On T lymphocyte alloreactivity

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Zusammenfassung

Alloreaktive αβ-T-Zellen erkennen fremde MHC Moleküle, wobei die Erkennung durch Determinanten des MHC-Moleküls und/oder des Peptides vermittelt wird. Ein Teil der peptidabhängigen alloreaktiven T-Zellen, die allorestringierten T-Zellen, ist spezifisch für ein bestimmtes Peptid, das durch das allo-MHC-Molekül präsentiert wird. Diese allorestringierten T-Zellen stellen ein Reservoir an peptidspezifischen T-Zellen dar, die für einen adoptiven Immuntransfer eingesetzt werden können. HLA-A2-allorestringierte CTL Klone von hoher Avidität, die spezifisch für Peptidbibliotheken sind, können aus HLA-A2-negativen Spendern generiert werden. Hier wurde diese Technik dazu benutzt, den Einfluss engverwandter selbst-HLA-Moleküle auf das Repertoire an alloreaktiven T-Zellen zu untersuchen. PBLs von HLA-A*0205-positiven Spendern wurden stimuliert mit HLA-A*0201-positiven Zielzellen, die mit einer Bibliothek von HLA-A*0201-Peptiden beladen worden waren. Wir beobachteten keinen erhöhten Anteil an peptidspezifischen CTL im alloreaktiven Repertoire von HLA-A*0205-positiven Spendern verglichen mit HLA-A2-negativen Spendern. Ein Vergleich der alloreaktiven T-Zellantwort zweier Spender mit ähnlichem HLA-Haplotyp zeigte, daß das allorestringierte T-Zellrepertoire starke individuelle Unterschiede aufweist.
Allorestringierte T-Zellen, die spezifisch für tumor-spezifische Antigene sind , können in vitro generiert werden. Allerdings ist in vitro eine Unterscheidung zwischen der Erkennung der MHC-Struktur und peptidspezifischer Erkennung schwierig. In dieser Promotionsarbeit wurde zum ersten Mal gezeigt, daß allorestringierte T-Zellen in der Lage sind, spezifisch HLA-Peptid-Tetramerkomplexe zu binden. Dies war zuvor nur für klassische antigenspezifische T-Zellen bekannt. Es wurde gezeigt, daß fluoreszierende HLA-Peptid-Tetramerkomplexe dazu geeignet sind, allorestringierte CTL gegen ein bestimmtes Peptid zu isolieren und zu klonieren. Weiterhin wurden mit Hilfe von HLA-Peptid-Tetramerkomplexen alloreaktive T-Zellen nachgewiesen, die eine peptidselektive Konformation des allo-MHC-Moleküls erkennen.
γδ T-Zellen erkennen, im Gegensatz zu αβ T-Zellen, ein breites Spektrum strukturell nicht verwandter Antigene. Vγ9/Vδ2 T-Zellen, die die überwiegende Mehrheit der γδ T-Zellen in der humanen Peripherie darstellen, werden beispielsweise durch nichtpeptidische Phosphoantigene aktiviert. Für eine Untergruppe von VγI/Vδ1 T-Zellen konnte dagegen vor kurzem die Erkennung von CD1c nachgewiesen werden. In dieser Promotionsarbeit wird der erste alloreaktive Vγ9/Vδ2 T-Zellklon (B18) beschrieben, der spezifisch ein HLA Klasse I-Molekül erkennt. Es wurde gezeigt, daß die Erkennung des HLA-Moleküls durch γδ T-Zellen unabhängig von der Expression und Funktion von NK–Rezeptoren (NKR) ist. Obwohl dieser Klon B18 den Vγ9/Vδ2 TCR exprimiert, wurde erstaunlicherweise weder eine Aktivierung durch bakterielle Phosphoantigene noch die Expression eines der bekannten NKR beobachtet. Der Vγ9/Vδ2 TCR benutzt das Cγ2 Gensegment, daher sind die γ- und δ-Ketten nicht durch Disulfidbrücken verbunden, wie es auch für den VγI/Vδ1 TCR der Fall ist. Für γδ T-Zellrezeptoren, die keine Disulfidbrücken aufweisen, ist daher eine Konformation wahrscheinlich, die die Erkennung von Molekülen der MHC Superfamilie erlaubt.

Summary

Alloreactive αβ-T cells recognise framework or peptide-dependent determinants on foreign MHC molecules. Among peptide-dependent alloreactive T cells, a significant proportion is specific for one particular peptide presented by the allo-MHC molecule, the allorestricted T cells. This pool of allorestricted T cells provides a reservoir of peptide-specific T cells that can be used in immuno-adoptive transfer. High avidity HLA-A*02 allorestricted CTL clones specific for peptide libraries can be generated from HLA-A*02 negative donors. We made use of this technique to study the role of closely related self-HLA molecules on the shaping of the alloreactive T cell repertoire. PBLs from HLA-A*0205 individuals were stimulated by HLA-A*0201 targets pulsed with an HLA-A*0201 peptide library. We did not observe a bias towards peptide-specific CTLs in the alloreactive repertoire of HLA-A*0205 donors compared to HLA-A*02 negative donors. Comparison of the alloreactive T cell response between two donors having similar HLA haplotypes demonstrates that the allorestricted T cell repertoire is largely different between individuals. Allorestricted T cells specific for tumour associated antigens can be raised in vitro, however it is difficult to differentiate *in vitro* between framework and peptide-specific recognition. We provide in this thesis the first evidence that allorestricted T cells can bind specifically HLA-peptide tetrameric complexes, as nominal antigen-specific T cells would do. We demonstrate that fluorescent HLA-peptide tetrameric complexes can be used for sorting and cloning of allorestricted CTLs specific against a peptide of interest. We also show by the mean of HLA-peptide tetramers the existence of peptide-selective alloreactive T cells that recognise a conformation on the allo-MHC.

In contrast with αβ T cells, γδ T cells recognise a broad variety of structurally unrelated antigens. Vγ9/Vδ2 T cell that represent the vast majority of γδ T cells in the human periphery are activated by non-peptidic phosphoantigen. A subset of VγI/Vδ1 T cell have been recently shown to recognise the CD1c molecule. In this thesis, we describe the first alloreactive Vγ9/Vδ2 T cell clone (called B18) specific for an HLA class I molecule. We provide evidence that the recognition of HLA molecules by γδ T cells is independent of NK-receptor (NKR) expression and function. Remarkably, although expressing the Vγ9/Vδ2 TCR, the B18 clone was not activated by bacterial phosphoantigen and does not express any known NKR. Importantly, the Vγ9/Vδ2 TCR of the B18 clone used the Cγ2 gene segment, was therefore non-disulphide-linked and very similar to the TCR of the VγI/Vδ1 T cell subset. Our results suggest that the particular conformation adapted by non-disulphide-linked γδ TCRs is more prone to react with molecules of the MHC superfamily.

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General introduction

Parts have been published previously:

Moris A., Rothenfusser S., Meuer E., Hangretinger R. and Fisch P.
Role of γδ T cells in tumour immunity and their control by NK receptors. *Microbes and Infection 1999, 1, 227-234.*

Fisch P., Moris A., Rammensee H.G. and Handgretinger R.
Inhibitory MHC class I receptors on γδ T cells in tumour immunity and autoimmunity. *Immunology Today 2000, 21(4), 187-191.*

The immune system

Our environment contains a great variety of infectious microbes – viruses, bacteria, fungi, protozoa and multicellular parasites. These can cause disease, and if they multiply unchecked they will eventually kill their host. Cells of our organism can undergo malignant transformations leading to development of cancer, a life-threatening disease. Most infections and cell transformations in normal individuals are short-lived and leave little permanent damage. This is due to the immune system, which combats infectious and malignant agents. Any immune response involves, firstly, recognition of the pathogen or foreign material, and secondly, mounting a reaction against it, to eliminate it. Broadly speaking, to protect the organism the immune system has developed two arms that are mutually interactive: the innate and the adaptive (or acquired) immunity. This separate but interdependent pathways work in harmony to identify, contain and eliminate harmful agents (Figure 1). This interplay is critical for successful detection and elimination of infectious pathogens and tumour.

Adaptive immunity

The important differences between innate and adaptive immunity are that an adaptive immune response is highly specific for a pathogen. Moreover, the response improves with each successive encounter with the same pathogen due to the establishment of a long term-antigen-memory. The humoral response of the acquired immunity, mediated by αβ T helper cells and B lymphocytes, and the cell-mediated response, mediated by the cytotoxic and inflammatory αβ T lymphocytes, rely on specific antigen receptors expressed by these cells specialised for pathogen recognition (1, 2). The B lymphocyte subset is stimulated through antigen binding to membrane-bound immunoglobulin that serves as B cell receptor. Upon activation and T cell help, B cells proliferate and differentiate into antibody-secreting cells (1). The secreted antibody molecules bind to intact structures on pathogen which results either in neutralisation of the virus and bacteria or destruction through recruitment of cells from the innate immunity. The B cell receptors and the antibodies are therefore specialised in the recognition of extracellular pathogen or antigenic determinants at the surface of affected or malignant cells (3).

In contrast, αβ T cells can detect cells infected with intracellular pathogens because such cells display on their surface peptide fragments derived from the pathogens' proteins (4). These foreign peptides are delivered to the cell surface by specialised polymorphic glycoproteins, the Major Histocompatibility Antigen (MHC) molecules (5). The antigen recognition is based on a dual self-MHC/peptide recognition mediated through the T cell receptor (TCR) (6). The basis for this MHC-restriction became clear with the cloning of the T cell receptor (7, 8), the elucidation of the crystal structure of the MHC molecules (9) and later the determination of the crystal structure of the TCR bound to the MHC-peptide complex (10). The antigenic peptide is accommodated in the peptide binding groove of the MHC molecule. Polmorphic residues on the α1, α2 helices and β-pleated sheet of the MHC molecule, that determine the peptide binding groove, alter the organisation and the specificity of the pockets, thereby conferring different requirements for the peptide to be bound (11). This holds true for both types of MHC molecules, class I and class II (12). MHC class I molecules bind a selection of peptides that result from protein degradation in the cytosol and lumen of the endoplasmatic reticulum (ER). Proteins in the cytosol are processed into peptides by the 20S proteasome and by the 26S proteasome that cleaves ubiquitinated proteins. Peptides are then transported into the ER by the peptide transporter TAP (transporter associated with antigen processing) where they bind nascent MHC class I heavy chain and light chain (β2-microglobulin, β2-m) (review in (13)) (Figure 2). MHC class I molecules have a narrow peptide binding groove that can only accommodate short peptides, 8-11 amino-acids, that contain an allele specific motif usually incorporating two anchor residues (11). In contrast, MHC class II molecules bind

peptides whose length is less restricted and that follow less defined anchor motifs because they have a different peptide binding groove (12). Antigens to be presented by MHC class II molecules are internalised in the endocytic pathway. Nascent MHC class II molecule, consisting of α and β chain, are targeted to specialised compartments involved in antigenic peptide loading, referred to as MHC class II compartments or MIICs (14).

MHC class I molecules are expressed on virtually all nucleated cells. Their primary function is to present antigenic peptides from intracellular protein to stimulate the lytic functions of the αβ $CD8^+$ cytotoxic T lymphocytes (CTLs). αβ $CD8^+$ T cells are the sentinel of the acquired immune system capable of eradicating virus-infected or malignant tumour cells. MHC class II molecules present peptides from ingested proteins and are expressed only at the surface of cells specialised in antigen presentation (antigen presenting cells - APC): dendritic cells, monocytes/macrophages, B cells and thymic epithelial cells. Upon ingestion of intracellular bacteria or toxin for example, they will stimulate αβ $CD4^+$ T cells that can differentiate into two types of effector T cells: inflammatory T cells (T_H1) that activate infected macrophages to destroy intracellular pathogens and helper T cells (T_H2) that activate B cells to produce antibodies and help for the induction of the CTLs. After encounter with their antigen, B cells and αβ T lymphocytes expand, exert their effector phase (antibody production, cytokine secretion, lysis of infected cells, etc) and differentiate into memory cells. This unique property of the adaptive immunity ensures the establishment of an immunological memory that allows an increased responsiveness to previously encountered pathogens.

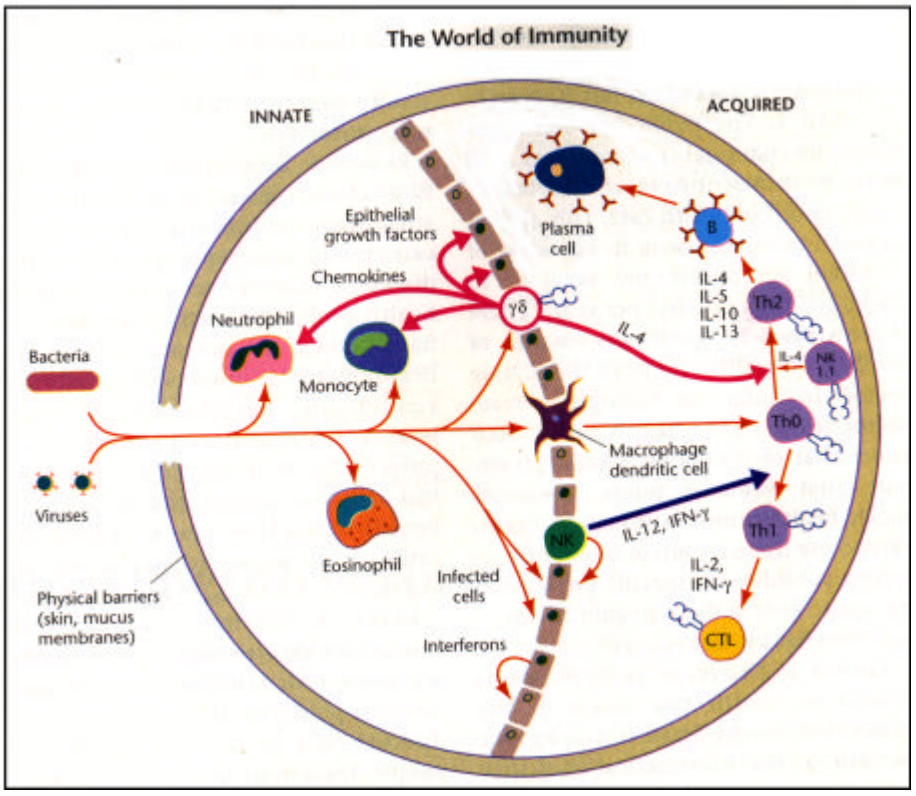

Figure 1: The world of immunity.
As pathogens cross the physical barriers of the host and gain to the body, both the innate and acquired immune systems are activated. The innate response composed of neutrophils, monocytes and interferons is responsible for limiting the spread of infection whereas the acquired response, with its humoral and cell-mediated arms, recognises and dispatches pathogens and elicits memory cells. Recent findings suggest that γδ T cells and NK cells may coordinate the interplay of acquired and innate immunity. (IL, Interleukin; T_H, T helper cell; IFN, interferon; B, B cell; CTL, cytotoxic T lymphocyte; NK, natural killer cell; γδ, gamma-delta T cell). Reprinted from Mak, T.W. and D.A. Ferrick. 1998. *Nat Med 4*:764.

Innate immunity

Innate immunity, although not pathogen specific, is largely responsible for containing and limiting the spread of infection. Macrophages, neutrophils and oesinophils are involved in the first line of defence through rapid recognition of generic molecular patterns such as surface carbohydrates of pathogens (15). Natural Killer cells (NK) and γδ T cells, after recognition of transformed or infected cells, secrete cytokin that play a critical role in coordinating the early defence by innate and later by the acquired immune system. Many viruses have developed strategies to escape the acquired immunity, like the down regulation of MHC class I molecules (16). NK cells, however, express at their cell surface inhibitory receptors (killer inhibitory receptors - KIR) that upon interaction with MHC class I molecules block the lytic machinery as well as cytokine secretion by NK cells (17). A soon as MHC class I molecules are down regulated at the surface of infected cells NK cells are activated. They lyse the infected cells and secrete cytokin that enhance the inflammatory responses and attract and stimulate cells involved in acquired immunity (18).

γδ T cells belong to the T lymphocyte subset and express a γ- and a δ-chain to form the γδ T cell receptor. In contrast to NK cells that do not express any antigen specific receptor, γδ T cells can be specifically activated through their TCR by "stress antigens" that are markers of cell infection or transformation (19). Circulating γδ T cells in humans can be stimulated by low-molecular-mass compounds that are widespread in nature: phosphoantigens (PAgs) (20) and antigenic alkyl amines (21). PAgs are very abundant among bacteria and activate γδ T cells to lyse target cells and to secrete cytokines. These particular antigens that do not require any kind of processing, could confer on γδ T cells a capacity to "see" myriads of bacteria, protozoa, and infected host cells (19). γδ T cells could be therefore part of a first line of defence with the NK cells, macrophages, neutrophils and oesophils that do not respond to a diversity of microbial antigens, but rather to particular molecular pattern and "stress antigens", marker of infections or transformations (Figure 1).

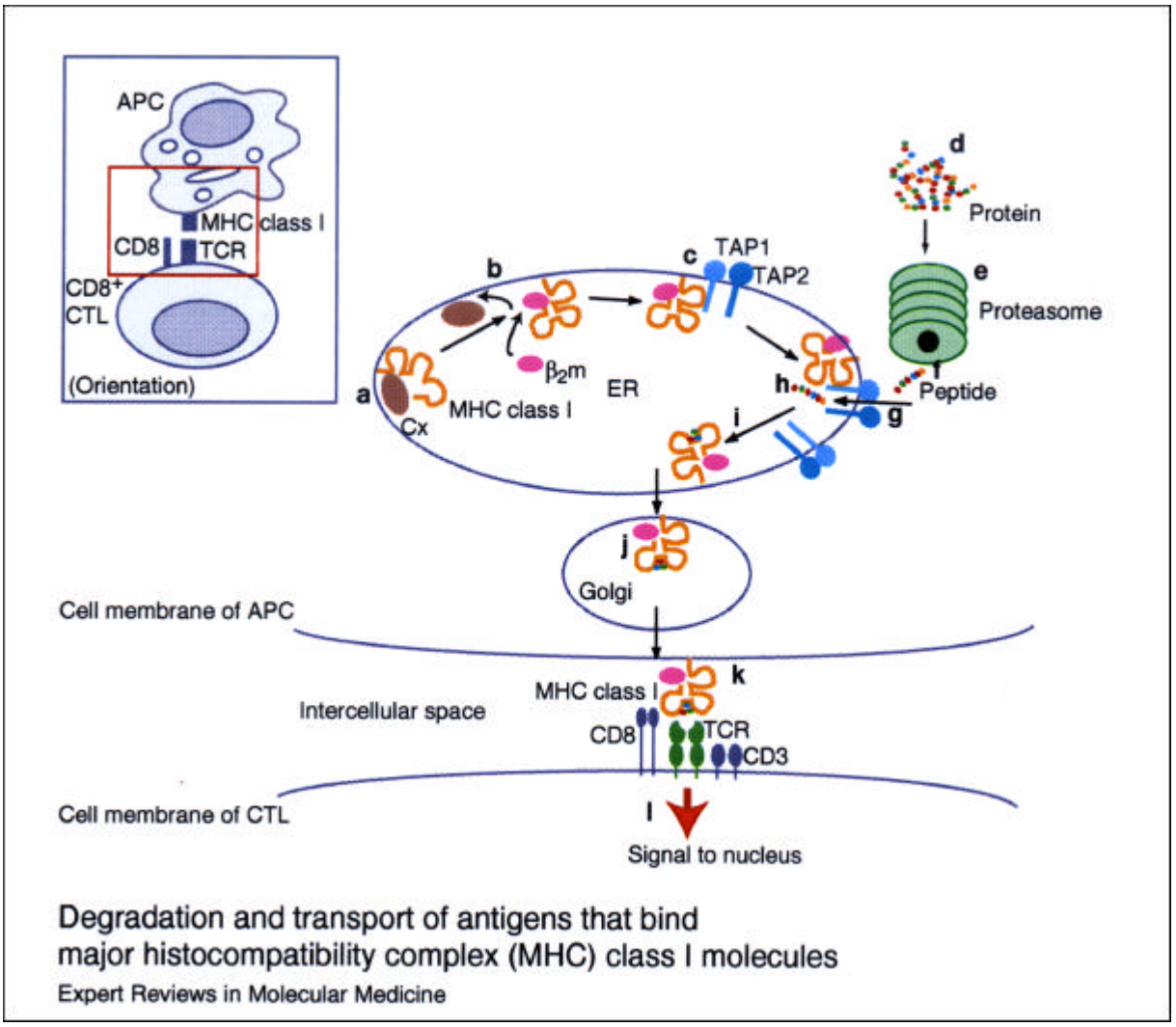

Figure 2: Degradation and transport of antigens that bind major histocompatibility (MHC) class I molecules.
(a) In an antigen-presenting cell (APC) newly synthesised MHC class I molecules bind to calnexin (Cx), which retains them in a partially folded state in the endoplasmatic reticulum (ER). (b) Binding of MHC class I molecules to β2-microglobulin (β2m) displaces Cx and allows binding of chaperonin proteins (calreticulin and tapasin; not shown). (c) The MHC class I-β2m complex binds to the TAP complex, which awaits the delivery of peptides. (d) Peptides (e.g. antigens) are formed from the degradation of cytosolic proteins (self-, pathogen- and tumour-derived proteins in the cytoplasm). (e) These are degraded by the proteasomes into (f) short peptides. (g) Peptides are transported into the ER by the TAPs, where they meet the MHC class I-β2m complex (h). This peptide binding in the antigenic groove of the MHC stabilises the structure of the MHC class I molecule and (i) releases the TAP complex. (j) The fully folded MHC class I molecule with its peptide is transported to the cell surface via Golgi apparatus. (k) Recognition of the MHC-peptide complex by the T cell receptor (TCR) of the antigen-specific cytotoxic T lymphocyte (CTL) takes place and (l) a signal transduction event activates effector functions in the MHC class I restricted T cell. Reprinted from Expert Reviews in Molecular Medecine (http://www-ermm.cbcu.cam.ac.uk).

αβ T cell alloreactivity

An overview of the history of immunology

The basis for recognition of alloantigen is one of the most significant issues in the history of immunology. Characterisation of the molecular mechanisms involved in alloreactivity lead to the discovery of the MHC genes and T lymphocytes.

Although vaccination was already discovered 200 years ago by Edward Jenner and developed in the 19th century by Koch and Pasteur, the molecular mechanisms of the acquired immunity were only recently revealed. At the beginning of the 20th century, pioneer scientists were working on tumour transplantation and described the exquisite strength of graft rejection between members of a species having different genetical background (5). Only years later, in 1936, Peter Gorer, then working on blood group typing, identified serologically the target molecules involved in the allograft rejection that he named the mouse histocompatibility genes (H-) (22). In the following years Georg Snell went on isolating a large number of such H genes that could be grouped into those causing fast skin graft rejection and those taking many weeks. The H genes with a strong effect were called major, as opposed to the minor H genes (5). The major H genes were later shown to be located in a cluster on the mouse chromosome 17 that we now call the Major Histocompatibility Complex (5). The first evidence that the allograft rejection was the consequence of foreign MHC molecule recognition by lymphocytes came in the 1950' (5). The development of new cell culture techniques, such as mixed lymphocyte reaction (MLR) (23) and chromium release assay (24) then allowed *in vitro* studies of graft rejection and the characterisation of the two main T cell subsets: the proliferating helper T cells and the cytotoxic T lymphocytes.

Modern theories on αβ T cell Alloreactivity

Graft rejection and its *in vitro* correlate, the MLR, is the manifestation of T cell reactivity on the foreign MHC molecules. It is characterised by a vigorous T cell response, as many as 1-10% of T cells respond to an alloantigen. Since the discovery of the MHC-restriction, the molecular basis of this high frequency of allorecognition remains enigmatic. Peter Doherty and Ralf Zinkernagel demonstrated in the 70s that T cells have a dual "specificity": they recognise the foreign antigen at the surface of the target cell in an self-MHC restricted manner (6). Under physiological situation the frequency of naive T cells that will expand upon specific antigen stimulation in the context of self-MHC molecule is very low (less than 1 for 100 000) (12). In contrast, the frequency of responder cells that recognise allogeneic MHC molecules is 10 to 100-fold higher (25). Over the years a number of theories have been proposed to explain this paradox.

