens met een ander HLA antigeen. Een T cel die aanvankelijk getraind is om een virus te bestrijden kan zodoende een "vergissing" maken en een donorcel aanvallen. Doordat zulke virus-specifieke T cellen een geheugenfenotype hebben, zou zo'n vergissing dus relatief snelle en sterke anti-donorreactiviteit tot gevolg kunnen hebben.

Dit proefschrift

Zoals gezegd is er nog veel onduidelijk over de rol van virus-specifieke T cellen die kruisreageren met allogeen HLA in transplantaatafstoting. In dit proefschrift probeer ik meer inzicht te genereren in de rol van deze cellen in orgaantransplantatie.

In **Hoofdstuk 1**, de algemene inleiding, wordt achtergrondinformatie gegeven over de geschiedenis van de orgaantransplantatie en tevens worden de basisprincipes van het immuunsysteem geïntroduceerd.

Hoofdstuk 2 schetst een overzicht van studies die het effect van virus-specifieke T cellen die kruisreageren met allogeen HLA in een transplantatiesetting onderzocht hebben. Waar in muizen een duidelijk verband kan worden gelegd tussen virus-specifieke T cellen en transplantaatafstoting, is dit bij mensen tot op heden nog niet zo eenduidig. Ook blijkt het aantal klinische studies dat tot op heden uitgevoerd is, erg beperkt te zijn. Er dient zodoende meer kennis vergaard te worden over heterologe immuniteit van virus-specifieke cellen, zodat een inschatting gemaakt kan worden of dit fenomeen een risico vormt voor transplantatiepatiënten.

Dit blijkt echter makkelijker gezegd dan gedaan. De huidige experimentele technieken waarmee virus-specifieke T cellen die kruisreageren met allogeen HLA gedetecteerd kunnen worden,

gaan alle gepaard met zowel voor- als nadelen. In **Hoofdstuk 3** worden hun mogelijkheden en valkuilen besproken. Het is belangrijk te realiseren dat de meeste methoden lang niet alle kruisreacties kunnen detecteren, waardoor vele zo onder de radar blijven. Daarnaast kunnen vals-positieve resultaten optreden doordat omstander T cellen, die niet zelf met allogeen HLA kunnen kruisreageren, geactiveerd raken door het inflammatoire milieu dat gecreëerd wordt door toedoen van "echte" kruisreagerende T cellen. Door een kleine aanpassing door te voeren in een bestaande experimentele techniek, bleek de detectie van echte kruisreactiviteit tegen allogeen HLA sterk verbeterd te kunnen worden.

In **Hoofdstuk 4** waren we in staat om aan te tonen dat een infectie met een enkel virus een breed assortiment aan kruisreactieve T cellen kan doen ontstaan die zowel virus als allogeen herkennen. Op basis van proliferatie-experimenten, waarin virus-specifieke T cellen werden blootgesteld aan een verzameling van donorcellen met de meest voorkomende HLA antigenen, bleken T cellen die hetzelfde virus herkennen kruisreactief te zijn tegen verscheidene allogene HLA antigenen. Zo kan een infectie met een enkel virus ervoor zorgen dat diens gastheer een afweerreactie ontwikkelt tegen meerdere donor HLA antigenen, zonder eerder aan die antigenen te zijn blootgesteld.

In tegenstelling tot het mogelijk nadelige effect van kruisreactiviteit in een transplantatie-setting, hebben we in **Hoofdstuk 5** aangetoond dat heterologe immuniteit ook positieve effecten kan hebben. Door allogene (donor)cellen juist in te zetten om T cellen te stimuleren, kunnen goed functionerende antivirale reacties opgewekt worden. Door deze "omweg" te nemen zou antivirale immuniteit bevorderd kunnen worden in immuun-gecompromitteerde patiënten (denk bijvoorbeeld aan HIV patiënten).

Daarnaast hebben we in **Hoofdstuk 6** gevonden dat meerdere ongerelateerde personen identieke kruisreacties tegen allogeen HLA kunnen ontwikkelen, zogenaamde "publieke" kruisreactiviteit. Dat personen met diverse HLA achtergronden precies dezelfde T-cel receptoren tot expressie kunnen brengen is al een klein wonder – wat verklaart waarom men voor lange tijd in de veronderstelling was dat publieke kruisreactiviteit extreem zeldzaam is. Echter, het feit dat eerder al een publieke kruisreactiviteit tegen allogeen HLA was gedocumenteerd van T cellen die het veel voorkomende Epstein-barr virus (EBV) herkennen, toont aan dat publieke kruisreactiviteit wellicht toch niet zo zeldzaam is. In dit proefschrift hebben we maar liefst drie additionele publieke kruisreacties kunnen identificeren, namelijk van cytomegalovirus-specifieke T cellen (CMV B35/IPS tegen allogeen HLA-B57/58 en HLA-B51), influenza-specifieke T cellen (FLU A2/GIL tegen allogeen HLA-B38) en varizella zoster virus-specifieke T cellen (VZV

A2/ALW tegen allogeen HLA-B55). Deze bevinding toont aan dat publieke kruisreactiviteit veel vaker voorkomt dan aanvankelijk voor mogelijk werd gehouden. Indien de klinische relevantie van publieke kruisreacties zorgvuldig bestudeerd wordt, kan deze kennis ingezet worden om alloreactiviteit te voorspellen en een risicoanalyse te maken voor transplantaatafstoting bij individuele donor-ontvanger combinaties.