In one of the earliest attempts to explain the basis for the high frequency of alloreactive cells, Jerne proposed that the alloreactive TCRs were germ line encoded. The alloreactive repertoire would be separated from the repertoire used in recognition of antigen (26). For each MHC molecule in the species there would be a complementary TCR. However the TCR repertoire involved in antigen recognition would be derived by somatic mutation from the receptor specific for the self-MHC molecule. Although some preferential Vβ TCR usage have been reported in the alloresponse against HLA-B*27 for example (27), it is now readily demonstrated that the TCR usage in an alloresponse against a single MHC molecule is heterogeneous (25). Since the discovery of the thymic positive selection of T cells on self-MHC molecules (28) it was assumed that the allorepertoire consisted of cross-reactive antigen specific T cells. Once it was possible to clone and expand *in vitro* antigen-specific CTL (29), it appeared that clones restricted by self-MHC could also display alloreactivity (30, 31). Thus, the antigen specific and allospecific repertoires are one and the same. However, a major prediction from the model proposed by Jerne was that the diversity of the TCRs used in the

recognition of antigen is ensured through somatic modification of germ line encoded genes. This was later confirmed by the cloning of the T cell receptor genes (2).

Matzinger and Bevan suggested that the high frequency of T cells directed to a particular foreign MHC was the consequence of the recognition of polymorphic antigens associated with the MHC (32). This model was based on the observation that allo-CTLs could be obtained between MHC identical strains that differed only from each other with respect to the expression of polymorphic proteins, the minor H antigens (28). The polymorphic antigens have been demonstrated to be antigenic peptides presented by the MHC molecule (4, 11, 33). The high frequency of alloreactive T cells would be therefore the consequence of the fact that MHC molecules display more than 10 000 different peptides on the cell surface (34), giving rise to a large number of potential targets. Various modifications of this theory have focused on whether the TCR contacts both the MHC molecule and the peptide or either the peptide or the MHC molecule only (25). On the one hand, although not involved in the contact with the TCR, the bound peptide could profoundly influence the conformation of the MHC residues comprising the epitope (35). Several studies have suggested the existence of such peptide-dependent but not peptide-specific CTLs (36-39). Indeed, experiments combining the use of TAP-deficient cell lines and the newly established HPLC fractionation of MHC bound peptides revealed the existence of alloreactive CTL clones that recognised several peptide fractions (36). This could indicate that more than one peptide can be recognised by a single allospecific clone. These alloreactive T cells might be sensitive to a conformation of the MHC that is adapted when particular, but unrelated, peptides are bound to the MHC molecule (25). On the other hand, studies focusing on MHC class II molecules showed that the alloreactive response could be tissue specific. Cells expressing the appropriate MHC class II molecule were unable to stimulate alloreactive T clones (40, 41). These result suggested that the specific MHC class II molecule was necessary but not sufficient for the stimulation of alloreactive T cells. Direct evidence for peptide involvement in allorecognition was first obtained for MHC class I. Several studies demonstrated that recognition by alloreactive CTL clones was dependent upon addition of specific peptide fraction from naturally processed peptide extracts (36, 42-45) or cytoplasmatic proteins cleaved using cyanogen bromide (33). In the mean time, evidences have accumulated showing that peptide-specific alloreactive T cells, also called allorestricted T cells, could recognise the MHC/peptide complexes as nominal antigen-specific T cells would do (46-49).

The previous theories were based on the assumption that the magnitude of the alloresponse was due to the large number of epitopes present on the allogeneic target. In contrast, Bevan reconsidering his own model proposed that the antigenic strength of the alloantigen would explain the high frequency of alloreactive T cells (50). This theory suggested that the T cell receptors would recognise directly allelic polymorphism on the MHC molecules. The expression of such epitopes at high density on the cell surface would result in stimulation of T cells bearing high and low affinity receptors for the allogeneic MHC molecule. This would contrast with foreign antigens present at low number on the surface of the target cell and that would stimulate only high affinity TCR (50). However, only one report so far described an alloreactive clone with significant lower affinity for the allo-MHC than for the self-MHC molecule (51). The principal data supporting this model consists of studies describing CTL clones that recognise the MHC molecule in the absence of bound peptide (52, 53). Using a skin graft model Smith and colleagues have reported that up to 20 % of primary and essentially all secondary alloreactive T cells were specific for the MHC class I molecules produced in insect cells even after acid wash treatment. However, to generate this peptide-independent CTL, they used manoeuvres selecting CD8-indepedent T cells, thus of high affinity (53).

The nature of the determinants involved in the alloreactive T cell recognition appears therefore to be very diverse. However, it was found in mouse and human that most of the epitopes recognised by the alloreactive CTLs depend on the presence of endogenous peptides (54, 55). It is nowadays generally believed that there is an overall dominance of peptide-dependent recognition among alloreactive CTLs (56).

The use of allorestricted CTL in immunotherapy

Many tumours overexpress mutated and normal proteins thereby modifying the set of self-peptides associated with MHC class I molecules. This phenomenon allows triggering of tumour specific CTLs. The identification, in the past 10 years, of such tumour-associated antigens recognised by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Different groups of cancer-associated antigens have been described as targets for cytotoxic T lymphocytes *in vitro* and *in vivo*: 1) cancer-testis antigens, which are expressed in different tumours and normal testis (MAGE, BAGE, GAGE, NY-ESO-1); 2) differentiation antigens (Melan-A, gp100, gp75 and Tyrosinase-A); 3) point mutations of normal genes (CEA, Ras); 4) antigens that are overexpressed in malignant tissues (WT1, md2m, GATA1, p53); and 5) viral antigens (E6/E7 from HPV) (review in (57)). It is not really surprising that the immune system can mount a response against antigens, e.g. cancer-testis, mutation or viral antigens, that are normally not accessible to the effector cells. However, the CTL should be tolerant to differentiation antigens. Indeed, CTLs undergo negative selection and peripheral tolerance mechanisms that diminish the number or eliminate self-peptide-specific CTLs (58). The CTL repertoire may not be tolerant to these proteins for several reasons. Firstly, those proteins are not normally expressed in the thymus, and secondly, these antigens might be normally expressed in immunologically privileged sites. Furthermore, the relevance for the course of the disease of differentiation-antigen-specific CTLs is not clear. Studies exploiting the MHC-tetramer technology have shown that these CTLs can be found in healthy donors (59) and in patients (Teichgräber V., Moris A. et al. manuscript in preparation). Moreover, it has been reported that a TyrA-specific CTL clone from a particular melanoma patient was anergic (60). The *in vivo* functionality of these tumour-specific CTLs is therefore a matter of debate. Nevertheless, these new informations on cancer-specific antigen have been used to develop new protocols for immunotherapy, e.g. peptide vaccination, dentritic cell based vaccines and adoptive transfer. However, the generation for adoptive immunotherapy of such tumour-specific CTLs *in vitro*, as *in vivo* in the mouse model, has proved to be tidious and poorly successful. In mice for example, p53 gene-knockout mice had to be used to generate CTL with high affinity for a p53 peptide to circumvent tolerance (61). For humans, it could be possible to circumvent tolerance by the use of allorestricted CTLs.

In mice as well as in humans, the diversity of the allorestrited T cell response has been investigated in two recent studies (47, 49). Splenocytes or peripheral blood lymphocytes (PBLs) were stimulated with synthetic peptide libraries loaded on the allogeneic molecules. The resulting CTLs were found to differ widely in their peptide specificity. These results showed that the allorestricted T-cell repertoire is highly diverse (47, 49). Several reports have focused on the avidity of alloreactive T cells receptors (62, 63). Using the well characterised 2C alloreactive clone it was shown that the avidity of the 2C TCR for H-2L^d presenting the relevant peptide was particularly high compared to the avidity of TCRs measured so far (62). A ten fold higher avidity for the allogeneic MHC than for the self-MHC was also reported using a different allorestricted CTL clone (63). These results demonstrated that the affinity for the allo-MHC/peptide complexes of allorestricted T cells is probably at least as high as the affinity of autologous antigen specific T cells. Studies defining both the self-MHC-peptide

and allo-MHC-peptide ligands for individual T-cell clones have shown that the molecular basis for T cell alloreactivity follows similar rules as self-MHC-peptide recognition (64). First, it is noteworthy to stress that MHC molecules differ primarily in amino acids that bind the peptide rather than at positions predicted to be directly accessible to the TCR (12). X-ray crystallographic data confirmed that the majority of the contacts between a TCR and the MHC are with highly conserved MHC residues (65). Furthermore, the crystal structure of self-MHC and the allo-MHC molecules involved in the 2C TCR system revealed that the orientation of the TCR-MHC-peptide complexes was analogous in each case (64). The allo-MHC target antigen might be therefore sufficiently similar to the MHC molecule involved in positively selecting the T cell, at residues in contact with the antigen receptor, to allow peptide-specific cross-reactivity (66).

The existence in the diverse repertoire of allorestricted T cells of high avidity CTLs (46-49) that recognise the MHC/peptide complexes as nominal antigen-specific T cells would do, raises the possibility of generating CTLs reactive against low-copy-number peptides bound to non-self MHC molecules. In two reports high affinity allorestricted CTLs could be generated *in vitro* using peptide libraries (47, 49). Using PBLs from HLA-mismatched donors, allorestricted CTLs could be generated against epitopes from the transcription factor WT1 (67) and the cyclin-D1 protein (48), overexpressed in leukaemia cells and in breast cancer cells respectively. These allorestricted CTLs specifically lysed tumour cells overexpressing the relevant proteins (48, 67). Moreover, allorestricted CTLs, specific for an mdm-2-derived epitope were reported to specifically lyse allogeneic tumour cells expressing mdm-2 *in vitro* but also to inhibit tumour growth *in vivo* (46). More recently, a study demonstrated for the first time that allorestricted CTLs specific for an epitope of the vesicular stomatitis virus (VSV) could lyse virus infected target cells (66). Altogether, these data demonstrate that allorestricted T cells are a new source of peptide-specific CTLs and promising reagents for adoptive immunotherapy.

MHC-related alloreactivity

More recently, the domain of allorecognition has been extended to non MHC-restricted cytotoxic effectors: NK and γδ T cells. Both cell types when activated by interleukin-2 (IL-2) display MHC-related alloresponses in the presence of allogeneic cells (68, 69).

Natural killer cells

NK cells were first characterised for their ability to lyse the MHC class I negative lymphomas (70). Klaus Kärre explained this observation by the "missing-self hypothesis" (68). This described the inverse correlation of HLA class I expression with the sensitivity to cytolysis by some human lymphocytes. However, the mechanisms explaining why some NK clones killed particular allogeneic tumour target cells (68) and why some NK clones killed normal allogeneic lymphocytes from some, but not all donors (71) remained unclear. Then, the discovery of natural killer cell receptors (NKRs) for HLA class I provided an explanation for both phenomena, the "missing-self" and the alloreactivity of NK clones. In contrast to classical alloreactive T lymphocytes which bear TCR that are positively triggered through interaction with the allogeneic MHC molecules, most of the NKR function as killer inhibitory receptors. The interaction of NKR with the MHC molecules was shown to deliver an inhibitory signal to the lymphocyte, allowing the target cells to escape lysis (72). Studies of human NK cells showed that the expression of specific HLA-A, -B or –C molecules could confer resistance to various NK cells (17). NKRs include indeed different types of killer inhibitory receptors, members of the immunoglobulin superfamily (KIR) and the C-lectin-type receptors (CD94/NKG2-) each molecule having a fine specificity for certain HLA alleles. The KIRs having two (KIR2D) and three (KIR3D) immunoglobulin-like domains interact with HLA-C and HLA-B, respectively (17). The CD94/NKG2-A and -B heterodimers bind the non classical MHC class Ib molecule, HLA-E (73). It appears that natural killer cells with strong cytolytic potential have at least one NKR specific for self-HLA class I molecules, so that autologous cells generally remain undamaged (74). NK cells express on average 6 NKRs that are randomly expressed during NK cell maturation until one or more NKR interacts with self-HLA class I molecule, thereby delivering a negative signal to the lytic machinery of the killer cells (75). NK cells may therefore have NKRs for which there is no autologous ligand. This mechanism may turn particular NK clones "tolerant" against some, but not other allogeneic target cells. NK cells will destroy autologous cells that have lost expression of all MHC class I or that have lost a class I molecule which is most inhibitory for a given NK clone (74). Similarly, NK clones will kill allogeneic cells that lack the relevant autologous HLA class I allele which determines inhibition by the particular clone. Other NKRs, designated as killer activatory receptors (KARs) may directly activate the killer cells by binding to a foreign class I allele and therefore induce lysis of the allogeneic cells by the NK cells (17). These receptors belong to the immunoglobulin superfamily (KIR2S, KIR3S) or the C-lectin-type receptors (CD94/NKG2-C). The overall lytic activity against a specific target cell by a given cytotoxic NK clone will be the result of a balance between these inhibiting and activating signals.

gd T cells

Soon after the discovery of the γδ T cell receptor on human T-lymphocytes, most γδ T cell clones were described as displaying strong MHC-unrestricted cytotoxicity against divers tumour cells (69). This cytotoxic activity, by IL-2 activated γδ T cell, is reminiscent of NK cells that lyse target cells which have lost MHC class I expression or kill allogeneic cells. However, it had been speculated that γδ T cells might be more specific for distinct tumour than NK cells (76). Nevertheless, there is still controversy as to whether the γδ TCR is

directly involved in the recognition of tumour cells by most γδ T cell. In part it is due to the fact that the target molecules for γδ T cells on tumour cells remain undefined. Besides the TCR, the NKRs expressed at the cell surface of γδ T cells might determine the cytotoxic specificity of individual clones and explain the preferential lysis of certain targets by γδ T cells (77).

Two main subsets of γδ T lymphocytes can be detected in human blood and intestine. Most circulating human γδ T cells use the same combination of variable regions to form their TCR, Vγ9 and Vδ2 (78). Vγ9/Vδ2 T cells use the Cγ1 gene segment and therefore express a disulphide-linked TCR (79). The vast majority of γδ T cells in tissues such as intestine and spleen uses the Vδ1 gene segment which is mainly paired with a Vγ segment, distinct from Vγ9, belonging to the VγI family (78). VγI/Vδ1 T cells use the Cγ2 gene segment, and therefore express a non disulphide-linked TCR (79). NKR are expressed by the vast majority of human Vγ9/Vδ2 T lymphocytes, but rarely by γδ T cells of the VγI/Vδ1 subset (80).

Antigen recognition by Vg9/Vd2 T lymphocytes

The vast majority of Vγ9/Vδ2 T cells proliferate *in vitro* when fresh human PBLs are stimulated with the β2-microglobulin (β2-m) deficient Burkitt's lymphoma Daudi (81, 82), allogeneic B cell lymphomas (83) and some bacterial extracts (84). These bacterial antigens have been identified as non-peptidic organic molecules that are divided into two groups: phosphorylated antigen, designated phosphoantigens (PAg) (20, 85), and alkyl amine antigens (AAg) (21). These compounds are very abundant in mycobacterial supernatants (84) (Figure 3). Vγ9/Vδ2 T cell clones produce cytokines to PAgs, AAgs and Daudi lymphoma (77). Cytokine release and cytotoxic activity to allogeneic B cell lymphomas are very much dependent on the pattern of NKR expressed by the Vγ9/Vδ2 T cell clone and the HLA haplotype of the B cells (80, 84). However, the recognition of Daudi and B cell lymphomas is not only a consequence of the NKR expression on γδ T cells, solid tumour such as melanoma and colon carcinoma failed to induce expansion of Vγ9/Vδ2 T cells *in vitro* (80). Incubation of these resistant targets with PAgs *in vitro* induced cytolysis by Vγ9/Vδ2 T cells (86). A similar specificity of γδ T cells for Daudi and PAgs has been observed in chimpanzees (87). However, it is remarkable that no human γδ T cell specificities are obviously conserved in mice.

All PAgs and Daudi-reactive Vγ9/Vδ2 T cells express a Vγ9-JP-C1γ chain and bear marked junctional variability of the γ- and δ-chains of their TCRs. However, despite their heterogeneity, almost all V2J-δ sequences, derived from peripheral but not thymic Vγ9/Vδ2 clones were shown to carry a distinct motif consisting in the presence of a strongly hydrophobic residue (Val, Leu, or Ile) at a conserved position (position 97) of the CDR3-δ region (88). This raises the possibility that the Vγ9/Vδ2 peripheral population is selected and expanded by similar and restricted ligands *in vivo.* Although there is no formal proof of direct binding of PAgs on the TCR, the involvement of the TCR in the recognition of PAgs and Daudi cells has been demonstrated (89). TCR transfer experiments showed that this recognition is dependent on a particular junctional region of the Vγ9-chain and on the pairing of both Vγ9 and Vδ2 chains (90). These experiments suggested that ligands related to PAgs might be recognised by Vγ9/Vδ2 T cells on Daudi cells. However, this Daudi ligand has not yet been identified molecularly and recognition of the Daudi cells by the Vγ9/Vδ2 TCR transfectants was relatively weak (89). The involvement of the γδ TCR in Daudi recognition is therefore a matter of debate (77).

There is no evidence of a presenting element for phosphoantigens and alkyl amine antigens. PAgs could be presented by a novel extracellular pathway that does not require any antigen uptake and processing (84). However, cell-cell contact is required for stimulation of the γδ T

cells, suggesting that there is recognition of a cell surface complex (86). Experiments using blocking antibodies, mutant APC, showed that HLA class I, HLA class II molecules or MHC-like CD1a, 1b, 1c are not involved in PAgs presentation to the Vγ9/Vδ2 TCR (91, 92). Nevertheless, HLA restriction has not been formally ruled out because self-presentation of PAgs by the HLA molecules of γδ T cells remains a possibility, though very unlikely. Alternatively, PAg and AAg that are small and flat molecules could interact directly with the Vγ9/Vδ2 TCR and cell-cell interaction could only provide necessary costimulatory signals (77) (Figure 3).

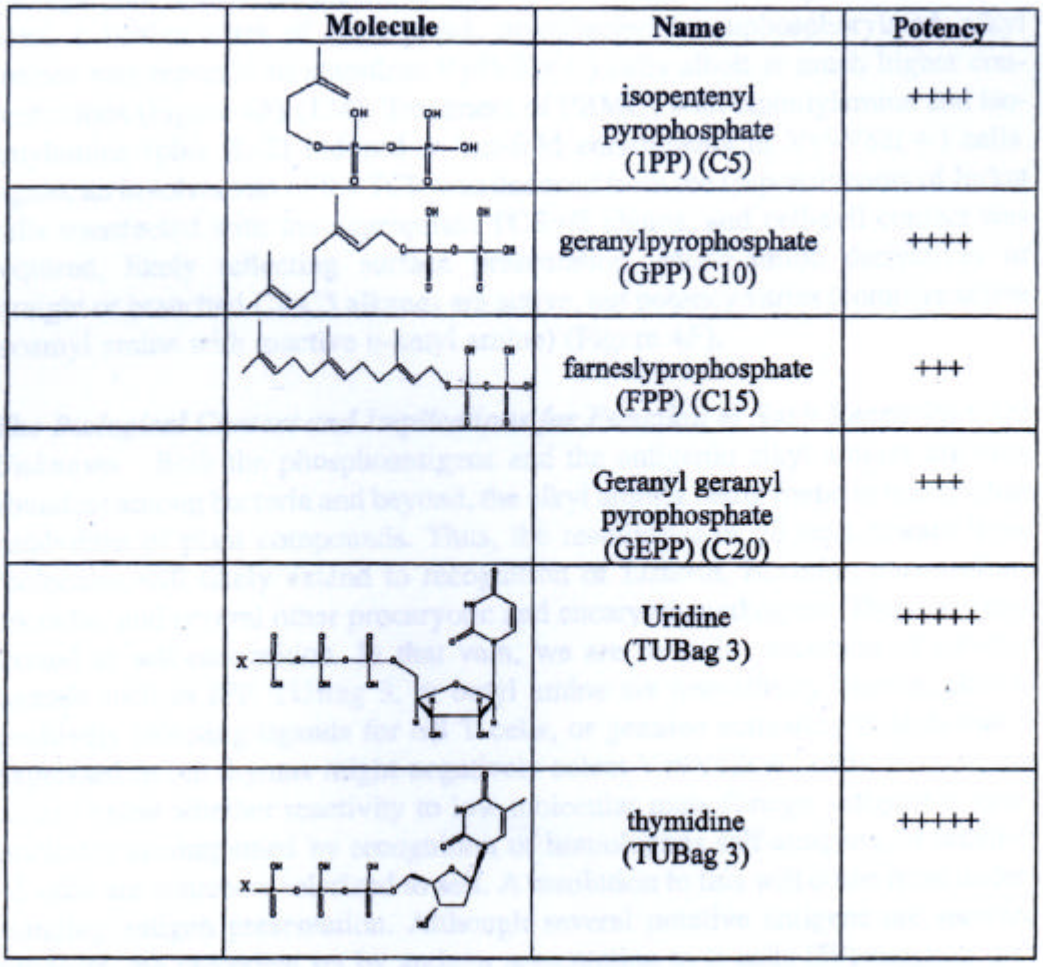

Figure 3: Natural ligand for Vg9/Vd2 T cells.
These phosphoantigens are highly abundant in mycobacterial supernatants or extracts. The structure of the phosphoantigens follows stringent chemical requirements. Simple modifications, e.g. addition of β-OH group, β-carboxylic group, or a β-amino group, reduced antigenicity by 100 times. The phosphate group and the unsaturated bond in the longer chain pyrophosphates are prerequisite for activation of Vγ9/Vδ2 T cells. Reprinted from Hayday, A.C. 2000. *Annu Rev Immunol 18*:975.

Antigen recognition by VgI/Vd1 T lymphocytes

VγI/Vδ1 T cells represent the large majority of γδ intraepithelial T lymphocytes (IEL) in the intestine and spleen. Until recently, the nature of the antigen recognised by the VγI/Vδ1 TCR remained poorly characterised. Newly, the VγI/Vδ1 T cells were found to recognise the CD1c molecule (93). CD1 molecules represent a separate lineage of antigen presenting molecules that can present non-peptidic antigens to nonclassical αβ T cells (94). The structure of the CD1 molecules is very similar to that of MHC class I molecules. The CD1c molecule is a member of the non polymorphic CD1 molecules (95). VγI/Vδ1 TCR mediated recognition of CD1c was readily demonstrated and was shown to be independent of the presence of exogenous foreign antigen (93). VγI/Vδ1 T cells proliferate, lyse target cells and release Th1 cytokines upon interaction with CD1c (93). The expression of CD1c can be induce in dendritic cells in response to GM-CSF, during infection or inflammation (96). CD1c specific VγI/Vδ1 T cells could therefore play a role in co-ordinating the αβ T cell response in the early phase of infection (93).

γδ T cells of this subset were previously found to recognise the MHC-encoded proteins MICA and MICB (97). MICA and MICB are distantly related to MHC class I molecules but are functionally distinct. Their truncated peptide binding groove can not accommodate any peptide and the expression of these molecules is dependent upon a heat-shock inducible promoter (98). They may function as stress inducible self-antigens (98). In contrast to the CD1c recognition, the contribution of the VγI/Vδ1 TCR in the MICA reactivity remains unclear. The only evidence that the VγI/Vδ1 TCR could play a direct role in the recognition of MICA/B expressing cells by the VγI/Vδ1 T cells, is based on a blocking experiment using an antibody against Vδ1 (97). However, the authors in this experiment did not use a Fab antibody fragment (97). Therefore, it could not be excluded that the antibody itself delivered a negative signal to the γδ T cells (82). Furthermore, there are now compiling evidences that the recognition might be due to an activating NKR, NKG2-D (99). NKG2-D belongs to the activatory receptor family NKG2 but in contrast to NKG2-A and -C it does not form any heterodimer with CD94 (99, 100). NKG2D is expressed at the cell surface of virtually all NK, αβ and γδ T cells (101). Using soluble MICA molecules and NKG2D transfectants it was clearly demonstrated that NKG2D is the receptor for MICA (99). MICA engagement with NKG2D at the surface of the γδ T cells, induces the γδ T cell lytic machinery (100). The contribution of the VγI/Vδ1 TCR in this mechanism is unclear. MICA/B expression has been detected on several intestinal cell lines and to be inducible when the cells were rapidly growing or when they were heat shocked (97). A broad tumour-associated expression of MICA/B has also been observed (97). These results raised the possibility that VγI/Vδ1 IELs might be a first line of defence during infection in the intraepithelial layers in different organs and might play a role in controlling malignant T cell transformations.

In mice the recognition of the non-classical MHC molecules T10/T22 by the γδ G8 clone (102) and by a small subset of γδ T cells (103) is reminiscent of the CD1c and MICA/B recognition in human.

MHC-restricted gd T cells

A few mouse γδ T cell clones have been shown to be specific for classical MHC class I and MHC class II molecules. These clones were obtained from nude mice after allogeneic stimulation and exhibited broad cross-reactivity on MHC class I or Class II (104, 105). An H-2^d specific allogeneic clone was described (105). The allogeneic recognition of H2-E^k, H2-E^b, H2-E^s but not H2-E^d by the mouse γδ LBK5 clone was shown to be independent of the peptide and of intracellular antigen processing (106). This reactivity was probably of low affinity and similar to IgM recognition of native protein (107). Stimulation of LBK5 by H2-E^k was not dependent on glycosylation, but could be influenced by the glycosylation of the H2-

Eα domain (107). Since infected, stressed or transformed cells often change the posttranlation modifications of their surface glycoproteins, it has been proposed that changes in glycosylation pattern could be a new way in which γδ T cells antigen recognition could be regulated (107). However, since the MHC class I or class II specific allogeneic clones were obtained from an athymic nude mice after strong allostimulation, the *in vivo* significance of these recognitions remains unclear. Furthermore, it has been readily demonstrated that a population of murine γδ T cells is specific for the non-classical MHC molecule T10 (103). The clones described above might have been selected *in vitro* on the basis of their cross-reactivity with classical MHC molecules.

Several human γδ T cell clones were shown to recognize HLA-A*02 (108), HLA-A*24 (109), HLA-A*09 (110), HLA-B*27 (111), HLA-DR molecules (112-115) and HLA.-DQA1/DQB1 heterodimers (116). Furthermore, a γδ T cell clone specific for a tetanos toxoid antigen and restricted by its autologous HLA-DR4 molecule was also reported. All the γδ T cell clones characterised in these reports used the VγI/Vδ1 TCR, except for a small fraction using the Vδ3 gene segment (114). However, most of the clone display also a strong NK-like cytotoxic activity against NK-sensitive cell lines. Although γδ T cells of the Vδ1 subset usually do not express NK receptors, we have recently reported the involvement of a VγI/Vδ1 T cell clone expressing NKR in autoimmune disorder (117). Therefore, one can not exclude an NKR contribution in the Vδ1 subset lytic functions. Especially one could not exclude an involvement of activatory NKR in the MHC class I recognition by VγI/Vδ1 T cells. Furthermore, a direct involvement of the TCR in the HLA recognition was never thoroughly demonstrated.

Outline of this thesis

The studies described in this thesis are focused first of all on the extension of the promising results with allorestricted T cells obtained in mice to human T cells and secondly on the study of the molecular mechanism of γδ T cell allorecognition in humans.
Several methods have been used so far to generate allorestricted T cells *in vitro*. In mouse and human, TAP-deficient cell lines in combination with peptide libraries have been used successfully(49). However, complex peptide libraries do not allow to generate allorestricted T cells against an individual peptide of interest. Multiple rounds of Splenocyte or PBL stimulations on different TAP-deficient cell lines loaded with tumour peptides to raise allorestricted T cells against tumour epitopes were also used. Although shown to be successful, this procedure is time consuming and does not allow an efficient separation of framework-specific T cells from alloreactive T cell lines. In *chapter 2*, we describe a new method to generate, separate and amplify *in vitro* allorestricted T cells against a peptide of interest. We made used of the newly developed technology based on HLA-peptide tetrameric complexes.
A better understanding of the recognition pattern by alloreactive T cells is also of great interest. In *chapter 2*, we demonstrate, using the HLA-peptide tetrameric complexes, that peptide-selective alloreactive T cells exist. This particular alloreactive T cells could generate problems when one tries to raise allorestricted T cell to be used in immunotherapy. In *chapter 3*, we study the repertoire of allorestricted T cells. We particularly were interested in looking at the influence of the self-HLA molecules in the shaping of these repertoires. We show that the human alloreactive repertoire is largely different between individuals.
In *chapter 4*, we made used of the alloreactive recognition of B cell lymphomas to study the molecular recognition pattern of γδ T cells. We describe the isolation the first Vγ9/Vδ2 T cell clone specific for a classical HLA class I molecule.
Finally, *chapter 5* contains a general discussion on allorestricted T cells, immunotherapy and γδ T cells.

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2

Characterization of allorestricted and peptide-selective alloreactive T cells using HLA-tetramer selection

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The vast majority of alloreactive T cells recognize foreign MHC molecules in a peptide-dependent manner. A subpopulation of these peptide-dependent alloreactive T is peptide-specific and contains T cells that are of interest for tumor imunotherapy. Allorestricted T cells (i.e., peptide-specific and alloreactive) specific for tumor associated antigens can be raised in vitro. However, it is technically difficult to distinguish between peptide-specific and peptide-non-specific alloreactive T cells by functional assays in vitro. Here we show for first time that allorestricted T cells specifically bind HLA-peptide tetrameric complexes, as nominal antigen-specific T cells would do. In consequence, fluorescent HLA-peptide tetrameric complexes can be used for sorting and cloning of allorestricted CTLs specific for a peptide of interest. We also show by the mean of HLA-peptide tetramers the existence of peptide-selective alloreactive T cells that recognize a conformation on the foreign-MHC brought about by some but not all peptides bound.

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Introduction

Allograft rejection and graft versus host disease (GvHD) are the clinical manifestations of T cell reactivity to foreign MHC molecules (1). The molecular basis of the allorecognition has been extensively studied (2, 3). The nature of the determinants involved in the alloreactive T cell recognition appears to be very diverse (3). The target of alloreactivity, the MHC class I molecules, bind 8-10 amino acid peptides from intracellular sources and display them at the cell surface. CTL clones that seem to recognize allogeneic molecule in a peptide-independent fashion have been reported (3-5). Several studies have indicated the existence of peptide-dependent but not peptide-specific CTLs (3, 6). This fraction of alloreactive T cells might be sensitive to the conformation of the MHC that is adapted when particular, but unrelated, peptides are bound (2). The existence of peptide-specific alloreactive T cells has been clearly demonstrated (3, 7-10). These allorestricted CTLs can recognize specific peptide/MHC complexes just like nominal antigen-specific T cells do (11-13). By using peptide libraries, our group has recently shown that the mouse as well as the human allorestricted T cell repertoire is broad and diverse (11, 13).

Many tumors overexpress normal proteins thereby modifying the set of self-peptides associated with MHC class I molecules. This phenomenon allows triggering of tumor specific CTLs. However, CTLs undergo negative selection and peripheral tolerance mechanisms that diminish the number or eliminate self-peptide-specific CTLs. This is an obvious limitation to generate *in vitro* tumor-specific cytotoxic T cells to be used in adoptive immunotherapy. The existence of the allorestricted repertoire raises the possibility of generating CTLs reactive against synthetic self peptides bound to non-self MHC molecules, since tolerance to self-antigens is self-MHC restricted (14). Thus, it should be possible to produce *in vitro* CTL against self-antigens that are expressed in tumor cells for adoptive immunotherapy (14, 15). Indeed, it has been shown recently that allorestricted CTLs specific for mdm-2 wild type peptide, can be a successful reagent for immunotherapy in mice (12). These CTLs can engraft and retain specificity in the host without causing GvHD (16). We and others (15) evaluate the possibility to isolate allorestricted CTLs that originate from HLA-A*02 negative donors and recognize specifically HLA-A2-peptide complexes. However, the *in vitro* generation of such allorestricted T cells remains problematic because of the difficulty to separate between the large pool of alloreactive (i.e. recognizing foreign MHC) and the small fraction of allorestricted (i.e. restricted for a particular peptide on foreign MHC) CTL activities.

Here, we investigated whether allorestricted T cells can bind HLA tetrameric complexes specifically, as antigen-specific T cells would do, and whether HLA tetramers can be used for sorting and cloning of allorestricted CTLs specific against a peptide of interest.

Materials and methods

Cells

The human EBV-transformed lymphoblastoid B cell lines (LCL) 721 (HLA-A*0201,-A*01,-B*05, -Cw1) (17), the TAP-deficient cell line T2 (HLA-A*0201^{low}, -B*5^{low},-Cw1^{low}) (18), the β2-microglobulin deficient Burkitt's Lymphoma Daudi (HLA^{-}) (19) and the breast carcinoma cell line KLHE (HLA-A*02^{-}) (kindly provided by Dr. B. Gückel, Tübingen, Germany) were used in ^{51}Cr release assays or for T cell stimulation.

Generation of CTLs

PBL from healthy donors registered in the Blood Bank (Tübingen, Germany) were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). PBLs (10^7) were stimulated with (10^6) irradiated T2 cells (200 Gy) pulsed with peptide. Peptide loading was performed for 4 h at room temperature with 10 µM peptide in serum free medium (20). After 5 days of culture, IL-2 (20 U/ml) was added to the culture medium (Proleukin, Chiron, Ratingen, Germany). After 10 days of culture, the bulk cultures were analyzed by FACS using tetramers and restimulated with (10^6) irradiated T2 cells pulsed with peptide in IL-2 containing medium. After sorting, the cells were seeded at 1 cell/well in 96-well plates (Costar, Bodenheim, Germany) with 4 x 10^4 irradiated syngeneic PBL (30 Gy) and 2 x 10^4 irradiated T2 cells pulsed with peptide. The cultures were restimulated weekly in the same fashion and tested in ^{51}Cr release assays against T2 and 721 target cells. Wells containing CTLs preferentially recognizing peptide-loaded targets or binding tetramers were expanded. All T cell cultures were performed in IMDM (Life Technologies, Eggenstein, Germany), 10 % human serum (Pel Freez, Mast-diagnostica, Hamburg, Germany), 20 U/ml IL-2 (Proleukin or Lymphocult, Biotest, Dreieich, Germany), 2 mM glutamine (BioWhittaker, Verviers, Belgium) and 50 U/ml penicillin/ 50 µg/ml streptomycin solution (BioWhittaker).

HLA-peptide Tetrameric complexes and Flow Cytometry.

HLA-peptide tetrameric complexes were produced as previously described (21). In brief, the HLA heavy chain was modified by deletion of the transmembrane domain and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site (21). The HLA-A2 heavy chain and β2-microglobulin were produced using a prokaryotic expression system (pET/HLA-A*0201, pET/β-2m plasmids and bacteria kindly provided by Dr. Vincenzo Cerundolo), purified and refolded *in vitro* by limiting dilution with the HLA-A*201 binding peptides. The HLA-A*201 binding peptide used were *Influenza* Matrix$_{58-66}$ GILGFVFTL (22), CEA$_{694-702}$ GVLVGVALI (23) and TyrosinaseA$_{369-377}$ YMDGTMSQV (24). The refolded complexes were purified by gel filtration (Superdex 75 Pharmacia, Uppsala, Sweden) using FPLC, biotinylated by BirA (Avidity, Denver, CO) in the presence of biotin (Sigma Chemical, Deisenhofen, Germany), ATP (Sigma Chemical) and $Mg2^+$ (Sigma Chemical). The biotinylated product was separated from free biotin by gel filtration and ion exchange (MonoQ Pharmacia) using FPLC. Tetramers were assembled by mixing biotinylated protein complexes with streptavidin-PE (Molecular Probes, Eugene, OR) at a molecular ratio of 4 to 0.8.

4 x 10^6 cells from the *in vitro* allostimulations or 2 x 10^5 CTL clones were incubated on ice or 37ºC with 10 µg/ml of tetrameric complexes. After 15 min of incubation, cells were washed extensively with PBS containing 1 % FCS. CD8 antibody (Caltag Laboratories, Burlingame, CA) and CD4 antibody (Immunotech, Marseilles, France) were added and the samples were incubated on ice for further 15 min. After extensive washing, samples were fixed with PBS containing 2 % formaldehyde. Triple-color analysis was performed with Tetramer-PE, CD8-Tricolor and CD4-FITC, using a FACScalibur (Becton Dickinson, Heidelberg, Germany) and

CellQuest software (Becton Dickinson). Sorting was performed without fixation and using a FACSvantage (Becton Dickinson).

Cytotoxicity assay
Targets were labeled with 1.85 MBq of $Na_2{}^{51}Cr0_4$ for 1 h at 37 °C, with or without preincubation with peptide (50 µM) for 1-2 h at room temperature in serum free medium. Labeled targets were incubated for 4 h with the CTLs in RPMI (Life Technology), 10 % FCS (Sigma Chemical), 2 mM glutamine (BioWhittaker) and 50 U/ml penicillin/ 50 µg/ml streptomycin solution (BioWhittaker). Subsequently, 50 µl of the supernatant was harvested. Percent specific lysis was calculated as (cpm experimental counts- cpm media control)/(cpm detergent- cpm media control) x 100 %. Medium controls were between 10 and 15 % of detergent samples.

Results and Discussion

PBLs from an HLA-A*02 negative healthy donor (HLA-A*01/*24, -B*08, -Cw7) were stimulated *in vitro* with the TAP-deficient cell line T2 loaded with the *Influenza* matrix protein MP (MP_{58-66}) peptide as a model antigen (22). The low level of peptides in this TAP-mutant T2 cell line causes most MHC class I molecules to remain empty or to associate with low-affinity peptides (25). By the external addition of peptides, empty molecules can be stabilized and low-affinity peptide replaced (20), leading to stimulation of the PBLs with a high number of a single peptide/MHC complex.
To separate between alloreactive CTL and allorestricted CTL activities, we constructed HLA-A*0201-peptide tetrameric complexes based on the *Influenza* matrix protein MP (MP_{58-66}) peptide. HLA tetrameric complexes have been developed by Altman et al. (21) in order to study peptide-specific $CD8^+$ T cells. They have been used to follow the fate of the immune response after viral or bacterial infections (26, 27). They allowed characterization of cell surface antigens expressed by auto-antigen specific T cells in autoimmune disorders and in tumor patients (26, 28). HLA-peptide tetrameric complexes bind to antigen-specific CTLs with high specificity and show no cross-reactivity on CTLs specific for an irrelevant peptide. Tetramer binding is known to correlate with both peptide specific cytolytic functions and cytokine secretions (26). Furthermore, even down to very low frequencies of antigen-specific T cells, HLA-peptide tetramers allow direct isolation of tetramer-positive cells by FACS (29).
By staining the bulk alloreactive culture with an HLA-A*02-MP tetramer that stained specifically a MP_{58-66} specific CTL clone (Figure 1A), we observed a very low but significant frequency of allorestricted CTLs specific for the MP_{58-66} peptide (Figure 1D). This specific staining was seen only among the T cell blast population (Figure 1D). No staining was observed in the T cell population that was gated for the unstimulated small and round T cells (Figure 1C) and in the fresh PBLs of the same donor (data not shown, see also (29)). Furthermore, using an irrelevant HLA-A*02-peptide tetrameric complex folded with the $CEA_{694-702}$ peptide, no staining was detectable in both the unstimulated and the blast T cell population (data not shown). A striking observation in this experiment is that the vast majority of alloreactive T cells can not bind the HLA-peptide tetramers (Figure 1). Among mouse and human alloreactive CTLs, a dominance of peptide-dependent recognition has been described (30, 31). The determinant recognized by the majority of those alloreactive CTLs that do not bind the HLA-peptide tetrameric complexes in our culture could therefore be dependent on TAP-independent peptides presented by the HLA-A*02 molecule on T2 cells. An alternative explanation would be that these alloreactive CTLs are of low affinity and need a high density of MHC/peptide complexes to be activated *in vitro* (32) and that we are not providing enough HLA-peptide complexes to stain these low affinity T cells.

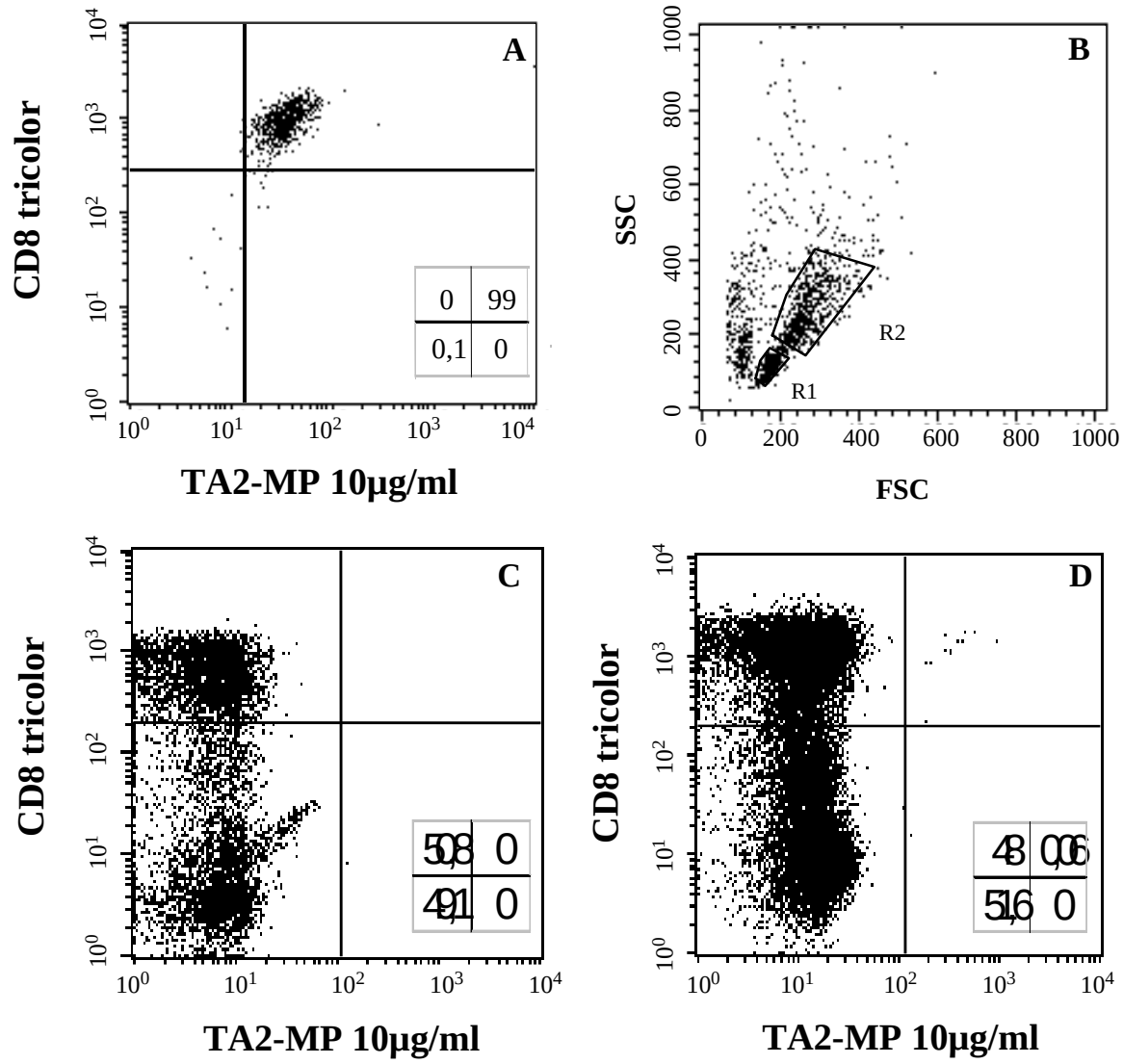

Figure 1: HLA-A*02 tetramer staining of allorestricted CTLs.
HLA-A*0201-MP_{58-66} specific CTL clones (A) or alloreactive bulk cultures (C, D) were stained with HLA-A*02-MP tetramer (TA2-MP) and CD8 antibody. Percentage of positive cells is given in each quadrant. (B) the cells from the alloreactive *in vitro* bulk cultures were gated on size (FSC) and granularity (SSC): small and round cells (R1), and blasts (R2). (C) is the staining of the unstimulated R1 cells and (D) is the staining of the activated R2 cells. To discriminate between activated (R2) and non-activated (R1) cells was necessary to detect a specific-tetramer staining. In C and D, cells that were unspecifically stained by the CD4 antibody were gated out to reduce background.

We further examined the peptide-specificity of the allorestricted CTLs by sorting the tetramer-positive cells using FACS. Tetramer-sorted cells were plated at 1 cell/well and expanded *in vitro* using T2 cells loaded with peptide and autologous feeder cells in IL-2 containing medium. The ability of the CTL clones to lyse peptide-pulsed target cells was then assessed (Figure 2). The $CD8^+$ HLA-A*02-MP $tetramer^+$ clones were able to lyse peptide pulsed HLA-$A*02^+$ target. In contrast, no significant activity was observed on T2 pulsed with an irrelevant peptide (Figure 2). These data confirmed that the tetramer-binding cells in Figure 1C were allorestricted CTLs specific for the MP_{58-66} peptide.

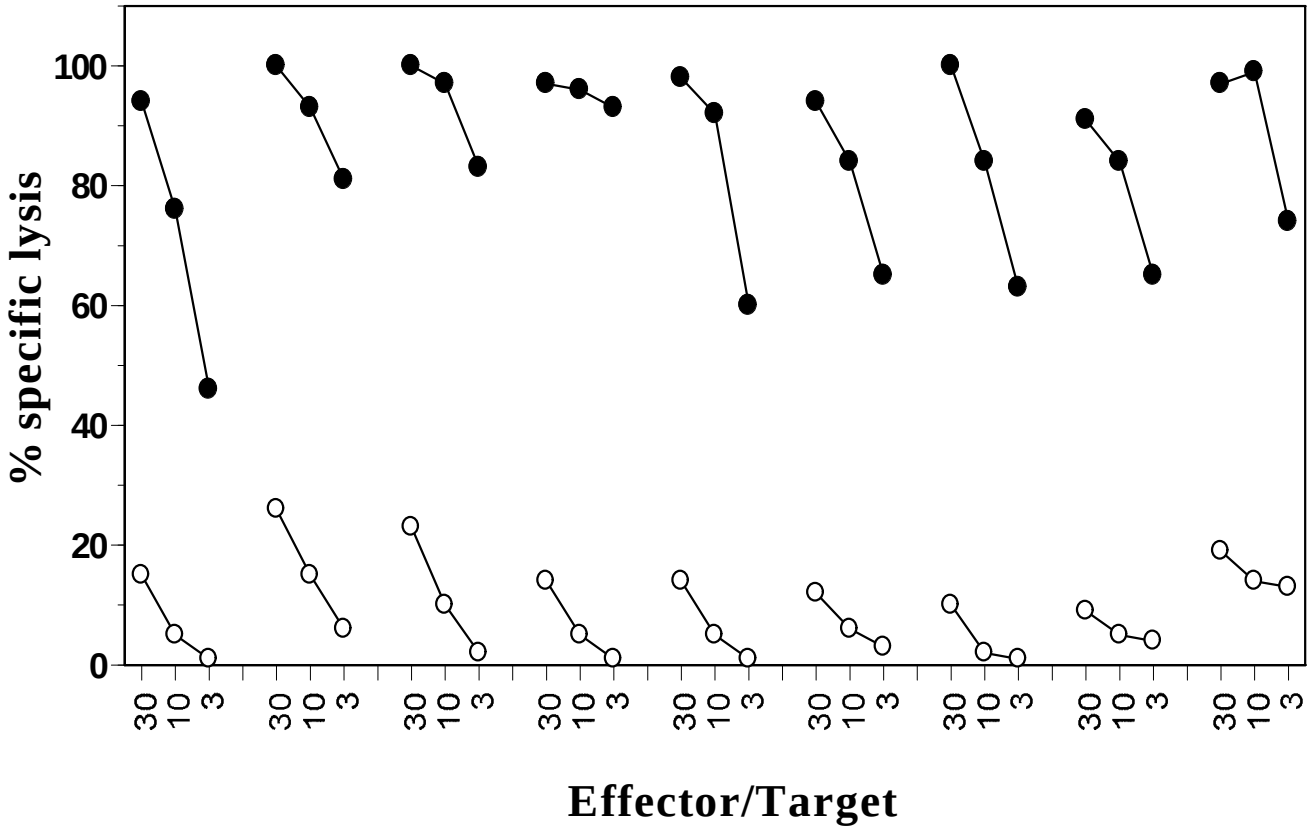

Figure 2: Cytotoxic activity of HLA-A2-MP-sorted alloreactive CTL clones.
The nine different clones were tested in a chromium release assay against the HLA-A*0201 positive LCL 721 pulsed with MP_{58-66} peptide (filled circles) or a control peptide from the RNA-helicase p72 YLLPAIVHI (empty circles).