Niet alle kruisreactieve T cellen zijn in staat om een volwaardige afweerreactie tegen allogene (donor)cellen te verwezenlijken. Een T cel wordt namelijk pas geactiveerd als de interactie tussen de T cel en zijn doelwit-cel sterk genoeg is ("T-cel aviditeit"). In **Hoofdstuk 7** hebben we aangetoond dat alloreactiviteit afhankelijk is van de expressie van het allogene pHLA complex (het "allo-epitoop") op het celoppervlak - en dat de T-cel aviditeit zelfs hoger uit kan vallen voor het allo-epitoop dan voor het het virale epitoop (het lichaamseigen HLA dat een virus peptide presenteert). Dit laatste suggereert dat een alloreactie een vergelijkbaar immuunpotentieel kan hebben als een antivirale reactie. Daarnaast vonden we dat de alloreacties die we in dit proefschrift bekeken hebben afhankelijk waren van hulp van een extra receptor op het oppervlak van de T cel (de "CD8 co-receptor"), die aan de zijkant van het HLA molecuul bindt en zo de interactie tussen de T-cel receptor en het allogene pHLA complex versterkt. De reactie van dezelfde kruisreactieve T cellen voor hun virale pHLA complex was niet afhankelijk van deze hulp. Of dit ook gegeneraliseerd kan worden voor alle kruisreactieve virus-specifieke T cellen moet nog worden onderzocht.

Tenslotte worden de bevindingen van dit proefschrift in **Hoofdstuk 8** samengevat en in een breder perspectief geplaatst. Met name de queeste om het allogene peptide te vinden dat bij een kruisreactie betrokken is (het "allopeptide") komt uitgebreid aan bod, aangezien kennis over de aard van dit peptide onmisbaar is om de klinische relevantie van (al dan niet publieke) kruisreactiviteit te bepalen. Tot op heden bestaat er geen universele werkwijze voor de identificatie van deze allopeptides. Echter, een veelbelovende nieuwe strategie is momenteel in ontwikkeling aan de Monash Universiteit in Melbourne, Australië, welke functionele T-cel assays met chromatografie en massaspectrometrie combineert. Als deze aanpak zijn belofte waarmaakt, ontstaan vele nieuwe mogelijkheden voor vervolgonderzoek en zouden we eindelijk de klinische relevantie van kruisreagerende virus-specifieke T cellen die donor-HLA herkennen in orgaantransplantatie kunnen bepalen.

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Abbreviations

ABCD3	ATP-binding cassette sub-family D member 3
Allo-HLA	allogeneic HLA
ATG	anti-thymocyte globulin
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CRA	^{51}Chromium-release assay
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CU	Cetus unit
DLI	donor lymphocyte infusion
EBV	Epstein-Barr virus
EBV LCL	EBV-transformed lymphoblastoid cell line
EFI	European Federation of Immunogenetics
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLU	influenza virus
FRET	Förster resonance energy transfer
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
GVM	graft-versus-myeloma
HLA	human leukocyte antigen
HS	human serum
HSCT	human stem cell transplantation
HUVEC	human umbilical vein endothelial cell
IFNγ	interferon γ
IL-7	interleukin 7
IMDM	Iscove's Modified Dulbecco's Medium
ITAM	immunoreceptor tyrosine-based activation motif
mAb	monoclonal antibody
ME	β-mercaptoethanol
mHAg	minor histocompatibility antigen
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
mTOR	mammalian target of rapamycin
NO	nitric oxygen
PBMC	peripheral blood mononuclear cell

PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohaemagglutinin
PLT	primed-lymphocyte test
pMHC	peptide-MHC
RPMI	Roswell Park Memorial Institute medium
SOT	solid organ transplantation
SPR	surface plasmon resonance
SSO	sequence-specific oligonucleotide
SSP	sequence-specific primer
TCR	T-cell receptor
TEC	tubular epithelial cell
Th1	type 1 helper T cell
Treg	regulatory T cell
VZV	varicella zoster virus

Dankwoord

Geen proefschrift kan tot stand komen zonder de hulp van anderen – en zo ook deze niet. Ik ben iedereen die heeft bijgedragen aan dit proefschrift dan ook enorm dankbaar. In het specifiek wil ik bedanken:

Frans, wat een geweldige begeleider ben je geweest en wat heb je een geduld gehad. Dank je wel voor al je goede adviezen en sturing in de juiste richting. Je “lab-embargo” was zonder twijfel de juiste zet.