In a second experiment PBLs from the same HLA-A*02 negative donor were stimulated *in vitro* with T2 cells as described before. As observed previously, $CD8^+$ HLA-A*02-MP $tetramer^+$ cells were detected after tetramer staining of the bulk culture (data not shown). Peptide-specificity of these putative allorestricted CTLs was then assessed again by sorting the tetramer-positive cells using FACS and performing a chromium release assay on peptide-pulsed target cells, as described before (Figure 3). A detailed analysis of one of these CTL clones BC19-3 is described in Figure 3 and -4. In contrast to what we observed previously, HLA-$A*02^+$ peptide pulsed T2 cells were lysed as well as the unpulsed cells (Figure 3). In order to exclude that the CTL clones were exhibiting an NK-cell-like activity, we performed a killer assay using the NK-sensitive HLA-deficient cell line Daudi and an HLA-A*02 negative target KLHE (Figure 3B). None of the cell lines were recognized by the clones (Figure 3), showing clearly that the lysis was MHC-specific and correlate with the expression of HLA-A*02 on the target cells. Thus, these CTL clones are either peptide-independent or dependent on TAP-independent peptides on T2. Since the wild type LCL 721 was equally lysed irrespectively of the peptide pulsed (Figure 3B), it was likely that these CTL clones were not specific for a particular TAP-independent peptide on T2.

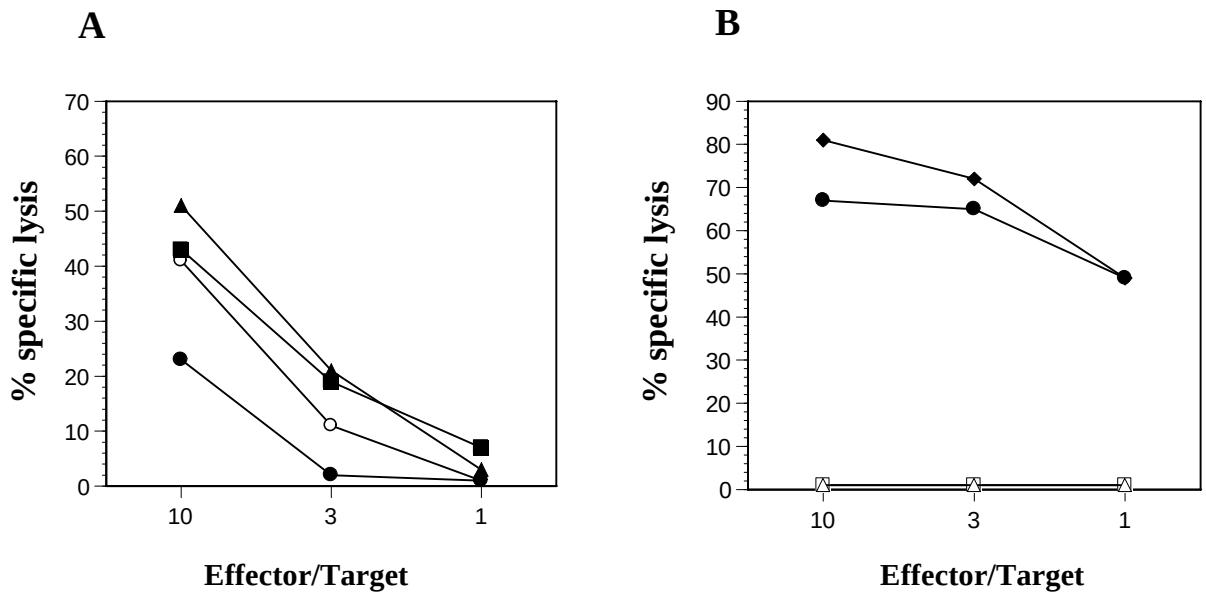

Figure 3: Cytotoxic activity of the HLA-A2-MP-sorted alloreactive CTL clone BC19-3.
(A) Targets: TAP-deficient T2 cell line unloaded (empty circles), loaded with MP_{58-66} (filled circles), $CEA_{694-702}$ (triangles) or $TyrosinaseA_{369-377}$ (squares). (B) Targets: LCL loaded with MP_{58-66} (filled circles) or $MelanA_{27-35}$ AAGIGILTV (diamonds), unloaded β2-microglobulin-deficient Daudi cells (empty squares) and the HLA-A*02 negative breast carcinoma cell line KLHE (empty triangles).

We examined whether the CTL clone described in Figure 3 would bind different MHC/peptide complexes using HLA-peptide tetrameric complexes folded with several peptides. As expected, the CTL clone could be stained by the HLA-A*02-MP tetramer that was used for the FACS sorting. However, this clone could also bind the HLA-peptide tetrameric complex folded with the $CEA_{694-702}$ peptide. In contrast it could not bind the HLA-peptide tetrameric complex folded with the $TyrosinaseA_{369-377}$ peptide (Figure 4). These particular HLA-peptide tetrameric combinations were used because for each of them a positive control was available consisting either of HLA-A*02 transgenic mouse CTLs or human CTL clones specific for the HLA-A*02-CEA (data not shown) or the HLA-A*02-TyrA tetramers respectively (data not shown and manuscript in preparation; Teichgräber et al.). This alloreactive CTL clone is therefore peptide-selective but not peptide-specific (Figure 4). It might be sensitive to the conformation of the HLA molecule that is adapted when particular, but unrelated, peptides are bound (2, 3). One or more peptides of this sort seem to be present at the surface of T2 and 721 as they are both recognized in a killer assay irrespectively of the peptide pulsed (Figure 3). Our group speculated on the existence of such alloreactive T cells in a previous study where the recognition of HPLC-fractionated K^b-extracted peptides by K^b-specific CTLs was analyzed (3). In that study a unique alloreactive CTL line that recognized multiple HPLC-fractions was described. Different groups obtained analogous results, which suggested that some clones may be peptide-dependent but not peptide-specific inasmuch as a variety of different peptides could promote their recognition of target cells (3, 33, 34). Thus, the present study shows for the first time directly that such alloreactive peptide-selective CTLs exist.

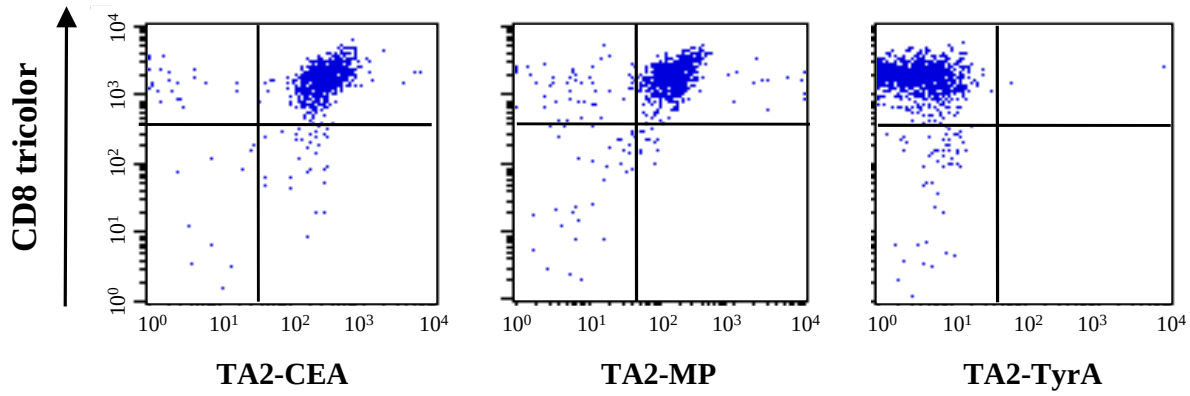

Figure 4: HLA tetramer staining of the alloreactive CTL clone BC19-3.
Double staining was performed using the HLA-A*02 tetramers and CD8 antibody: HLA-A*02-$CEA_{694-702}$ (TA2-CEA) (left panel), HLA-A*02-MP_{58-66} (TA2-MP) (middle panel), and HLA-A*02-$TyrA_{369-377}$ (TA2-TyrA) (right panel).

Using HLA-peptide tetrameric complexes, we have described a new approach to generate peptide-specific CTL that could be useful for clinical tumor immunotherapy. Allogeneic bone marrow transplantation as treatment of leukemia patients is often associated with GvHD which is detrimental for the patient, but also graft-versus-leukemia (GvL) which is positively correlated with the prognosis (35, 36). Both activities are attributed to alloreactive donor T cells (36). The transfer of donor allorestricted CTLs specific for a leukemia tumor antigen at the same time as stem cell transplantation could be of particular interest to enhance the GvL without causing GvHD (36, 37). A better understanding of the nature of the determinants recognized by GvL-mediating alloreactive CTLs is of obvious interest to establish new transplantation protocols. Importantly, undetected crossreactivity of tumor-specific allorestricted T cells could induce immunopathology such as GvHD. We have shown that HLA-peptide tetrameric complexes are a powerful tool that allows the characterization of recognition patterns by alloreactive T cells and new insights in the molecular basis of alloreactivity.

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3

The peptide-specific alloreactive human T cell repertoire varies largely between individuals

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Submitted for publication

*Alloreactive T cells recognise framework or peptide-dependent determinants on foreign MHC molecules. Among the peptide-dependent alloreactive T cells a significant proportion is specific for one particular peptide presented by the allo-MHC molecule as antigen-specific T cells would do. Such alloreactive, peptide-specific T cells are referred to as "allorestricted". High avidity HLA-A*02 allorestricted CTL clones specific for peptide libraries can be generated from HLA-A*02 negative donors. We made use of this technique to study the role of closely related self-HLA molecules on shaping of the alloreactive T cell repertoire. PBLs from HLA-A*0205 individuals were stimulated by HLA-A*0201 targets pulsed with an HLA-A*0201 peptide library. We did not observe a bias towards peptide-specific CTLs in the HLA-A*0201-directed alloreactive repertoire of HLA-A*0205 donors as compared to HLA-A*02 negative donors. Comparison of the alloreactive T cell response between two donors having similar HLA haplotypes demonstrated that the allorestricted T cell repertoire is largely different between individuals.*

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Introduction

Allograft rejection and graft versus host disease (GvHD) are the clinical manifestations of T cell reactivity to foreign MHC molecules (1). Although the molecular basis of allorecognition has been extensively studied in the past twenty years it remains enigmatic. A puzzling observation is that the frequency of cytotoxic T lymphocytes (CTLs) that recognise allogeneic MHC class I molecules is typically 10- to 100-fold higher than that of T cells recognising viral antigen presented by self-MHC molecules (2).
One model to explain this paradox suggested that T cell receptors directly recognise allelic polymorphism on the MHC molecules (3). The expression of such epitopes at high density on the cell surface would result in stimulation of T cells bearing low as well as high affinity receptors for the allogeneic MHC molecule. The principal studies supporting this model described CTL clones that recognise the MHC molecule in the absence of bound peptide (4,5). An alternative model suggested that T cells recognise many different cellular antigens, i.e. peptides, together with the foreign molecule. The strong alloreactive response would then be explained by multiple binary interactions between alloreactive T cell clones and new peptides not presented by self-MHC (6). In the meantime, existence of peptide-specific alloreactive T cells has been clearly demonstrated (7-11). These allorestricted CTLs can recognise specific peptide/MHC combinations just like nominal antigen-specific T cells do (12-14). Furthermore, the mouse as well as the human allorestricted T cell repertoire is broad and diverse, as shown by the use of peptide libraries (12,14). In addition, several studies have indicated the existence of peptide-dependent CTLs (8,15,16) that are not specific for a particular peptide. This fraction of alloreactive T cells might be sensitive to the conformation of the MHC that is adapted when particular, but unrelated, peptides are bound to the MHC molecule (2). The nature of determinants involved in the alloreactive T cell recognition appears therefore to be very diverse. Still, overall a dominance of peptide-dependent recognition among CTL has been reported in mice and human (17,18).
The existence of high avidity CTLs in the diverse repertoire of allorestricted T cells (12-14), raises the possibility of generating CTLs reactive against low-copy-number peptides bound to non-self MHC molecules. Many tumours overexpress normal proteins thereby modifying the set of self-peptides associated with MHC class I molecules. This phenomenon allows triggering of tumor specific CTLs. However, CTLs undergo negative selection and peripheral tolerance mechanisms that diminish the number of or eliminate self-peptide-specific CTLs. Since tolerance to self-antigens is self-MHC restricted (19), allorestricted T cells can be raised against self-antigen including tumour associated antigens (self as seen from the tumour's host) and presented by allogeneic MHC (foreign for the T cell) for adoptive immunotherapy (19,20). Indeed, it has been recently shown that allorestricted CTLs specific for mdm-2 wild type peptide were a successful reagent for immunotherapy (13) and that the CTLs engrafted and retained specificity in the host without causing GvHD (21).
Studying the influence of the self-MHC molecules on the shaping of the alloreactive T cell repertoire could therefore provide technical insights into the generation of allorestricted T cells *in vitro* but also into thymic positive selection of T lymphocytes. In a recent report, our group has demonstrated that the closer the foreign MHC molecule is related to the T cell's MHC, the higher is the proportion of allorestricted T cells versus peptide-independent T cells in the mouse (22). In the present study, we analysed this relation in human T cell population. We compared the influence of closely related HLA alleles on the allorestricted human T cell repertoire by stimulating and testing PBLs from HLA-A*0205 positive and HLA-A*02 negative donors with the TAP-deficient T2 cell line pulsed with a HLA-A*0201 peptide library.

Materials and Methods

Cells

The human EBV-transformed lymphoblastoid B cell line (LCL) 721 (HLA-A*0201,-A*0101, -B*5101, -B*0801, -Cw1) (23) and the TAP-deficient cell line T2 (HLA-A*0201^{low}, -B*5101^{low},-Cw1^{low}) (24) were used in ^{51}Cr release assays or for T cell stimulation. Cell lines were maintained in RPMI 1640 (Life Technology, Eggenstein, Germany), 10 % FCS (Sigma Chemical, Deisenhofen, Germany), 2 mM glutamine (BioWhittaker, Verviers, Belgium) and 50 U/ml penicillin/ 50 µg/ml streptomycin solution (BioWhittaker).

Generation of CTLs

PBL from healthy donors registered in the Blood Bank (Tübingen, Germany) were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). PBLs were plated in limiting dilution in round-bottom 96-well plates (Costar, Bodenheim, Germany), starting with 10^5 cells/well and dilution steps of 1:2. In each well the effector cells were stimulated with 2 x 10^4 irradiated T2 cells (200 Gy) pulsed with the synthetic HLA-A*0201 peptide library. Pulsing was performed overnight at room temperature with 10 µM peptide library in serum free medium (25). After 7 days of culture, the effector cells were restimulated with 2 x 10^4 irradiated T2 cells pulsed with peptide and 4 x 10^4 irradiated syngeneic PBLs (30 Gy) as feeder cells in IL-2 containing medium (Proleukin, Chiron, Ratingen, Germany). The cultures were tested 5-7 days later in a split-well ^{51}Cr release assay against T2 and 721, as described (12,14). Wells containing CTL preferentially recognising peptide-loaded targets were then expanded in 6 wells of 96-well plates with 4 x 10^4 irradiated syngeneic PBLs and 2 x 10^4 irradiated T2 pulsed with peptide library. All T cell cultures were performed in IMDM (Life Technologies), 10 % human serum (Pel Freez, Mast-diagnostica, Hamburg, Germany), 20 U/ml IL-2, 2 mM glutamine (BioWhittaker) and 50 U/ml penicillin/ 50 µg/ml streptomycin solution (BioWhittaker).

Cytotoxicity assay

Targets were labelled with 1,85 MBq of $Na_2{}^{51}CrO_4$ for 1h at 37°C, with or without preincubation with the peptide library (50 µM) for 3 h at room temperature in serum free medium. Labelled targets were incubated for 4 h with the CTLs in RPMI (Life Technology), 10 % FCS (Sigma Chemical), 2 mM glutamine (BioWhittaker) and 50 U/ml penicillin/50 ug/ml streptomycin solution (BioWhittaker). Subsequently, 50 µl of the supernatant was harvested and radioactivity was measured in a microplate format scintillation counter (1450 Microbeta Plus, Turku, Finland), using solid phase scintillation (Luma Plate-96, Packard, Dreieich, Germany). Percent specific lysis was calculated as (cpm experimental counts- cpm media control)/(cpm detergent- cpm media control) x 100 %. Medium controls were between 10 and 15 % of detergent samples.

Peptides and Peptide library

The synthetic HLA-A*0201 peptide library was already described (14). In brief, the peptide library was produced by applying the Fmoc-amino acids in threefold excess in the respective position, using a peptide synthesiser 423A (Applied Biosystems, Weiterstadt, Germany). The library was analysed by reverse phase HPLC (System Gold, Beckman Instruments, Munich, Germany) and mass spectrometry (G2025A, Hewlett-Packard, Waldbronn, Germany). The library contains 34 560 peptides. The peptide called p68 used as negative control in the experiments was derived from the RNA-helicase p72 (YLLPAIVHI).

Results

Comparison of the peptide-specific versus peptide-independent alloreactive T cell repertoire between two unrelated donors.

To compare the influence of HLA-A*02 alleles on the repertoire of alloreactive T cells, PBLs from an HLA-A*0205 positive donor (HLA-A*0205/A*11, -B*07/*49, -Cw7) and an HLA-A*02 negative donor (HLA-A*01, -B*08/44, -Cw2/w7) were stimulated against T2 loaded with the HLA-A*0201 peptide library under limiting dilution condition. The PBLs were seeded at 10^5 cells/well as starting concentration and diluted in 1:2 steps down to 6 x 10^3 cells/well, a cell number giving rise to a high probability of clonal $CD8^+$ T cells (14). After one round of restimulation, each individual well was tested in a split-well chromium release assay on T2 cells loaded with p68 peptide (YLLPAIVHI) as a control and T2 cells loaded with the HLA-A*0201 peptide library (Figure 1). Each well's activity is represented as a dot. Dots close to the diagonal are considered to represent framework-specific and not allorestricted or peptide-dependent CTLs (Figure 1). At the lowest cell concentration of HLA-$A*0205^+$ PBLs, in no case was there a deviation from the diagonal, indicating that the great majority of the alloreactive CTLs were framework-specific (Figure 1, B). At the highest cell concentration, only few CTL lines from the HLA-A*0205 donor showed signs of peptide specificity (Figure 1, A). In contrast, several wells from the HLA-A*02 negative PBL showed clones (Figure 1, D) and lines (Figure 1, C) with potential peptide specificity.

The lines and clones from both donors exhibiting at least some peptide-specificity were expanded and tested in a chromium release assay at different effector to target ratios (E/T) (Figure 2). Two lines (2-35 and 2-59) out of 4 (Figure 2, B) and 1clone (10-63) out of 4 (Figure 2, C) of the HLA-A*02 negative donor showed specificity for the HLA-A*0201 peptide library loaded on T2. However, out of the 2 lines expanded from the HLA-A*0205 positive donor only one showed a very weak peptide specificity, if any. The results presented in Figures 1 and 2 are representative of the highest and lowest number of PBL seeded per well. The experiment was performed in duplicates, and for each dilution the complete split-assay was performed. Furthermore, lines or clones were expanded and tested again in a ^{51}Cr release assay. All these data are summarised in Table I. Overall, the HLA-A*0205 positive donor did not give rise to a higher number of peptide-specific or peptide-dependent alloreactive CTLs than the HLA-A*02 negative donor, as we would have expected from our previous mouse data. In a different experiment using a second HLA-A*0205 donor and another HLA-A*02 negative donor (HLA-A*03/24,-B*07/50, -Cw2/w7), significant differences were not observed either (data not shown). These results suggested that the HLA-$A*0205^+$ PBL repertoire is not more prone to react in a peptide-dependent manner towards HLA-A*0201 than the HLA-A*02 negative PBL repertoire.

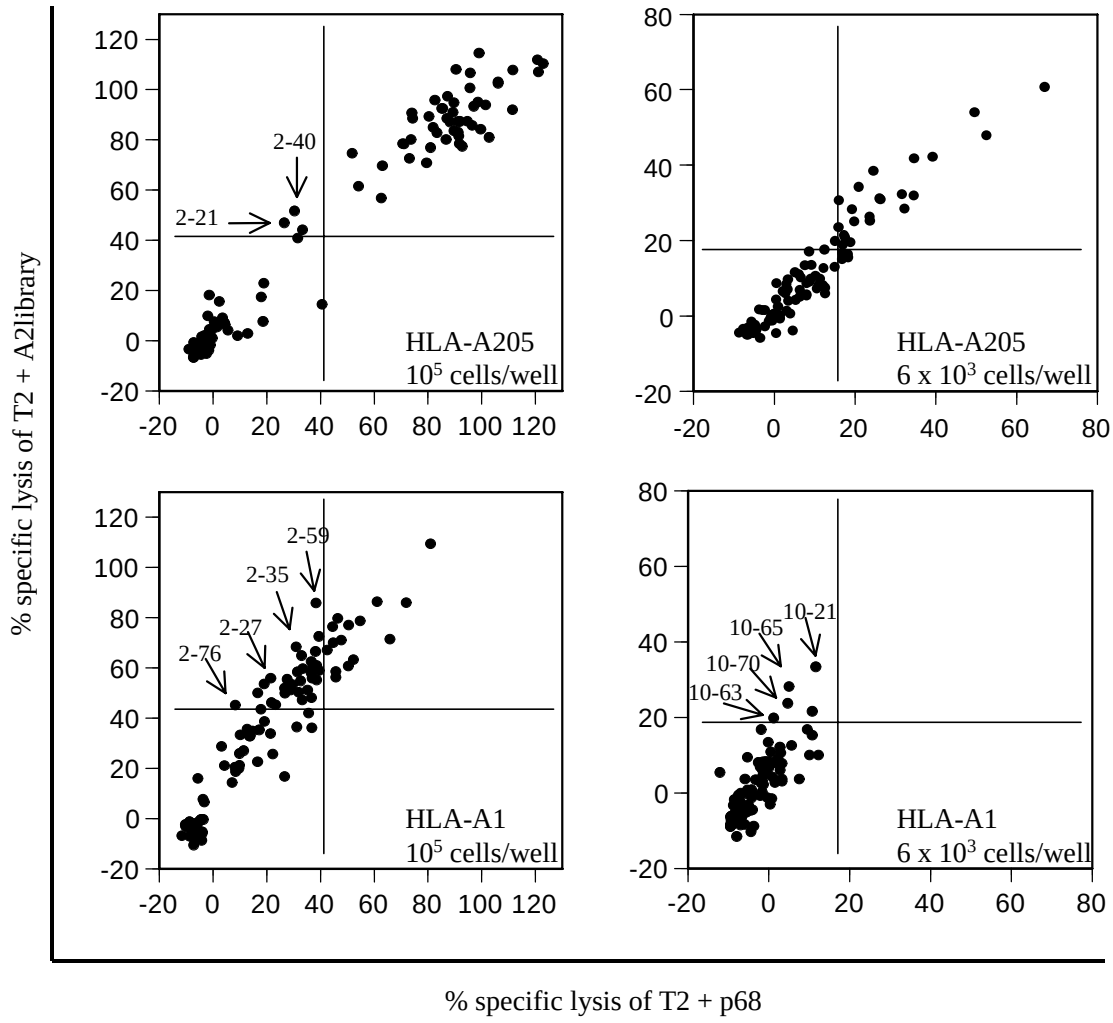

Figure 1: Peptide specificity of the PBL stimulations.
Individual limiting dilution wells from the HLA-A*0205^{+} responder (A-B) and the HLA-A*01^{+} (C-D) were assayed against T2 pulsed with a control peptide, called p68, from the RNA-helicase p72 (YLLPAIVHI) and T2 pulsed with the HLA-A*0201 peptide library. PBL stimulations with the highest cells/well ratio (A-C) and with a high probability of clonality (B-D) are presented. Wells containing peptide library specific CTLs were defined as those negative for lysis of T2+p68 and positive for lysis of T2+library and are in the upper left region of the arbitrarily chosen quadrant. Arrows indicated peptide-specific wells that were expanded.

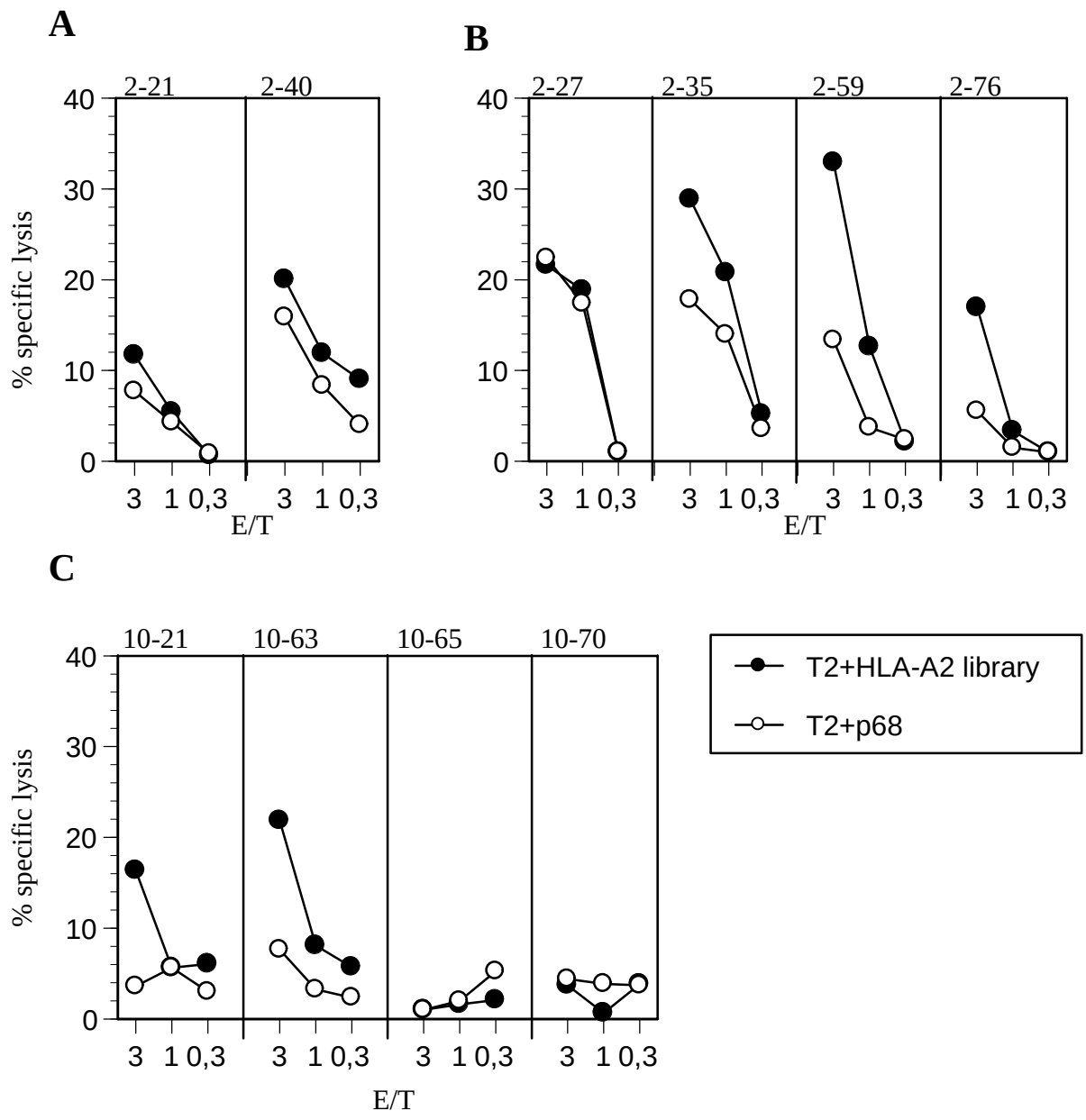

Figure 2: Alloreactive CTLs expanded from the PBL stimulations.
Cell lines expanded from the 10^5 cells/well plates (A, B) and clones from the 6 x 10^3 cells/well plates (C) were tested for peptide specificity in a killer assay using different effector to target ratio (E/T). (A) HLA-A*0205$^+$ CTLs and (B, C) HLA-A*01$^+$ CTLs. p68 control peptide in the figure legend corresponds to RNA-helicase p72 (YLLPAIVHI).

Table I: Reactivity patterns against peptide library of alloreactive CTLs from limiting dilution cultures.

	appeared peptide specific		peptide-specific CTL lines or clones	
Cells/well	A*0205	A*01	A*0205	A*01
10^5	0	7	NA	3
10^5	3	5	1	3
5.10^4	0	7	NA	5
5.10^4	3	9	NA	0
$2,5.10^4$	2	5	1	0
$2,5.10^4$	6	7	0	1
$1,25.10^4$	2	2	0	0
$1,25.10^4$	5	8	0	1
6.10^3	0	8	NA	0
6.10^3	0	4	NA	0
Total	21	62	≥2	13

Limiting dilution cultures were set up as described in Methods. At the end of 12-15 days, individual culture wells were split into two aliquots and tested in a ^{51}Cr-assay against T2 and T2 loaded with the HLA-A*0201 peptide library. Wells displaying a significant peptide-specificity (% lysis of T2 pulsed with the library at least 10 % higher than % lysis of T2 pulsed with control peptide, see Figure 1) were expanded and tested in a second ^{51}Cr-release assay on T2 pulsed with the library or pulsed with control peptide. The responder cell doses lower than 2,5 x 10^4 had a high probability of clonality, i.e.<30% of cultures were specific for HLA-A*0201. HLA types are as follow (HLA-A*0205/*A11, -B*07/*49, -Cw7) and (HLA-A*01, -B*08/44, -Cw2/w7). (NA: not applicable)

Comparison of the peptide-specific versus peptide-independent alloreactive T cell repertoire between two donors with similar HLA haplotypes.

T cells are selected in the thymus for their ability to bind self-MHC molecules and are negatively selected in order to be tolerant to self-MHC molecules associated with self-antigens. For this negative selection mechanism the up to 6 classical HLA class I allele products in heterogenous individuals all contribute in shaping the T cell repertoire. Thus, we speculated that the lack of dramatic differences in the alloreactive T cell repertoire in the previous experiment could be due to the heterogeneous HLA context of the donors masking a potential influence of HLA-A*0205. To test this hypothesis, we repeated the PBL stimulations, as described before, but with two donors having a similar HLA haplotype, at the exception of one HLA-C and one HLA-A allele. One donor was HLA-A*0205 and the other HLA-A*24 (see Table II). The results of the split-well chromium release assay on T2 cells loaded with control peptide or the HLA-A*0201 peptide library are shown in Figure 3. As we observed before, the majority of the CTL lines from both donors showed specificity for a structure on T2 independent of the peptides added. Furthermore, for the HLA-A*0205 PBLs there was no well displaying significant peptide specificity neither at high (Figure 3, A) nor low (Figure 3, B) cell number per well. PBLs of the HLA-A*02 negative donor showed only borderline peptide specific lysis at 10^5 cells/well (Figure 3, C). In contrast, at lower cell number per well some CTLs showed peptide specificity (Figure 3, D). Still, for all dilutions, we decided to expand CTLs that lysed T2 pulsed with the HLA-A*0201 peptide library at least 10 % more than they lysed T2 pulsed with control peptide (Figure 3). The CTL 1-66, for example, that lysed T2 pulsed with the library with 69 % specific lysis and T2 pulsed with control peptide with 47 % specific lysis was selected, expanded and tested in a killer assay at different E/T ratio (Figure 4). Out of the 5 lines of the HLA-A*02 negative donor tested, only one (2-12) showed a significant specificity for the HLA-A*0201 peptide library loaded on T2 (Figure 4, C-D). However, out of the 6 lines expanded from the HLA-A*0205 positive none was clearly peptide specific.

These results suggested again that even when the HLA background of the two donors is largely overlapping, the HLA-A*0205$^+$ PBL repertoire seems not to be more prone to react in a peptide-dependent manner towards HLA-A*0201 as compared to the HLA-A*02 negative PBL repertoire.

Table 2: Comparison of the HLA class I haplotypes of the two donors used in Figure 3.

	HLA molecules					
	A		B		C	
HLA-A*02 $^+$ donor	***0205**	*03	*07	*50	w7	**w6**
HLA-A*02 $^-$ donor	***24**	*03	*07	*50	w7	**w2**

Differences in HLA class I expression are shown in bold.

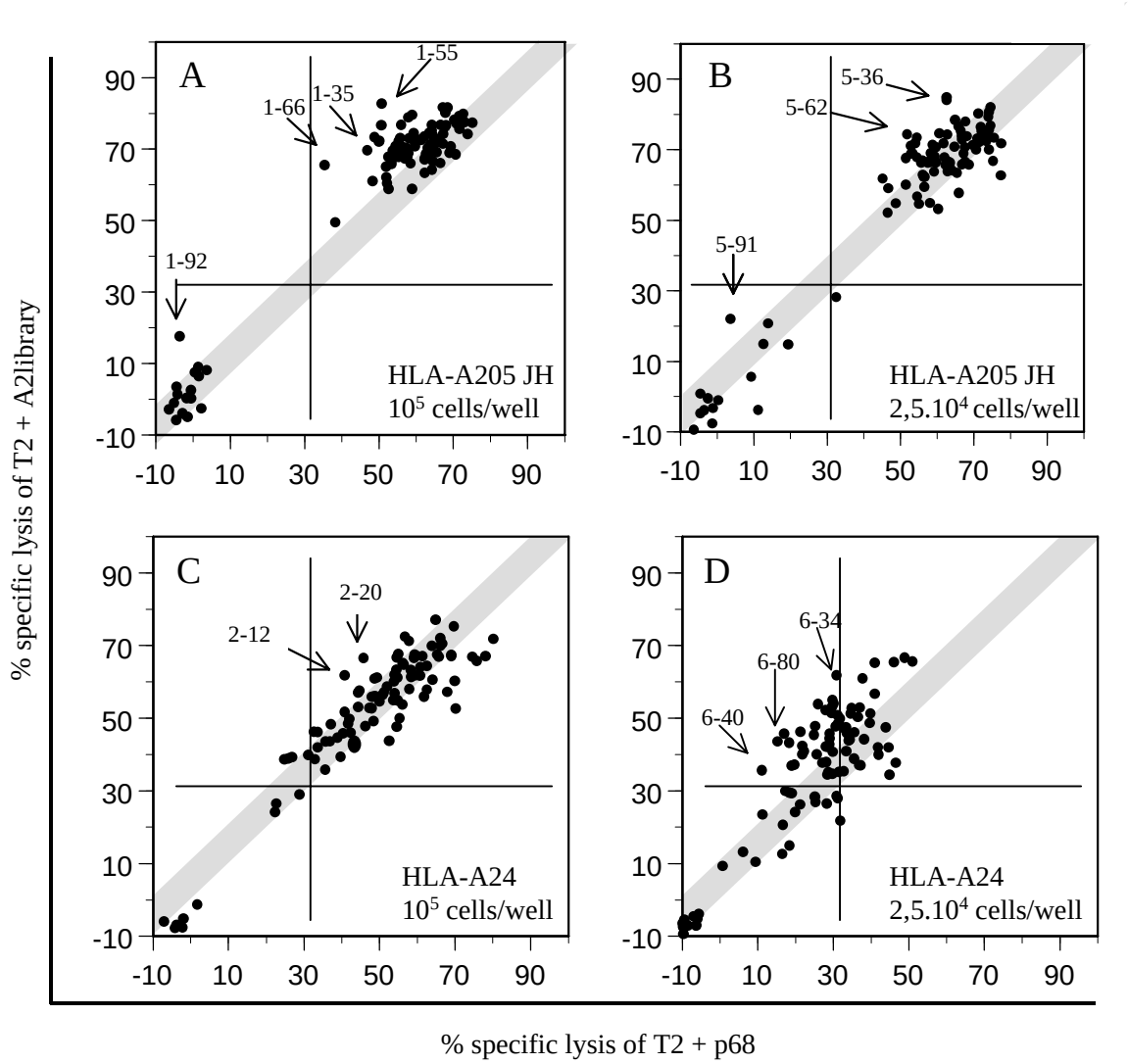

Figure 3: Peptide specificity of the PBL stimulations from two similar HLA haplotypes. Individual limiting dilution wells from the HLA-A*0205^{+} responder (A-B) and the HLA-A*24^{+} (C, D) were assayed against T2 pulsed with a control peptide RNA-helicase p72 (YLLPAIVHI) and T2 pulsed with the HLA-A*0201 peptide library. PBL stimulations with the highest (A, C) and a lower (B, D) cells/well ratio are presented. Data presented are representative of the limiting dilution panel. Wells containing peptide library specific CTLs were defined as those negative for lysis of T2+p68 and positive for lysis of T2+library and are defined in the upper left region of the arbitrarily chosen quadrant. However, as in the majority of the stimulations (A, B, C and data not shown) no culture with significant peptide-specificity was observed, we considered expanding lines that showed at least 10 % more lysis against T2 pulsed with the library (located above the grey line). We assumed that those lines were a mix of peptide-specific and structure-specific cells. Arrows indicated peptide-specific wells that were expanded.

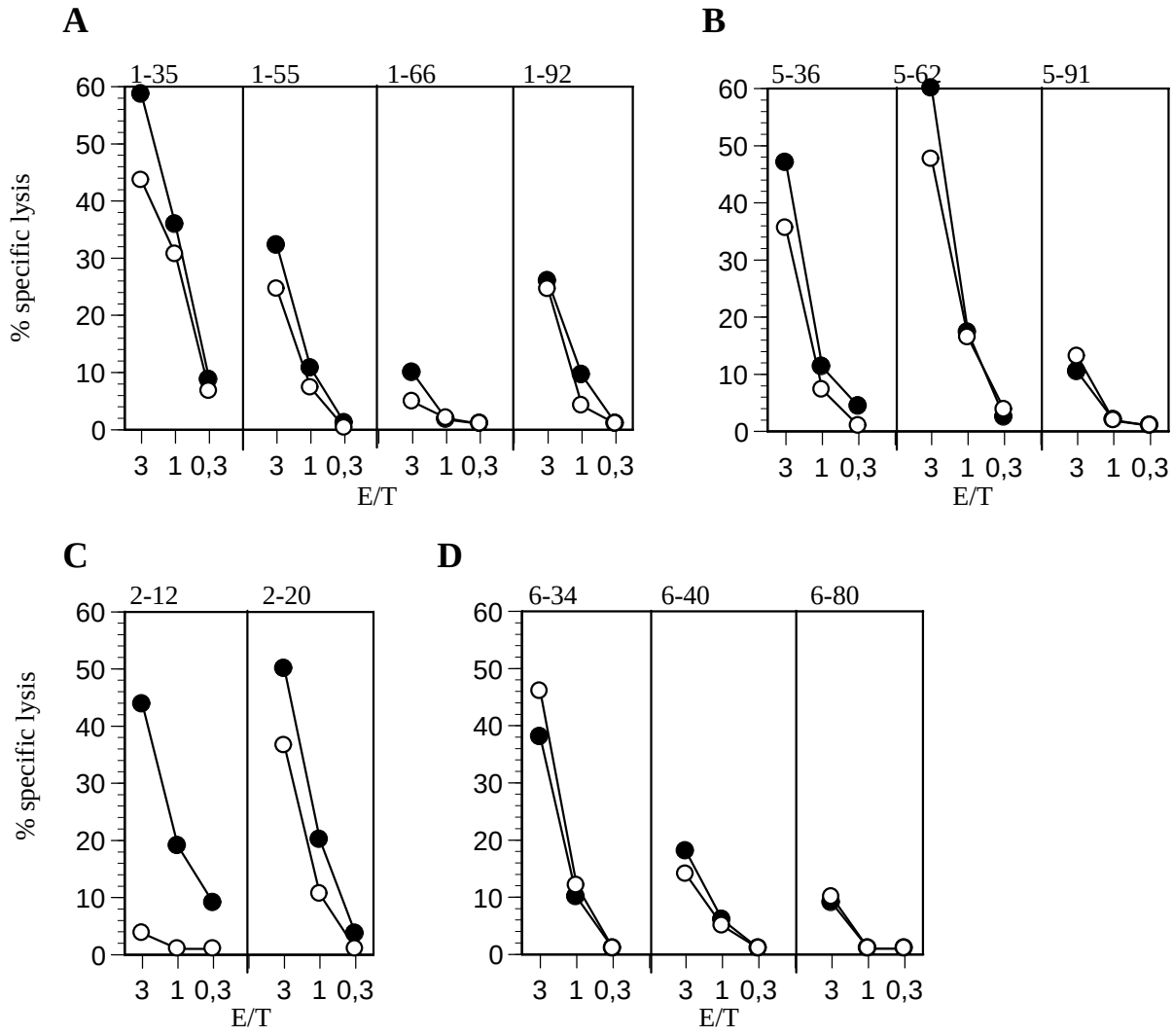

Figure 4: Alloreactive CTLs expanded from the PBL stimulations of two similar haplotype. Cell lines expanded from the 10^5 cells/well plates (A, C) and lines from the 2,5 x 10^4cells/well (B, D) were tested for peptide specificity in a killer assay using different effector to target ratio (E/T) against T2 pulsed with the peptide library (full circle) and pulsed with a control peptide RNA-helicase p72 (YLLPAIVHI) (empty circle). (A, B) HLA-A*0205$^+$ CTLs and (C, D) HLA-A*24$^+$ CTLs.

Discussion

Our group has previously shown that high avidity HLA-A*02 CTL clones specific for peptide libraries could be generated from HLA-A*02 negative donors (14). In the present report, we made use of this technique to study the role of closely related HLA molecules on the shaping of the alloreactive T cell repertoire. Although we could generate allorestricted CTLs, we did not find a bias towards peptide-specificity in the alloreactive T cell repertoire of three HLA-A*0205 donors, in comparison with HLA-A*02 negative donors, when stimulated by T2 cells loaded with an HLA-A*0201 peptide library. In the human system an alloresponse against a related HLA molecule seems not to contain more peptide-specific T cells than a response against an unrelated one. This appears to be in contrast to our previous observations with the mouse alloreactive T cell repertoire (12).

The T cell repertoire is reactive to gross as well as subtle changes in MHC-peptide complexes. T cells can react against self-peptide when presented in a new context, i.e. amino acid substitutions in the α-helices at positions that point towards the TCR can stimulate T cells (26,27). T cells can be raised in a peptide-specific way against completely allogeneic MHC molecules, having allelic differences in the peptide-binding groove as well as in the helices (12-14). Furthermore, mutations in the peptide-binding groove that change only the spectrum of bound peptides can elicit an alloreactive response (22,28). We speculated that this latter constellation should allow an easier generation of allorestricted CTLs against self-, viral or tumour-associated antigens. Indeed, in the mouse model our group found that alloreactions against MHC molecules carrying groove mutations only were clearly dominated by peptide-specific CTLs (22). In contrast, alloresponses directed against molecules with both groove mutation and α-helices substitutions were more prone to give rise to structure-specific T cells (22). In the present study, we made use of the natural HLA-A*02 polymorphism to test this hypothesis with human T cells. The HLA-A*0201 molecule is found in all populations studied and shows a strong predominance in the Caucasian population (>95% of HLA-A*02 individuals). The majority of the amino acid substitutions that discriminate the HLA-A*02 alleles clusters around the peptide-binding groove of the MHC class I molecule. The HLA-A*0205 allele that was used in this study has 4 amino acid substitutions: Tyr^{9} (Y) and Trp^{156} (W) affect the pocket B,C and D,E respectively of the peptide-binding groove and therefore modify the array of peptide presented by the molecule (29-31); Arg^{43} (R) and Leu^{95} (L) do not interfere with the peptide (31). None of these amino acids was identified as a potential binding site for the T cell receptor on the class I molecule (for review see (32)). The HLA-A*0205 allele was therefore the perfect candidate to study the T cell alloreactive repertoire against HLA-A*0201. However, we could not see a domination of the allorestricted towards structure-specific T cell response compared to an unrelated donor expressing HLA-A*01 (Figure 1), which has multiple amino acid substitutions affecting the peptide-binding groove, the helices and the TCR contacts (31,32). In our previous report we used K^{b} mutants having amino acid substitutions on the K^{b} molecule but having otherwise strictly the same class I (D^{b}), class II molecules and non-MHC molecules (22). We therefore performed the stimulation by HLA-A*0201 molecules with PBLs of HLA-A*0205^{+} and HLA-A*24^{+} donor having almost an identical set of remaining HLA class I allelic products (Table II). Although we could generate allorestricted CTLs, no significant difference in the alloreactive repertoire of the two donors was observed either.

We concluded earlier from our mouse experiments that the allorecognition is readily influenced by the selecting MHC molecule and might mirror the resemblance between self and foreign. The T cell repertoire is indeed shaped by positive and negative selection on self-MHC molecules, in the thymus and by peripheral mechanisms (for review see (33)). Our present data do not exclude an influence of the selecting MHC on the alloreactive T cell

repertoire. A single peptide/MHC complex can select a large panel of $CD8^+$ T cells (33), however the overall contribution of the up to 6 different class I molecules in the selection of one $CD8^+$ T cell is not clear so far. One possible explanation for our data could be that we did not find a donor having a permissive HLA context that would allow to study the influence of the selecting HLA-A*0205 molecule alone on the shaping of the alloreactive repertoire directed against HLA-A*0201. The other HLA molecules could have shaded the influence of HLA-A*0205 molecule by negatively selecting potential peptide-specific alloreactive T on HLA-A*0201. Furthermore, we focused on the classical MHC molecules but other polymorphic HLA molecules (HLA-E, G), or MHC-like molecule (MICA/B) (34) and also non-MHC molecules could also play a role in the selection mechanisms. Moreover, the history of infection with immunogenic viruses might also have influenced the alloreactive repertoire of the donors (35). An alternative explanation is that in the human system allorecognition is not influenced by the selecting HLA molecules, but we consider this as being unlikely.

Allogeneic bone marrow transplantation as treatment of leukaemia patients is often associated with GvHD which is detrimental for the patient, but also graft-versus-leukaemia (GvL) which is positively correlated with the prognosis (36). Both activities are attributed to alloreactive donor T cells. The transfer of donor allorestricted CTLs specific for a leukaemia tumour antigen at the same time as stem cell transplantation could be of particular interest to enhance the GvL without causing GvHD (37). A better understanding of the mechanism shaping the repertoire of alloreactive T cells is therefore of great interest to establish new protocols for the generation of allorestricted CTL against tumour or viral epitope to be used in immunotherapy.