Ellen, wat had ik zonder jou gemoeten? De experimenten leken soms wel eindeloos, het kweekwerk stapelde zich maar op (is er misschien nóg een incubator vrij?) maar samen konden we het aan. Dank je wel voor al je goede adviezen, je steun en gezelligheid en dat je mijn paranimf wilde zijn.

Gezellig was het ook in de “bieb”: dames, ik vond het een eer dat ik deel van de groep uit mocht maken en ontzettend bedankt voor alle goede tips en adviezen. Paula, je zit inmiddels alweer heel wat jaartjes in Australië, maar ik zal je hulp nooit vergeten. En het was geweldig om te zien hoe je je leven hebt opgebouwd in Perth. Marry, je was een geweldige buurvrouw en ik had geen betere kunnen wensen. Els, dank je wel voor al je lieve zorgen; dat glazen oog is me mooi bespaard gebleven door jou. Yvonne, Yvonne Z, Manon, Juliette, Ouassima, Hanneke: jullie waren topcollega’s, keep up the good work! Jacqy, dank je wel voor je hulp bij het sequencen van al die (vele…) T-cel klonen.

Lieve mede-PhD’ers, jullie gezelligheid en mentale steun zullen me altijd bijblijven. Gonca, je was een geweldige side-kick en ik mis onze LUMC-PhD-frietjes nog steeds - om maar niet te spreken over alle overheerlijke zelfgemaakte Turkse lekkernijen. Yang, I know you did not have the time to catch up with your Dutch, so I’ll write in English: I think it is truly inspiring how hard you worked to come to where you are now. And thank you so much for bringing Sissi to the lab! Sissi, you were a fantastic help, even though I know that you will not admit it yourself. Now it is in writing. I wish you guys and Zhe all the best in China. Cynthia en Moniek, wat fijn dat we op het laatst eindelijk een echte “PhD-kamer” konden delen - Cynthia, als je nog eens om een reviewer verlegen zit dan sta ik paraat. Vincent, de wijntjes op je balkon smaakten altijd extra goed na een lange werkweek. Anita, ook al deelden we zelden dezelfde geografische coördinaten, heb ik genoten van het sparren over ons onderzoek. En uiteraard dank aan alle andere PhD studenten; Els, Anna-Sophia, Frederieke, Li Na, Gijs, Jan-Willem, Tetje, Niels, Angela, Lisa, Tess… en uiteraard alle anderen.

Michael, Sebas, dank jullie wel voor jullie creatieve wetenschappelijke input – en natuurlijk voor jullie leuke gezelschap bij de vele congressen. Dave, ik heb een hoop van je geleerd over heterologe immuniteit en je kennis heeft me enorm geholpen. Geert, dank je wel voor je altijd waterdichte statistiek! Arend, dank voor je adviezen en je triviakennis ten tijde van de Claas Quiz. Ilias, dank je wel voor de motiverende werkbesprekingen aan het begin van het promotietraject. Moshe, you are a true inspiration, not just as a scientist but also as a human being. I hope to see you once again in Tel Aviv. Iedereen van het typ- en screeningslab: de gezelligheid bij de koffie was elke dag een klein feestje. Anouk, dank je wel voor het oplossen van alle secretariële vraagstukken en de vrolijke manier waarop je dat deed.

Daarnaast wil ik iedereen van het Allovir consortium en in het speciaal Ineke en Kirstin, bedanken voor alle waardevolle input en het intensieve meedenken. Cees en Jurjen, dank jullie wel voor jullie expertise en vernieuwende ideeën. We hebben mooi onderzoek gedaan en de Allovir meetings waren altijd goed voor nieuwe inspiratie!

Naast het werk wil ik ook mijn lieve vriend(innet)jes bedanken. Meiden van de Ruysdael, lieve Aaf, Ling, Miki, Yannouck, Krista: jullie gezelligheid, positiviteit en waardevolle adviezen hebben me er steevast doorheen geholpen. Dank jullie wel voor de mooie tijd! Joke, Frouke, Ilse, Odette, José: we zijn samen de reis aangegaan van onbevangen eerstejaarsstudenten naar echte “Grote Mensen” en ik had me geen betere reisgenoten voor kunnen stellen. Steef, Su, Pau, onze vriendschap gaat door landsgrenzen heen en zal dat altijd blijven doen - op naar de volgende 28 jaar! Nina, Joep, Ambi en Renske, hoewel onze jubileum-etentjes soms wat (jaartjes) later waren dan gepland, waren ze altijd memorabel.

Pap en mam, er zijn natuurlijk geen woorden voor hoeveel ik jullie zou willen bedanken. Jullie zijn er altijd onvoorwaardelijk en dat is onbetaalbaar. Lieve Mariëtte, bedankt voor je steun aan je kleine zus.

Lieve Cyril, wat is de wereld leuk met jou. Dank je wel voor je nimmer aflatende steun en je geloof in mij.