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4

Allo-MHC class I recognition by human Vγ9/Vδ2 T lymphocytes

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Submitted for publication

In contrast to αβ T cells, γδ T cells recognize a broad variety of structurally unrelated antigens. Vγ9/Vδ2 T cell that represent the vast majority of γδ T cells in the human periphery are activated by non-peptidic phosphoantigen (PAgs). A subset of Vγ1/Vδ1 T cell have been recently shown to recognize the CD1c molecule. In this report, we describe the first Vγ9/Vδ2 T cell clone -B18- specific for an HLA class I molecule. We provide evidences that the recognition of HLA molecules by γδ T cells is independent of NKR expression and function. Remarkably, although expressing the Vγ9/Vδ2 TCR the B18 clone was not activated by bacterial phosphoantigen and does not express any known NKR. Importantly, the Vγ9/Vδ2 TCR of the B18 clone used the Cγ2 gene segment, was therefore non-disulfide-linked and very similar to the TCR of the Vγ1/Vδ1 T cell subset. Our results suggest that the particular conformation adapted by non-disulfide-linked γδ TCRs is more prone to react with molecules of the MHC superfamily.

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Introduction

Cytotoxic T cells (CTL) play a major role in immuno-surveillance against tumor cells and in controlling viral and bacterial infections. CTL express either the T cell receptor (TCR) -α/β or TCR-γ/δ, two distinct receptor heterodimers that recognize antigen and confer T cell specificity. In mice, γδ T cells expand in response to mycobacteria, listeria, salmonella (1). Mice lacking γδ but not αβ T cells die when infected with large inoculate of mycobacteria (2;3). In humans, γδ T cells dramatically expand up to 50% of all T cells in the peripheral blood following infection with bacteria or parasites (4). γδ T cells also display a strong cytotoxicity against tumor cells when activated with IL-2 (5). This cytotoxic activity is reminiscent of natural killer (NK) cells that preferentially lyse target cells which have lost MHC class I expression (6), a frequent occurrence in the course of tumor progression (7).

TCR-α/β is used by the majority of human PBLs. It is extremely diverse and recognizes antigenic peptides presented in the context of MHC class I or class II molecules (8). In contrast, TCR-γ/δ is expressed by a minor population in human PBLs and is encoded by only a limited number of V and J gene segments (4). Most circulating human γδ T cells use the same combination of variable regions, Vγ9 and Vδ2 to form their TCR (4). Vγ9/Vδ2 T cells use the Cγ1 gene segment and therefore express a disulfide-linked TCR (9). Vγ9/Vδ2 T cells are specifically activated by non-peptidic phosphorylated antigens derived from Mycobacteria (10;11), primary alkylamines found in bacterial supernatants also naturally occurring in edible plants (12) and by poorly characterized ligands expressed by most B-cell lymphomas (13). TCR transfection experiments have showed that the recognition of these short non-peptidic antigens is mediated via the TCR (14) and is critically dependent on the CDR3 sequence of the γδ TCR (15). The mechanism by which these antigens are presented is not known, but it does not involve the MHC class I or MHC Class II antigen presentation and processing machinery (16). Vγ9/Vδ2 T cells were never found to be specific for any MHC or MHC-like molecule.
Vγ9/Vδ2 T cells upon activation by IL-2 display a strong MHC unrestricted cytotoxicity against tumor cells (17). This cytotoxic activity as well as cytokine secretion of the Vγ9/Vδ2 T cells, has been shown to be modulated by a family of receptors originally cloned on NK cells, the NK cell receptors (NKR) (18). NKR are divided in two different groups, the KIRs belonging to the immunoglobulin (Ig) superfamily and the CD94/NKG2-A, B, C heterodimers members of the C-type lectin glycoprotein superfamily (19). In both families, these receptors can either inhibit (KIRDL and CD94/NKG2A, B) or activate (KIRDS and CD94/NKG2C) the NK and T cell functions (19). While KIRDL receptors bind to HLA-A, B or C alleles (20), CD94/NKG2A and B bind to HLA-E (21), both interactions inhibiting the NK cells' cytotoxic machinery against target cells expressing normal MHC class I levels. However cells with reduced MHC class I expression will not engage binding with KIR at the surface of the NK cells and therefore will be more sensitive to cytolysis by NK cells. The contribution of the KAR in the NK lysis remains enigmatic but activation of the NK lytic machinery might be the result of a balance between activating (KAR) and inhibitory (KIR) signals. As in NK cells practically all human Vγ9/Vδ2 T cells were recently shown to express NKR (18;22-25).

The vast majority of γδ T cells in tissues such as intestine and spleen, uses the Vδ1 gene segment which is mainly paired with a Vγ segment, distinct from Vγ9, belonging to the VγI family (4). VγI/Vδ1 T cells use the Cγ2 gene segment, and therefore express a non disulfide-linked TCR (9). Although the nature of the antigen recognized by the VγI/Vδ1 TCR remains poorly characterized, its antigenic repertoire seems to be much more diverse than that of the Vδ2 subset. Recently γδ T cells were found to recognize the CD1c molecule (26). The CD1c

molecule is a member of the non polymorphic CD1 molecule, a separate lineage of antigen presenting molecules that can present non-peptidic antigens to T cells (27). The structure of the CD1 molecule is very similar to that of MHC class I molecules (28). VγI/Vδ1 TCR mediated recognition of CD1c was clearly demonstrated and was shown to be independent of the presence of exogenous foreign antigen (26). γδ T cells of this subset were previously found to recognize the MHC-encoded proteins MICA and MICB (29). MICA and MICB are distantly related to MHC class I molecules but are functionally distinct. These molecules have no role in the presentation of peptide antigens, but they may function as stress inducible self-antigens (30). Recognition might be through the activating NKG2-D molecule with , in contrast to the CD1c recognition, an unclear contribution from the VγI/Vδ1 TCR (31). In mice the recognition of the non classical MHC molecule T10/T22 by the γδ G8 clone (32) and by a small subset of γδ T cells (33) could be reminiscent of the CD1c and MICA/B recognition in human.
A few mouse and human γδ T cell clones have been shown to be specific for classical MHC class I and MHC class II molecules (34). The recognition of IE^k by the murine γδ LBK5 clone was shown to be independent of the peptide bound to IE^k (34) but influenced by the glycosylation of the IEα domain (35). Several human γδ T cell clones were shown to recognize HLA-A*02 (36), HLA-A*24 (37), HLA-A*09 (38), HLA-B*27 (39), HLA-DR molecules (40-43) or HLA.-DQA1/DQB1 heterodimers (44). All the γδ T cell clones characterized in these reports used the VγI/Vδ1 TCR, except for a small fraction using the Vδ3 gene segment (42). However most of the clone display also a strong NK-like cytotoxic activity against NK-sensitive cell lines. Although γδ T cells of the Vδ1 subset usually do not express NK receptors, we have recently reported the involvement of a VγI/Vδ1 T cell clone expressing NKR in autoimmune disorder (45). Therefore one can not exclude an NKR contribution in the Vδ1 subset lytic functions. Especially one could not exclude an involvement of the NKR in the MHC class I recognition by VγI/Vδ1 T cells.

In this report we investigated the function of NKR negative Vγ9/Vδ2 T cells. We describe the first Vγ9/Vδ2 T cell clone -B18- specific for an HLA class I molecule. Remarkably, the B18 Vγ9/Vδ2 T cell clone did not secrete cytokine when incubated with PAgs. The TCR of this clone was non-disulfide-linked and therefore similar to the TCR of the VγI/Vδ1 T cell subset.

Materials and Methods

Cell lines.
The human EBV-transformed lymphoblastoid B cell lines (LCL) 721 (HLA-A*0201,-A*01,-B*05, -Cw1) was used for T cell stimulation (46). The β2m-deficient Burkitt's lymphoma (BL) Daudi (47), the BLs Akuba, Silfere (HLA-B*5801^{+}) and the follicular B cell lymphoma Bjab (48), were used in killer and/or cytokine release assays. The class I^{+} Daudi variant E8.1 has been derived following transfection of human β2m genomic DNA (49). LCL 721.221, a derivative of LCL 721 that lack expression of HLA-A, -B, -C, -G and retains HLA-E intracellularly (50), β2m-transfectants of β2m-deficient colon carcinoma cell line HCT (51) and β2m-deficient melanoma cell line FO-1 (49) were used for transfection of HLA class I molecules and chromium release assay. All cell lines were maintained in culture in RPMI 1640 (Life Technology, Eggenstein, Germany), 10 % FCS (Sigma Chemical, Deisenhofen, Germany), 2 mM glutamine (BioWhittaker, Verviers, Belgium) and 50 U/ml penicillin/50 µg/ml streptomycin solution (BioWhittaker).

Immunofluorescence Analysis and mAbs
The phenotype of the T cell clones and the tumor cell lines was analyzed by standard techniques of direct or indirect immunofluorescence staining using a FACScalibur (Becton Dickinson, Heidelberg, Germany) and the CellQuest software (Becton Dickinson). The fluorochrome conjugated mAb were specific for TCR-γδ (FITC-conjugated anti-TCRδ1), TCR αβ (FITC conjugated anti-BMA031) (both from T Cell diagnostica Cambridge). The unconjugated mAbs specific for the V regions of the TCR γδ were directed against Vδ2 (anti-divVδ2a), Vδ1 (dTCS1) (both provided by Dr. M. Bonneville, Nantes, France) and Vγ9 (anti-TiγA provided by Dr. T. Hercend, Paris, France). The HLA class I specific mAbs were W6/32 (reactive with all β2m-associated HLA class I molecules) and B1.23.2 (specific for HLA-B and C molecules). The NKRs specific mAbs were EB6 (specific for p58.1 and p50.1, also called KIR2DL1 and KIR2DS1 respectively), GL183 (specific for p58.2 and p50.2, also called KIR2DL2 and KIR2DS2 respectively), Z27 (anti-p70 or KIR3DL1) and XA185 (anti-CD94). MHC class I and NKR specific mAbs were hybridoma culture supernatants.

T cell clones.
PBLs were obtained from healthy volunteer donors by Ficoll-Hypaque density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). PBLs were double stained with mAbs against the Vγ9 TCR region (using anti-TiγA) and one of the NKR specific mAbs using antibodies directed against p58.1, p58.2, p70, p140 and CD94. γδ T cells expressing one of the NKRs or lacking expression of CD94 were isolated into vial containing irradiated autologous PBL feeder cells, using a FACStar^{plus} cell sorter (Becton Dickinson, Erembodegen-Aalst, Belgium). Subsequently, the sorted cells were cloned at 0.5 cells/well with feeder cells in 96-well plates (Costar, Bodenheim, Germany) as described (52). The NKR and the V region gene expressions of selected clones was confirmed by FACS with specific mAbs. T cell clones were grown in complete culture medium [IMDM (Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 50 U/ml penicillin/50 µg/ml streptomycin solution, 25 mM Hepes Buffer (All from BioWhittaker, Verviers, Belgium)] supplemented with 10 % human serum (heat-inactivated AB serum, Pel Freez, Mast-diagnostica, Hamburg, Germany), 400 U/ml rIL-2 (Proleukin, Chiron, Ralingen, Germany) and 0.25 µg/ml purified PHA (Murex Diagnostika, Burgwedel, Germany). The clones were supplemented with fresh culture medium at least once a week and restimulated with irradiated feeders (2-4 x 10^4 PBLs) (30 Gy) plus irradiated LCL 721 (1-2 x 10^4 cells) (100 Gy) per round bottom microwell at 1-3 week intervals.

Chromium release assay.
Targets were labelled with 50 µCi of $Na_2{}^{51}Cr0_4$ for 1 h at 37°C, with or without preincubation with anti-HLA class I mAb for 30 min at 37°C. Labelled targets were incubated for 4 h with the CTLs in RPMI (Life Technology), 10 % FCS (Sigma Chemical), 2 mM glutamine (BioWhittaker) and 50 U/ml penicillin/50 ug/ml streptomycin solution (BioWhittaker). Subsequently, 50 µl of the supernatant was harvested and radioactivity was measured in a microplate format scintillation counter (1450 Microbeta Plus, Turku, Finland), using solid phase scintillation (Luma Plate-96, Packard, Dreieich, Germany). Percent specific lysis was calculated as (cpm experimental counts- cpm media control)/(cpm detergent- cpm media control) x 100 %. Medium controls were between 10 and 15 % of detergent samples.

HLA-B5801/02 cDNA cloning.
Daudi mRNA was extracted using the Quick-prep Micro mRNA Purification Kit (Pharmacia, Freiburg, Germany) and reverse transcribed into oligo-dT-primed cDNA (First-Strand cDNA Synthesis Kit, Pharmacia). The HLA-B heavy chains were amplified by PCR (1.5 min 94°C, 1.5 min 60°C, 2 min 72°C for 24 cycles) with the Vent-DNA-Polymerase (New England Biolab, Schwalbach, Germany) using primers flanking the coding sequence: sense primer 5'-GGGCAAGCTTGGACTCAGAATCTCCCCAGACGCCGAG-3' and anti-sense primer 5'-CCGCGGATCCCTGGGGAGGAAACACAGGTCAGCATGGGAAC-3' (the Hind III and BamH I restriction enzyme sites added for cloning are underlined). The PCR products were passed through Sephadex G50 columns (Pharmacia), digested with both enzymes (Boehringer Mannheim) , separated on LMP gels (Gibco BRL, Eggenstein, Germnay) and the bands were purified from gel slices (Sephaglass, Pharmacia). The eluted fragments were cloned into the pBluescript II KS^+ vector and positive colonies were identified by colony hybridization using the radioactively labeled PCR fragments (Oligolabelling Kit, Pharmacia) as probes. Single-stranded DNA obtained by single-stranded rescues with the M13KO7 helper phage, were sequenced using the Sequenase 2.0 DNA sequencing Kit (Amersham, Braunschweig, Germany) and the primers: 5'-GTTCGACAGCGACGCCGCGA-3' (177-196), 5'-TACGACGGCAAGGATTAC-3' (424-441), 5'-GGAGATCACACTGACCT-3' (705-721), 5'-GTGGTGGTGCCTTCTGGA-3' (811-828), 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' (Plasmid-DNA) and 5'-AGCGGATAACAATTTCACACAGGA-3' (Plasmid-DNA) (indicated are the primer positions in the HLA heavy chain cDNA-sequence). Sequences were compared with the described HLA class I cDNA sequences (http:// www.anthonynolan.com/HIG/data) and identified as HLA-B*5801 and HLA-B*5802. Using the Hind III and BamH I restriction sites the cDNA were subsequently cloned into the expression vector p636 (provided by Dr. W. Sugden, Madison, WI). p636 is an episomal vector that is able to replicate in EBV^+ cells by means of its oriP. It confers Hygromycin B resistance to eukaryotic cells in which the cDNA expression is under the control of the CMV promoter. The cDNA sequences were also blunt ligated in the pBOS vector that confers puromycin resistance (provided by Dr. T. Rabbitts, Cambridge, UK) for transfection in solid tumors.

HLA-B5801/02 Transfection in LCL and Solid Tumors.
The LCL 721.221 were transfectant by cell electroporation with the p636-B*5801 and p636-B*5802 plasmids. 10^7 cells were incubated with 20 µg DNA for 5 min at 4°C in serum free medium. Cells were electroporated at 960 µF and 230 V in 60 ms ($Gene^{pulser}$ II, Bio-Rad, Munich, Germany). The electroporated cells were allowed to rest for 48 h before addition of Hygromycin-B (200 µg/ml) (Boehringer Mannheim, Mannheim, Germany). Hygromycin-B resistant LCLs were tested for expression of HLA-B*5801 or HLA-B*5802 using mAbs B1.23.2 or W6/32 and sorted for high expression using $FACStar^{plus}$. The colon carcinoma

HCT-β2m and the melanoma FO1-β2m cell lines were transfected with pBos-B*5802 using the same protocol, except that the DNA was linearised prior to electroporation with KpnI (Boehringer Mannheim) and transfectants selected for Puromycin resistance (1 µg/ml) (Sigma Chemical, Deisenhofen, Germany). Puromycin resistant cells were tested for expression of HLA-B*5802 using mAb B1.23.2 and high expressing cells sorted.

TNF release assay

Supernatant TNF content was measured using the previously described cytotoxic assay against WEHI 164 clone 13 cells (53). At least one week after the last restimulation, T cells (2 x 10^3) were incubated in V-bottom 96-well plates (Costar, Bodenheim, Germany) with phosphoantigens or in U-bottom 96-well plates (Costar, Bodenheim, Germany) with non irradiated BLs (20 x 10^3) for 24 h in 100 µl of culture medium supplemented with 10 U/ml rIL-2 (Proleukin, Chiron, Ralingen, Germany). Isopentenyl pyrophosphate (Sigma Chemical, Deisenhofen, Germany) was used at 10 µM, TUbag-1 (kindly provided by Dr. J.-J. Fournié, Toulouse, France) at 1 mM and the *E.coli* phosphoantigen (kindly provided by Dr. Wilhelm, Würzburg, Germany) at 3.10^{-4} M. Subsequently, 50 µl of the supernatants were added to 50 µl of actinomycin D-treated (2 µg/ml, Sigma Chemical) WEHI cells (30 x 10^3 cells) in flat-bottom 96-well plates (Costar) and incubated for 24 h at 37°C. After incubation, 50 µl of tetrazolium salts (MTT, 2.5 mg/ml, Sigma Chemical) was added to each well and incubated for 4 h. Formazan crystals were solubilised with 100 µl of lysis buffer (1 vol. of N,N-dimethyl formamide, 2 vol. of 30 % SDS, adjusted at pH 4.7 with acetic acid). After 24 h, the plates were measured in an ELISA reader (Spectra^{max} 340 Molecular Devices, Munich, Germany) at 570 nm with 650 nm as control wavelength, percent dead cells were calculated and these values were converted into pg/ml using a calibration curve of serially diluted rhTNF-α (Boehringer Mannheim). Experiments were performed in triplicate determination and each supernatant was tested at two dilutions (1:1 and 1:10).

Generation of an EBV-transformed cell line

Epstein-Barr virus (EBV) was derived from the marmoset B95-8 cell line following standard protocols (54). EBV containing culture supernatant was incubated with 5 x 10^6 PBLs from the Vγ9/Vδ2 B18 clone blood donor for 1 h at 37°C. Subsequently, 8 ml culture medium containing 2 µg/ml cyclosporin A (Sandimmun, Sandoz, Basel, Switzerland) were added to the cells. After a week incubation the same culture medium volume was again added to the cells. Clumps of proliferating cells appeared 3 weeks after transformation.

TCR analysis by PCR

The mRNA of the Vγ9/Vδ2 T cells was extracted and reverse transcribed as described before. The Vγ9/Vδ2 TCRs were amplified by PCR (2 min 95°C, 2 min 60°C, 3 min 72°C for 24 cycles) with the Vent-DNA-Polymerase (New England Biolab) using primers flanking the coding sequences. The primer used to amplified the γ chain were: VG9F 5'-TAAGAGCTCGAGGCCATGCTGTCACTGCTCCACACATCAAC-3' and CG1B 5'-TAAGGTACCTCGAGATGGCCTCCTTGTGCCACCGTCTG-3'. The prime set used for amplification of the δ chain was: VD2F 5'-TAAGAGCTCGAGGCCATGCAGAGGATCTCCTCCCTCATC-3' and CDB 5'-TAAGGTACCTCGAGTGTAGCTTCCTCATGCCAGTCAG -3'. In the VG9F and VD2F primer sequences are underlined the Sac I and Xho I restriction enzyme sites, added for cloning, followed by the Kozak sequence. In the CG1B and CDB primer sequences is underlined the Kpn I restriction enzyme site added for cloned. The primer combinations were designed for the PCR products to be cloned into a Sac I-Kpn I restricted pBluescript II KS$^+$ vector, as described before. The CDR3 regions were sequenced as described using the appropriate primers.

Results

Selection of NKR negative Vγ9/Vδ2 T cells

Readily all Vγ9/Vδ2 T cells express NKRs at the cell surface. We pursued efforts to isolate NKR negative killer Vγ9/Vδ2 T cells. γδ T cells lacking the expression of the C-lectin type CD94/NKG2 receptor dimers were selected by FACS. Most of the expanded clones displayed no lytic activity with some having an NK-like activity when tested in a chromium release assay against the HLA class I negative Daudi cells and HLA class I positive BLs (data not shown). However, the γδ T cell clone B18 was selected for its striking capacity to lyse the HLA class I positive Daudi variant E8.1 (Figure 1, B). Surprisingly, the B18 clone did not react to the HLA class I negative Daudi cells (Figure 1, B). In contrast, the NKR^+ selected γδ T cell clones displayed strong lytic activity against Daudi (Figure 1, A and see(18)). Furthermore, preincubation of the E8.1 target with an HLA-B and -C specific antibody blocked lysis by the B18 clone (Figure 1, B). B18 clone did not show cross-reactivity, with or without the addition of HLA class I specific mAbs, on the HLA class I^+ B cell lymphomas Akuba and Bjab (Figure 1, B). These results suggested that the recognition was dependent upon specific interaction with Daudi's HLA-B or -C alleles. The lytic pattern of B cell lymphomas by the γδ B18 clone was strikingly different than the killing pattern of NKR^+ γδ T cell clones (Figure 1-A). NKR^+ γδ T cells reacted strongly against Daudi. However upon transfection of Daudi with β2-microglobulin (49), the T cell clone lytic machinery was inhibited (Figure 1, A and (18)). NKR^+ γδ T cells lysed the HLA class I^+ B cell lymphomas (Figure 1, A) but with a relatively lower efficiency as compared to Daudi. Full target lysis could be rescued by blocking the interaction between the NKR and the HLA-B or -C molecules using an HLA-B and -C specific antibody (Figure 1, A).

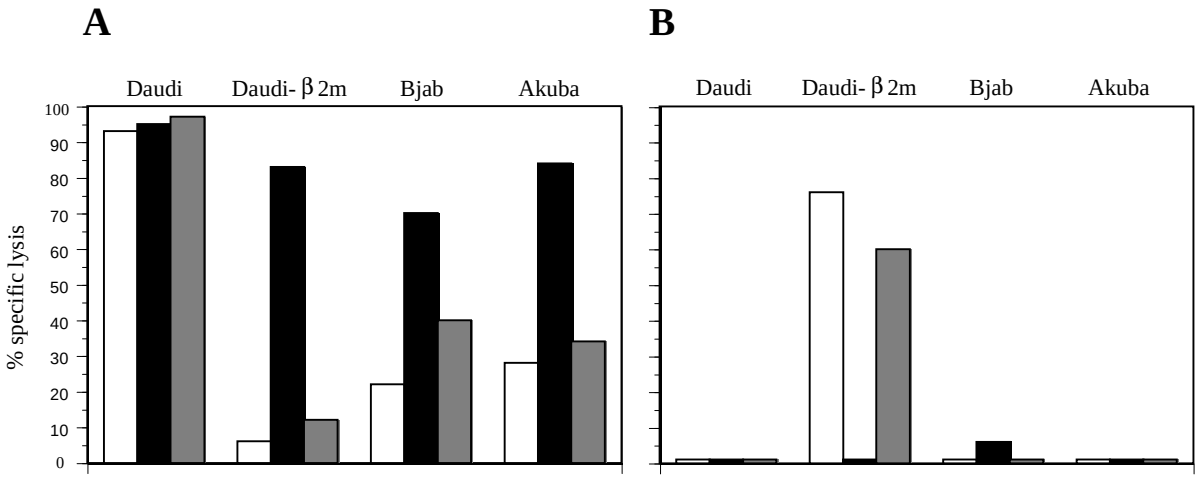

Figure 1: Recognition of B cell lymphomas by sorted NKR^+ and NKR^- $Vγ9^+$ T lymphocytes. NKR^+ clone A23 (A) and NKR^- clone B18 (B) were tested in a chromium release assay against HLA-deficient Daudi cells, β2m-transfected Daudi and two B cell lymphomas (E/T ratio 3:1). Target cells were either not preincubated (*white column*) or preincubated with the anti-HLA-B/C B1.23.2 antibody (*black column*) or the anti-HLA class I W632 antibody (*grey column*). W632 could not block B18 recognition of β2m-transfected Daudi, presumably because it did not bind the same epitope as B18, and was used as a negative control for the blocking experiments to exclude ADCC.

Vγ9/Vδ2 B18 clone uses a unique TCR and does not express NKR at the cell surface

The γδ T cells were selected for the expression of the Vγ9 TCR region. We therefore confirmed that the Daudi reactive γδ T cell clones were using the Vγ9/Vδ2 TCR heterodimer required for recognition of the BLs (15;18). All Daudi reactive γδ T cell clones tested indeed expressed the Vγ9 variable region in combination with the Vδ2 variable region (Figure 2, A). None of them expressed either the Vδ1 variable region or an αβ TCR heterodimer (Figure 2, A). Similarly, the B18 clone appeared to express the Vγ9 variable region in combination with the Vδ2 variable region, without residual expression of the Vδ1 variable region or the αβ TCR (Figure 2, A). Furthermore, Daudi reactive Vγ9/Vδ2 T cell clones and Vγ9/Vδ2 B18 clone did not express a second γ chain, as tested by Vγ chain specific antibody staining and PCR analysis (data not shown) (55).

We further analyzed by FACS the NKRs expression by the Vγ9/Vδ2 T cell clones. As expected, the Daudi reactive clones expressed at least one NKR (Figure 2, B). Vγ9/Vδ2 A23 clone was stained positively by EB6, Z27, XA183 mAbs, specific for the KIR2DL1/KIR2DS1, KIR3DL1 and CD94 receptors respectively (Figure 2, B). In contrast, Vγ9/Vδ2 B18 clone was not stained by any NKR specific antibody tested. Therefore, it does not express any Ig superfamily receptors either inhibitory KIR2DL1, KIR2DL2, KIR3DL1 (Figure 2, B) and KIR3DL2 (tested with mAbs Q66, data not shown) or activatory KIR2DS1, KIR2DS2 (Figure 2, B). It also does not express any CD94/NKG2A, B and C heterodimers (Figure 2, B).

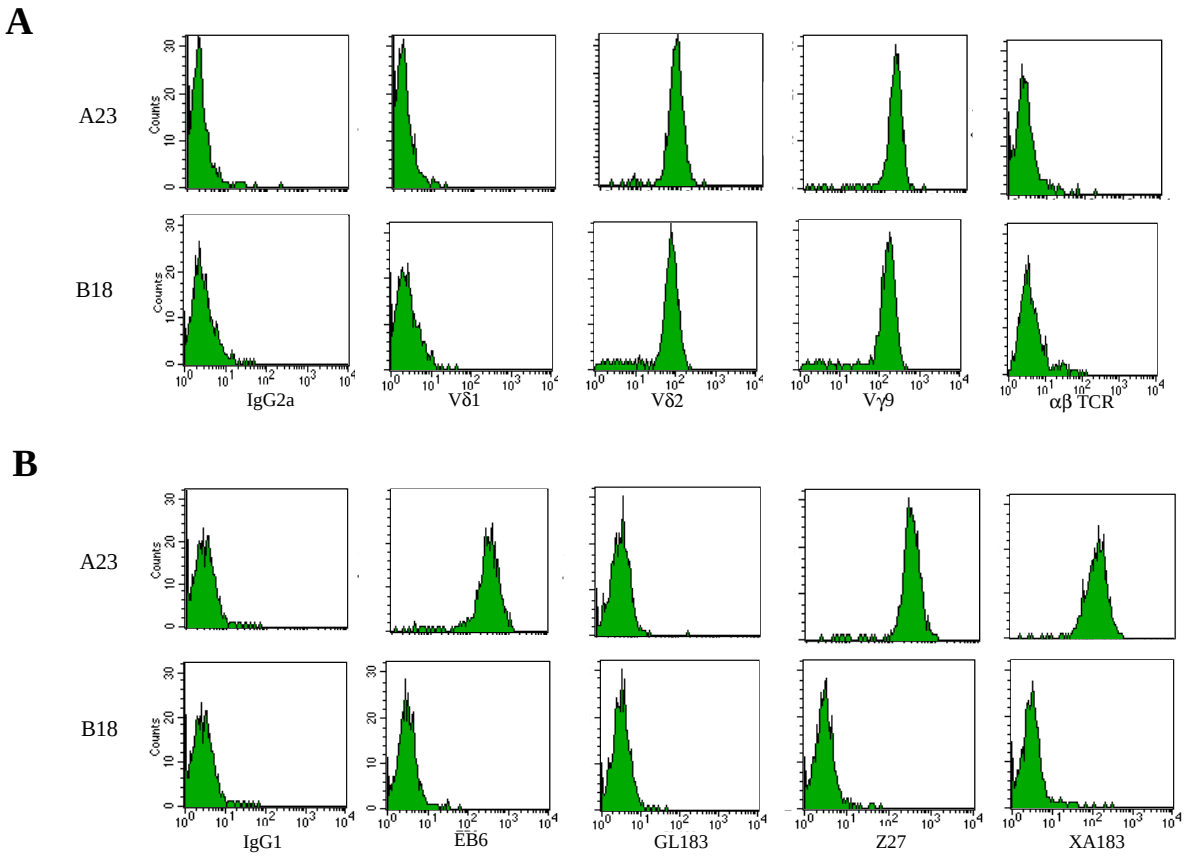

Figure 2: Flow cytometric analysis of the A23 and B18 clones.

The mAbs used were (A) IgG_{2a} (isotype control) and the anti-TCR antibodies dTCS1 (anti-Vδ1), div-Vδ2 (anti-Vδ2), anti-Tiγ9 (anti-Vγ9), BMA031 (anti-αβ TCR); (B) IgG_1 (isotype control) and the anti-NKR antibodies EB6 (specific for KIR2DL1 and KIR2DS1), GL183 (specific for KIR2DL2 and KIR2DS2), Z27 (specific for KIR3DL1) and XA183 (anti-CD94).

*Vγ9/Vδ2 B18 clone is specific for HLA-B*5801/02*

In order to further characterize the specificity of the Vγ9/Vδ2 B18 clone we extracted, reverse transcribed the mRNA of Daudi, and PCR amplified the cDNA encoding for the HLA-A, B and C molecules. The resulting cDNA fragments were sequenced, identified and cloned into the p636 expression vector. Subsequently, the HLA class I deficient LCL 721.221 (50) was transfected and the LCLs expressing the constructs selected by FACS using the pan HLA class I mAb W632 (data not shown). The transfectants were then used as target in a chromium release assay with the Vγ9/Vδ2 B18 clone (Figure 3). Vγ9/Vδ2 B18 clone did not react to the mock transfected LCL 721.221 (Figure 3), but lysed the LCLs expressing HLA-B*5801 or HLA-B*5802 (Figure 3, A and B). This lysis could be blocked by the addition of an mAb specific for HLA-B molecules (Figure 3, A). B18 did not recognize Daudi's HLA-A and C molecules (data not shown). Vγ9/Vδ2 B18 clone showed, therefore, a clear specificity for the Daudi's HLA-B molecules: HLA-B*5801 and HLA-B*5802. In contrast, NKR^+ Vγ9/Vδ2 T cell clones lysed the HLA deficient LCL 721.221 (Figure 3, A). HLA-B*5801 cDNA expression conferred resistance to the target cells, that could be blocked upon addition of an mAb specific for HLA-B molecules (Figure 3, A). In order to test if we were looking at an alloreactive recognition of the HLA-B*5801/02 by the Vγ9/Vδ2 B18 clone, we generated an autologous EBV transformed B cell line from the blood of the healthy donor. The Vγ9/Vδ2 B18 clone was unable to lyse the autologous LCL (Figure 3, B). HLA blood typing also confirmed that the donor was HLA-B*5801/02 negative. Vγ9/Vδ2 B18 clone was alloreactive against HLA-B*5801/02 molecules.

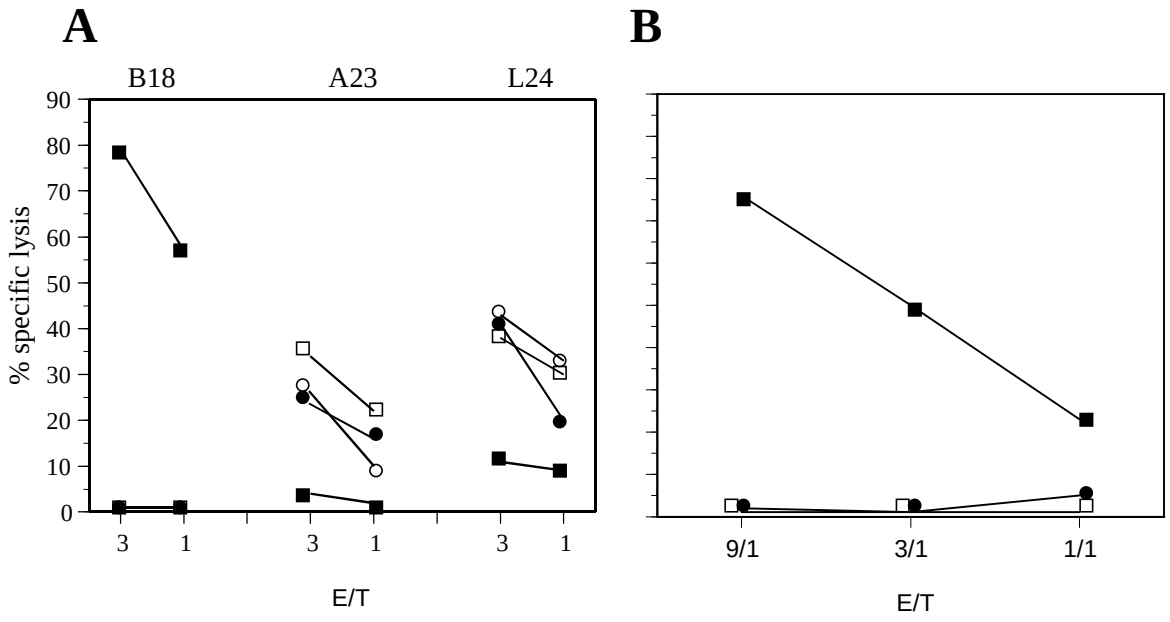

Figure 3: HLA-B*5801/02 alloreactive recognition by NKR^- Vγ9/Vδ2 B18 clone.
(A) 721.221 HLA-B*5801 expressing cells (*full squares*) are lysed be the NKR^- Vγ9/Vδ2 B18 clone in a chromium release assay. Upon incubation of the HLA-B*5801^+ 721.221 cells with the B1.23.2 anti-HLA-B/C (*empty squares*) recognition is abrogated. 721.221 mock transfectants (*empty circles*) are not recognised independently of the preincubation with B1.23.2 (*full circles*). A23 and L24 are two NKR^+ Vγ9/Vδ2 T cells used as control.
(B) Although reacting to 721.221 HLA-B*5802 expressing cells in chromium release assay (*full squares*) NKR^- Vγ9/Vδ2 B18 clone do not react to an autologous LCL line (*full circles*) and mock transfectants (*empty squares*).

Vγ9/Vδ2 B18 clone is not activated by PAgs

Virtually all Vγ9/Vδ2 T cells reacting against Daudi cells, can be stimulated by phosphoantigens (PAgs) that were originally found in mycobacteria supernatants (10;11). We investigated using a cytokine release assay if the Vγ9/Vδ2 B18 clone could be activated by three PAgs of different origins. $CD4^+$ Vγ9/Vδ2 T cell clone that do not express any NKR (18) were used as control in this experiment. Isopentenyl pyrophosphate (IPP) and TUbag-1, both isolated from mycobacterial supernatant (10;11), as well as a PAg isolated from *E.coli* were able to stimulate a $CD4^+$ Vγ9/Vδ2 T cell clone to secrete TNF-α (Figure 4). Coincubation of the $CD4^+$ Vγ9/Vδ2 T cell with the Daudi cells, used as a positive control (18), could as expected stimulate cytokine secretion. Although the $CD4^+$ Vγ9/Vδ2 T cell clone used in this experiment did not express any NKR, the HLA class I^+ BL Silfere, did not stimulate any cytokine secretion by the T cells (Figure 4). This observation was also made in a previous report (18). In contrast, the Vγ9/Vδ2 B18 clone did not secrete any cytokine after incubation with either the PAgs or Daudi cells (Figure 4). However, the Vγ9/Vδ2 B18 clone secrete more than 120 pg/ml TNF-α after incubation with the HLA-B*5801 positive BL Silfere. After incubation of the $CD4^+$ Vγ9/Vδ2 T cell or Vγ9/Vδ2 B18 clone alone, without PAgs or Daudi cells, no TNF release could be detected (Figure 4). These results confirmed the specificity of the Vγ9/Vδ2 B18 clone for HLA-B*5801 and demonstrated that this clone is not reactive to bacterial PAgs.

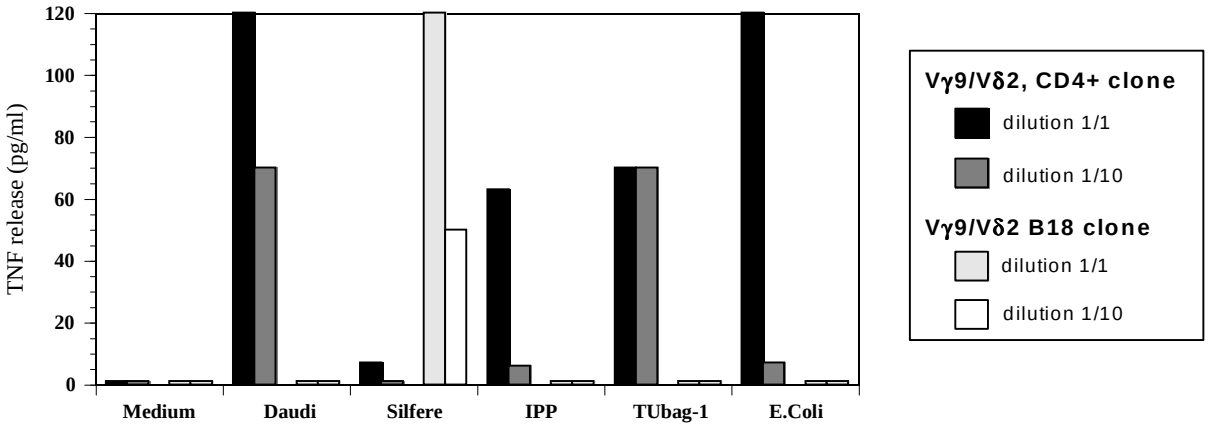

Figure 4: Vγ9/Vδ2 B18 clone is not activated by PAgs.

A Vγ9/Vδ2 $CD4^+$ clone and NKR^- Vγ9/Vδ2 B18 clone were tested in a TNF-α release assay for reactivity towards PAgs: IPP (Isopentenyl pyrophosphate), TUbag-1 (PAg purified from mycobacterial extracts (10)), E. coli (PAg purified from *E. coli* extracts). Daudi cells and the HLA-B*5801^+ cell line Silfere were used as positive controls for TNF-α release by the Vγ9/Vδ2 $CD4^+$ and the NKR^- Vγ9/Vδ2 B18 clones respectively.

*Vγ9/Vδ2 B18 clone recognizes HLA-B*5801/02 positive solid tumors*

In order to test if the Vγ9/Vδ2 B18 clone was specific of the HLA-B*5801/02 molecules only when expressed in B cell lymphomas, we transfected the HLA-B*5802 into the colon carcinoma cell line HCT-β2m and the melanoma cell line FO1-β2m. HCT-β2m and FO1-β2m expressing HLA-B*5802 were lysed in a chromium release assay (Figure 5). In contrast, cells transfected with a mock construct were not recognized (Figure 5). Therefore, Vγ9/Vδ2 B18 clone recognition of HLA-B*5802 expressing cells was not tissue specific.

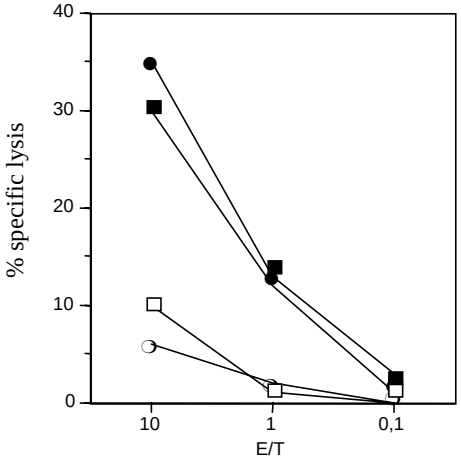

Figure 5: HLA-B*5802^{+} solid tumour recognitions by NKR^{-} Vγ9/Vδ2 B18 clone.
The colon carcinoma HCT-β2m (*full squares*) and the melanoma FO1-β2m (*full circles*) cell lines expressing HLA-B*5802 were lysed by the NKR^{-} Vγ9/Vδ2 B18 clone in a chromium release assay. In contrast, HCT-β2m (*empty squares*) and FO1-β2m (*empty circles*) transfected with a mock construct were not recognized.

Vγ9/Vδ2 B18 clone has a unique TCR combination.

To determine the nature of the γδ TCR CDR3 region involved in the recognition of the HLA-B*5801/02 molecules, we extracted, reverse transcribed Vγ9/Vδ2 B18 mRNA and we amplified the γ and δ chain using PCR. Subsequently the amplified cDNA fragments were cloned into pBluescript and the γ and δ CDR3 regions sequenced. These sequences were compared with those of Daudi reactive γδ TCRs (Figure 6). The NKR^{+} Vγ9/Vδ2^{+} A23 clone carried in its V2DJ1 δ sequence the distinctive functional motif of Daudi and PAgs reactive γδ T cells (Figure 6) (56). This motif consists in the presence of strongly hydrophobic amino acids at conserved position (i.e. V at position 97, see Figure 6) (56). The γ chain of this clone used V9JPC1 γ sequence with a limited "N" nucleotide additions and VJ trimming which are typical features for Daudi reactive γδ TCRs (18;56). In contrast, the Vγ9/Vδ2 B18 clone used the V9J2C2 γ sequence generally found among the Vγ9/Vδ1^{+} T cells but rarely in the Vγ9/Vδ2 T cell population (Figure 6). Furthermore, its V2DJ1 δ sequence did not carry any particular motif (Figure 6). The Vγ9/Vδ2 TCR used by the B18 clone was therefore highly different from the Vγ9/Vδ2 TCRs usually observed in the Vγ9/Vδ2 T cell population in the periphery.

		CDR3 regions						
Clone	**VJγ**	**CDR3γ**			**VDJδ**	**CDR3δ**		
		V	N	J		V	NDN	J
A23	V9JP	lwev	fg	elg	V2D3J1	cdt	vkigihalry	tdkl
B18	V9JP2	lwev		ykk	V2D3J1	cd	pgdlg	kl

Figure 6: CDR3 region comparison between Daudi reactive Vγ9/Vδ2 A23 clone and the NKR^{-} Vγ9/Vδ2 B18 clone. The deduced amino acid sequences are in the single letter code.

Discussion

Our group has previously shown the Vγ9/Vδ2 T cell recognition of B cell lymphomas was controlled by NKRs expressed at the cell surface of the T cells (18). The overall lytic activity against allogeneic B cells by a given Vγ9/Vδ2 T cell might be the result of a balance between inhibiting signals, delivered through the NKRs, and an undefined activating signal through the T cell receptor (57). In the present study, to obtained cytotoxic Vγ9/Vδ2 T cells from which the lytic activity would not be influenced by inhibiting signals, we have selected Vγ9/Vδ2 T cells that did not express any NKR. We have isolated and described a particular Vγ9/Vδ2 T cell clone specific for a classical HLA class I molecule, HLA-B*58. Compared with NKR^+ Vγ9/Vδ2 T cell, this Vγ9/Vδ2 B18 clone used a unique TCR and in contrast to Vγ9/Vδ2 T cell from the periphery it could not be activated by PAgs.

The best characterized antigens for γδ T cells are non-peptidic molecules naturally occurring in bacterial lysates or culture supernatants (10) and certain edible plants (12;58). These compounds are small aliphatic molecules linked to pyrophosphate moiety (PAgs) (10) or to primary amine (AAgs) (12). Vγ9/Vδ2 T cells from the periphery secrete cytokines and lyse target cells upon incubation with PAgs (Figure 4) (59). Virtually all PAg reactive Vγ9/Vδ2 T cells lyse and proliferate to the HLA-deficient BL Daudi and allogeneic B cell lymphomas (Figure 1) (18;52). However the nature of the antigen recognized at the surface of Daudi cells has not yet been thoroughly characterized. PAgs and B cell lymphoma recognition by Vγ9/Vδ2 T cells is controlled by NKRs (18;23). As illustrated in Figure 1, A with the NKR^+ Vγ9/Vδ2 A23 clone, the degree of responsiveness of a particular NKR^+ Vγ9/Vδ2 T cell clone towards different allogeneic targets is very much dependent on the nature of the HLA class I molecules express at the cell surface of the target cells (18).
However, this HLA-related reactivity is determined by inhibitory signals delivered through NKR-HLA class I molecules interactions and not through direct binding between the γδ TCR and the HLA class I molecules (13). In this report , we have characterized a Vγ9/Vδ2 T cell clone which recognition of Daudi variant E8.1 was dependent upon expression of HLA class I molecules (Figure 1). This particular Vγ9/Vδ2 B18 clone was specific for the HLA-B*5801/02 molecules as shown by transfection experiments (Figure 3). Furthermore, although expressing the Vγ9/Vδ2 TCR this clone did not respond to PAgs and Daudi cells either in cytokine release (Figure 4) or chromium release assays (Figure 1 and data not shown). NKR^+ Vγ9/Vδ2 T cells can express at the cell surface inhibitory and/or activatory receptors (19). Most of this receptors display a broad specificity for HLA class I molecules (19). The KIR3DL receptors for example, react with HLA-B allotypes possessing the Bw4 motif at residues 77-83 in the α1 domain of the HLA-B heavy chain (60). Strikingly the Vγ9/Vδ2 B18 clone did not express any known NKR and showed a defined specificity for the HLA-B*5801 and HLA-B*5802 molecules, that differ only in amino acid substitutions in the peptide binding groove (8). This suggested that an activatory receptor was not contributing in the HLA-B*5801 recognition. However, γδ T cells of the VγI/Vδ1 subset have been previously shown to kill target expressing stress-induced MICA and MICB MHC-encoded structures (29), through the NKG2D NK receptor (31). NKG2D is a C-type lectin-like NKR express at the cell surface of most T lymphocytes that does not form, in contrast to NKG2A or NKG2C, a heterodimer with CD94 (31). NKG2D could therefore contribute in the Vγ9/Vδ2 B18 clone killing pattern. However, MICA and MICB are not expressed at the cell surface of B cells and the Vγ9/Vδ2 B18 clone did not appear to recognize MICA, as this clone did not recognize the Hela cell line that expresses MICA (61) (data not shown). NKG2D involvement in the lytic activity of the Vγ9/Vδ2 B18 clone was therefore very unlikely.

All PAgs and Daudi-reactive Vγ9/Vδ2 T cells bear marked junctional variability of the γ- and δ-chains of their TCRs. However, despite their heterogeneity, almost all V2J-δ sequences, derived from peripheral but not thymic Vγ9/Vδ2 clones were shown to carry a distinct motif consisting in the presence of a strongly hydrophobic residue (Val, Leu, or Ile) at a conserved position (position 97) of the CDR3-δ region (Figure 6) (56). Furthermore, Vγ9/Vδ2 T cells express usually a unique Vγ9-JP chain with limited "N" nucleotide additions and VJ triming (56) and uses the C1 γ constant region, therefore use a disulfide-linked TCR. Transfer experiments of the Vγ9/Vδ2 TCR readily demonstrated that the recognition of PAgs is TCR mediated, depending on the Vγ9-JP chain and the pairing with V2J chain expressing the described motif. In contrast, the Vγ9/Vδ2 B18 clone expressed a non-disulfide linked TCR consisting of a Vγ9-JP2 chain and the C2 γ constant region. Moreover, its CDR3-δ region did not bear any characteristic of PAgs reactive Vγ9/Vδ2 T cells.

VγI/Vδ1 T cells constitute the majority of γδ T cells in tissue such as intestine and spleen. This subset of γδ T cells seems to focus on recognition of cell surface molecules related in structure to the classical MHC molecules. Spada et al have recently demonstrated that some members of this γδ T cell subset kill $CD1c^+$ targets, secrete cytokine and proliferate in response to $CD1c^+$ presenting cells (26). CD1c is one member of the nonpolymorphic CD1 molecule family expressed exclusively on antigen presenting cells. CD1 molecules are not encoded in the MHC but still belong to the MHC superfamily and bind β2-microglobulin (28). They present foreign lipid or glycolipid antigens to T cells (27). However, CD1c recognition by VγI/Vδ1 T cells was not dependent on the presence of foreign antigen. VγI/Vδ1 T cells reactivity towards CD1c was TCR mediated, as recognition was transferred by transfection of the γδ TCR (26). VγI/Vδ1 usually express a non-disulfide-linked γδ TCR (9). Strikingly, the TCR of the Vγ9/Vδ2 B18 clone described in our study was therefore much more similar to the VγI/Vδ1 TCR than the classical Vγ9/Vδ2 TCR. Firstly, B18 and VγI/Vδ1 TCRs were structurally related since they used a non-disulfide-linked γδ TCR. Secondly, the targets recognized by the Vγ9/Vδ2 B18 clone and VγI/Vδ1 T cells belong to the same MHC superfamily.
Several γδ T cell clones were shown to recognize classical HLA molecules. Most of them displayed an alloreactive response against foreign HLA molecules (36-38;40;42;44). γδ T cells reacting in a peptide-specific manner on autologous HLA class II molecules were also reported (41;43). Importantly, all the γδ T cell clones characterized in these reports used the VγI/Vδ1 TCR, except for a small fraction using the Vδ3 gene segment (42). Therefore, similarly to the Vγ9/Vδ2 B18 clone all the HLA specific γδ T cell clones characterized used non-disulfide-linked TCRs. These results and our characterization of a Vγ9/Vδ2 non-disulfide-linked TCR reactive to an HLA molecule, suggested that the particular conformation adapted by the non-disulfide-linked human γδ TCRs is more prone to react with MHC or MHC-like molecules.

Comparing the CDR3 length of immunoglobulins (Igs), αβ TCR and γδ TCR it was proposed that the γδ TCRs as a group are structurally more similar to Igs than the αβ TCR (62). The crystal structure of the δ chain of the HLA-A*02 reactive γδ T cell clone confirmed that γδ TCRs are structurally distinct from αβ TCRs. These results suggested that the recognition of certain antigens by γδ TCRs may resemble antigen recognition by antibodies (63). This could explain the capacity of γδ TCRs to recognize a large variety of ligands from small non-peptidic PAgs to MHC molecules (64). However, the fact that γδ TCR recognition is more Ig-like does not preclude that some γδ T cells may recognize MHC molecules in a peptide-specific way as do αβ T cells. Peptide-specific antibodies have been indeed reported (65).

The nature of the determinants involved in αβ T cell alloreactivity is very diverse (66). CTL clones that seem to recognize allogeneic molecule in a peptide-independent fashion have been reported (67;68). Several studies have indicated the existence of peptide-dependent but not peptide-specific CTLs (69;70) (and our unpublished data). Furthermore, the existence of peptide-specific alloreactive T cells has been clearly demonstrated (69;71-73). The HLA-B*58 recognition by the Vγ9/Vδ2 B18 clone seemed to be dependent on peptides bound to the HLA molecule since acid washed target cells were not recognized (data not shown). The broad Vγ9/Vδ2 B18 clone alloreactivity against tumor cells expressing HLA-B*58 suggested that the recognition was peptide-dependent but not peptide-specific (Figure 5). Furthermore, Vγ9/Vδ2 B18 specificity for both HLA-B*5801 and -B*5802 alleles that bind a different array of peptides tended to exclude peptide specificity (8). However, specificity for an ubiquitous peptide presented by both HLA-B*5801 and -B*5802 molecules could not be excluded.

We have characterized the first Vγ9/Vδ2 T lymphocyte specific for an HLA class I molecule. Strikingly, this particular Vγ9/Vδ2 T cell clone did not express any know NKR and expressed a non-disulfide-linked TCR. This clone might be a member of a γδ T cell subset expressing non-disulfide-linked TCR specific for MHC or MHC like molecules. The fact that γδ T cells recognize a very large variety of ligands from non-peptidic PAgs to MHC molecules or MHC-like molecules remains a puzzling question in immunology. Determination of the crystal structures of the disulfide-linked Vγ9/Vδ2 TCR specific for PAgs and the non-disulfide-linked Vγ9/Vδ2 B18 TCR specific for the HLA-B*58 molecule might provide insights in the function and recognition pattern of γδ T cells.

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5

General discussion

General Discussion

The basis for recognition of alloantigen remains a fascinating issue in immunology. The alloreactive response is a powerful model to study *in vitro* and *in vivo* the molecular basis of the immune response. The characterisation of αβ $CD8^+$ allorestricted T cells opened new perspectives in our understanding of the TCR/MHC interactions, T lymphocyte positive selection and in the establishment of new protocols for immunotherapy. Similarly, the existence of alloreactive HLA-specific Vγ9/Vδ2 T cell might provide new insights in the function and recognition pattern of γδ T cells.

Allorestricted T cell repertoire as a source of antigen-specific T cells.

Compiling evidences showing that the allorestricted T cells recognise the MHC/peptide complex as do nominal antigen-specific T cells have accumulated. First, the allorestricted T cell repertoire is broad and diverse, as shown with peptide libraries (1, 2). Second, allorestricted T cells have an affinity at least as high as peptide-specific autologous T cells for the MHC/peptide complex (3, 4). Third, allorestricted T cells specific against tumour and viral derived epitopes could lyse specifically tumour cell lines (5, 6) and infected cells (7) respectively.

Several methods have been used so far to generate *in vitro* allorestricted T cells. In mouse and human, TAP-deficient cell lines in combination with peptide libraries have been used successfully (1, 2). In mice a depletion of framework-specific alloreactive T cells, with antibodies against the transferrin receptor prior to incubation with the peptide library, was necessary (1). However, complex peptide libraries do not allow to generate allorestricted T cells against peptide of interests. Though, a combination of several peptides of interest might be used in the future (7). Sadovnikova et al., have used multiple rounds of splenocyte or PBL stimulations on different TAP-deficient cell lines loaded with tumour peptides to raise allorestricted T cells against tumour epitopes (5, 6, 8). Although shown to be successful this procedure is time consuming and does not allow an efficient separation of framework-specific T cells from alloreactive T cell lines (6).

We were considering developing a new protocol to separate accurately and rapidly human alloreactive T cells restricted against the HLA-A*0201 molecule and specific for a particular peptide from those T cells specific for MHC-frameworks. Several techniques have been developed recently to study peptide-specific T cells: the ELIspot assay is based on ELISA detection of cytokine after antigen-specific stimulation and computer processing (9); the intracellular cytokine staining is based on FACS detection of cytokines trapped in the Golgi apparatus after brefeldin A treatment and antigen-specific stimulation (10). Although allowing detection of antigen-specific T cells, these techniques do not permit separation of antigen-specific cells. A similar technique using cytokine secretion as marker of antigenic specificity that allows separation of cells has been more recently developed, the "Interferon-γ-sandwich cell separation" (Miltenyi Biotec. GmbH, Bergisch Gladbach, Germany). However, this method would not allow discrimination between allorestricted and framework-specific T cells as they both secrete cytokine upon stimulation. An alternative method for sorting of peptide-specific T cells is based on the direct staining of T cells with the antigen that they recognise, namely the MHC/peptide complexes. McHeyzer-Williams and Altman developed for that purpose tetramers of MHC/peptide complexes using the streptavidin/biotin chemistry (11, 12) (a related method using MHC-Ig chimeras has also been used (13)). HLA-peptide tetrameric complexes have been shown since to be a powerful tool to study peptide-specific T cells (Figure 1). They have been used to follow the fate of the immune response after viral or bacterial infections (14, 15). They allowed characterisation of cell surface antigens expressed by auto-antigen specific T cells in autoimmune disorders and in tumour patients (14, 16). Importantly, even down to very low frequencies of antigen-specific T cells, HLA-peptide

tetramers allow direct isolation of tetramer-positive cells by FACS (17). Indeed, HLA-peptide tetrameric complexes bind to antigen-specific CTLs with high specificity and show no cross-reactivity on CTLs specific for an irrelevant peptide. Furthermore, tetramer binding is known to correlate partially with both peptide specific cytolytic functions and cytokine secretions (14).

We therefore decided to apply the HLA-tetramer complex technology to study allorestricted T cells. PBLs from HLA-A*0201 negative donors were stimulated with the HLA-A*0201$^+$ T2 cell line pulsed with particular peptides (*Chapter* 2) or a peptide library (*Chapter* 3). The T2 cell line has a chromosomal deletion that led to the loss of one TAP gene (18). While peptide can be supplied to class I molecules by TAP-independent mechanisms (19), their contribution to the pool of class I binding peptides is low. Furthermore, TAP-independent peptides generally have a lower binding affinity for the class I molecules than peptides that are delivered via TAP. The low levels of peptides in T2 cause most class I molecules to remain empty or associate with low-affinity peptides. Class I molecules devoid of peptides are structurally less stable and are transported inefficiently to the cell surface, resulting in low class I surface expression in TAP-deficient cell lines (19). Unstable HLA-A*0201 molecules at the cell surface of T2 can be stabilised by the addition of external peptides (20).

Combining PBL stimulations with the TAP-deficient T2 cell line and HLA-peptide tetrameric complexes, we have shown in *chapter* 2 that allorestricted T cells could be identified. Indeed, after HLA-A*02$^-$ PBL stimulation with T2 loaded with the *Influenza* matrix protein MP_{58-66} epitope a very low but significant percentage of allorestricted T cells could be stained by the HLA*0201-MP tetrameric complex (*Chapter* 2). Furthermore, HLA*0201-MP tetramer positive cells could be sorted by FACS and expanded *in vitro*. The specificity of these allorestricted T cells for the MP_{58-66} epitope could be demonstrated in a chromium release assay (*Chapter* 2). A striking observation in this experiment was that the vast majority of alloreactive T cells could not bind the HLA-peptide tetramers. Among mouse and human alloreactive CTLs, a dominance of peptide-dependent recognition has been described (21, 22). The determinant recognised by the majority of those alloreactive CTLs that did not bind the HLA-peptide tetrameric complexes in the alloreactive cultures could therefore be dependent on TAP-independent peptides still presented by certain HLA-A*02 molecules. However, the existence of peptide-independent alloreactive T cells was also reported (23, 24). These data supported the model proposed by Bevan suggesting that the majority of alloreactive T cells would have low affinity framework specific TCRs (25). An alternative explanation could be therefore that these alloreactive CTLs needed a high density of HLA-A*02/peptide complexes to be activated in vitro and that we were not providing enough HLA-peptide complexes to stain these T cells. However, increasing the HLA-peptide tetramer concentrations led to an overall increase of unspecific staining on all cells in the bulk cultures, inclusive CD4$^+$ T cells, but not specifically of the CD8$^+$ alloreactive T cell staining. We might need to provide multimers of HLA-peptide complexes higher than tetramer to stain this alloreactive T cells.

Still, using HLA-peptide tetrameric complexes, we have described a new approach to generate peptide-specific CTLs that could be useful for *in vitro* studies in tumour biology and adoptive immunotherapy.

HLA-peptide tetrameric complexes as a tool to study the molecular basis of alloreactivity.

The HLA-peptide tetramer selection of allorestricted CTLs described in *chapter* 2 provided also further evidence that allorestricted T cells bind MHC/peptide complexes as nominal antigen-specific T cells do.

In *chapter* 2 we provided the first direct evidence that peptide-selective alloreactive T cell really exist. Rötzschke et al. speculated on the existence of such alloreactive T cells in a previous study where the recognition of HPLC-fractionated K^b-extracted peptides by K^b-

specific alloreactive CTLs was analyzed (26). In that study, a unique alloreactive CTL that recognized multiple HPLC-fractions was described, implying that it was specific for several peptides. However, in this work the relevant peptide fractions were not sequenced, therefore it could not be excluded that the fractions contained analogous peptides with N-terminal elongations. Guimezanes et al. have reported the ability of an antigenic peptide of the VSV nucleoprotein to sensitise the TAP-deficient RMA-S cells for recognition by an K^b specific alloreactive CTL clone which specificity for an endogenous peptide presented by K^b was shown (27). Bluestone et al. obtained similar results which suggested that some clones may be peptide-dependent but not peptide-specific inasmuch as a variety of different synthetic peptides could promote their recognition of targets cells (28).

Using the HLA-MP tetrameric complexes we have sorted and expanded alloreactive CTLs (*chapter* 2). Surprisingly, these T cells could lyse the TAP-deficient T2 cells and the LCL 721 independently of the pulsed peptides. We therefore examined whether the CTL clones could bind different MHC/peptide complexes using HLA-peptide tetrameric complexes folded with several peptides. As expected, the CTL clone described in *chapter* 2 could be stained by the HLA-A*02-MP tetramer that was used for the FACS sorting. However, this clone could also bind the HLA-peptide tetrameric complex folded with the $CEA_{694-702}$ peptide. In contrast it could not bind the HLA-peptide tetrameric complex folded with the $TyrosinaseA_{369-377}$ peptide. The two peptide recognised by the clone have a different sequence however they may produce similar conformational changes in the HLA-A*0201 molecule, thus resulting in the cross-reactive recognition observed. Similar observations were made by Chattopadhyay et al. when studying the contribution of peptide differences in the alloreactive response with K^b mutants (29). In this report, they described an alloreactive CTL clone recognising K^b but not the K^{bm8} mutant although the amount of peptide extracted from K^b expressing cells was much more less than K^{bm8} expressing cells. Because K^b and K^{bm8} molecules differ only in amino acid that contact the peptide but not the TCR, this data implied that the lack of K^{bm8} recognition was due to conformation differences (29, 30).

Thus, our study showed for the first time directly that alloreactive peptide-selective CTLs exist. Furthermore, we have shown that HLA-peptide tetrameric complexes are a powerful tool that allows the characterization of recognition patterns by alloreactive T cells and new insights in the molecular basis of alloreactivity.

Figure 1: Model of the HLA-B*08-peptide tetramer.
The complex is represented without fluorochrome. *Light blue* streptavidin; *dark blue* the four HLA-B*08 heavy chains and the peptides; *intermediate blue* beta-2 microglobulins. Reprinted from A.J. McMichael and C. A. O'Callaghan. 1998. *J. Exp. Med*. 187: 1367-1371.

Shaping of the allorestricted T cell repertoire by self-HLA molecules.

An alternative method to generate allorestricted T cells could be to use PBL from HLA-related donors. Indeed, Obst et al. have recently shown that an alloresponse against a related MHC molecule contains more peptide-specific T cells than a response against an unrelated one (7). In order to analyse the repertoire of CTL precursor specific for allogeneic MHC-peptide complexes in closely versus unrelated MHC combinations, Obst et al. used K^b mutants having mutations in the peptide-binding groove only or both groove and α-helical replacements. The data clearly demonstrated that mutations in the peptide-binding groove that changes only the spectrum of bound peptides could elicit an alloreactive response with the vast majority of allorestricted T cells. In contrast, alloresponses directed against molecules with both groove mutation and α-helices substitutions were more prone to give rise to structure-specific T cells. Furthermore, alloresponses against completely allogeneic molecules were dominated by framework specific or peptide-selective T cells (7). In addition, using the K^{bm1} mutant splenocytes, K^b-restricted CTLs with high specificity for self- as well as viral naturally presented epitopes could be generated without prior depletion of framework-specific alloreactive T cells (7).

We speculated that using PBLs from HLA-A*0205 donors should allow an easier generation of allorestricted CTLs against self-, viral or tumour-associated antigens presented by the HLA-A*0201 molecule. The HLA-A*0205 molecule has four amino acids replacements that do not contribute to the TCR/MHC interactions (for precise description see Discussion in *chapter* 3). The alloreactive response of the HLA-A*0205^+ PBLs was compared with PBLs expressing the HLA-A*01 molecule that bear multiple amino acid substitutions affecting the peptide-binding groove, the helices and the TCR contacts (31, 32). In contrast, to what was observed with the mouse alloreactive T cell repertoire (7), we could not see a domination of allorestricted towards structure-specific T cell response in both T cell repertoires (*chapter* 3). Using PBLs from an HLA-A*0205^+ donor and an HLA-A*024 donor with almost identical set of HLA class I allelic products, did not resolve this dilemma (*chapter* 3).

The mouse experiments suggested that the allorecognition was readily influenced by the selecting MHC molecule and might mirror the resemblance between self and foreign (7). The T cell repertoire is indeed shaped by positive and negative selection on self-MHC molecules, in the thymus and by peripheral mechanisms (for review see (33)). Our data with human cells did not exclude an influence of the selecting MHC on the alloreactive T cell repertoire. The overall contribution of the up to 6 different class I molecules in the selection of one $CD8^+$ T cell is not clear so far. One possible explanation for our results could be that we did not find a donor having a permissive HLA background that would allow to study the influence of the selecting HLA-A*0205 molecule on the shaping of the alloreactive repertoire directed against HLA-A*0201. The other HLA molecules could have shaded the influence of HLA-A*0205 molecule by negatively selecting potential peptide-specific alloreactive T on HLA-A*0201. Furthermore, we focused on the classical MHC molecules but other polymorphic HLA molecules (HLA-E, G) (31), or MHC-like molecule (MICA/B) (34) and also non-MHC molecules could also play a role in the selection mechanisms. Moreover, the history of infection with immunogenic viruses might also have influenced the alloreactive repertoire of the donors (35).

The mouse experiments were performed with laboratory inbred mice that differ only in few point mutations in the K^b gene. Comparing the selection of alloreactive T cells in humans or wild type mice might be much more complicated that what we observed with these inbred mice. Nevertheless, these observations pointed out that the alloreactive human T cell repertoire varies different between individuals.

The use of allorestricted T cells in immunotherapy.

There is now ample evidence that allorestricted CTLs are as peptide-specific as conventional peptide-specific CTLs. Allorestricted T cells might be promising reagents for adoptive immunotherapy.

However, the risk that the T cells could be recognised by the immune system of the recipient and therefore rejected before having eradicated the malignant cells is a serious limitation for the application of allorestricted CTL in adoptive transfer. Sadovnikova and colleagues reported that tumour protection after adoptive transfer of allorestricted CTLs in immunocompetent mice was limited to a short period of approximately two weeks (5). These results indicated that efficient tumour protection is dependent upon long-term survival of allorestricted CTLs in the recipient host (36). Therefore, this particular therapy should be privileged for immunosuppressive recipient.

The most efficient treatment for certain types of haematological malignancy is intensive radiochemotherapy followed by bone marrow transplantation (BMT) (37). When matched sibling donor or closely matched unrelated donor are lacking, transplantation can be carried out with bone marrow from an allogeneic donor (38). The transplanted cells contain on the one hand the hematopoietic stem cell (HSC) that repopulate the bone marrow and reconstitute the hematopoietic cell lineages. On the other hand, significant number of mature T lymphocytes and NK cells are cotransplanted. Peripheral expansion of these transplanted T and NK cells provides T and NK cell reconstitutions. In adults central production from HSC-derived precursors contributes notably less to the T and NK cell pools repopulation (39).

These T cells are responsible for both beneficial and deleterious clinical effects. The presence of donor T cells is associated with a reduction in the number and severity of post-BMT infections (40, 41). This graft-versus-infection (GvI) effect results from adoptive transfer of mature donor T cells, including memory T cells (42), which can recognise microbial antigens presented be the HLA molecules that are shared between the host and the donor. Donor T cell can also improve HSC engraftment possibly through alloreactive destruction of residual host immunocompetent cells and secretion of cytokines. Donor T cells provide a potent antileukaemic effect through the alloreactive response towards mismatched HLA molecules and the recognition of minor histocompatibility antigen (43). Importantly, leukaemia-antigen-specific CTLs have been recently detected using HLA-peptide tetrameric complexes in patient after BMT (44). In this study a strong correlation between the appearance of leukaemia-antigen-specific CTLs and cytogenetic disappearance of tumour was observed. This graft-versus-leukaemia (GvL) is therefore a major component of the therapeutic efficacy of BMT for leukaemia patients (44, 45).

However, donor T cell alloreactive response against host HLA molecules is responsible for a major life-threatening complication, graft-versus-host disease (GvHD) (45). GvHD is characterised by immunosuppression and severe multi-organ dysfunction. Effort to avoid GvHD have concentrated on the *in vitro* depletion of donor T cells from BM transplants or transplantation of highly purified peripheral blood CD34+ HSC (38). This procedure significantly reduced GvHD but might result in less efficient engraftment and reduction of GvI, increasing the risk of BM rejection and infections. Importantly, elimination of donor T cells might impair the therapeutic efficacy of BMT for leukaemia patient by reducing the GvL effect. Several patients suffering from leukaemic relapse and fatal infection were reported after purified CD34+ HSC transplantation (38, 46).

The severe immunosuppression required for successful bone-marrow transplantation could provide a unique window of opportunity for engraftment of allorestricted CTLs. The transfer of donor allorestricted CTLs specific for leukemia tumour antigens and viral antigens, at the same time as HSC transplantation could be of particular interest to enhance the GvL and GvI without causing GvHD. In a recent report using a mouse model for BMT, Hans Stauss' group

demonstrated that after adoptive transfer of allorestricted CTL clones specific for a tumour antigen, the CTLs could engraft and retain specificity in the host without causing GvHD (47). Nevertheless, our personal experience has shown that the selection of human allorestricted T cells is not such an easy task. Now that the principle as been proven, the HLA-peptide tetrameric complexes could be used for routine selection protocols in the clinic. However, the established of stimulation and culture conditions allowing a better expansion of allorestricted T cells early after the first encounter of the antigen will be a requirement for the future. We have indeed noticed that allorestricted T cells in a bulk culture are rapidly overgrown by alloreactive T cells and that after few antigenic restimulations they are not detectable anymore (data not shown). Furthermore, we have clearly described a limitation of the HLA-peptide tetramer technology: HLA-peptide tetramer can stain peptide-selective alloreactive T cells. Transfer of such T cells would certainly enhance GvHD. Strict controls will be highly recommended before any application of allorestricted T cells sorted by HLA-peptide tetramer in a clinical trial.

Allo-MHC recognition by gd T cells

As we briefly described in the introduction, efforts trying to characterised the molecular basis for the alloreactive response lead to major discoveries in the field of immunology. The allogeneic stimulation of PBLs is still used as a routine technique in many different areas, i.e. in the clinic it is used to monitor the strength of the recipient T cell response against donor cells prior to transplantation; in the laboratory it is often used as model when studying the influence of drugs, cytokines, chemokines, etc. on effector cells of the immune system. The *chapter* 4 of this thesis illustrates such an application of alloreactivity in research.

We have isolated the first Vγ9/Vδ2 T cell clone specific for a classical HLA molecule, HLA-B*58, while studying the allogeneic recognition of B cell lymphomas by γδ T cells. This Vγ9/Vδ2 B18 clone was, in terms of V gene usage, member of Vγ9/Vδ2 T cell subset. The Vγ9/Vδ2 T cells represent the vast majority of γδ T cells in the periphery of adults. These Vγ9/Vδ2 T cells seem to undergo an antigen driven expansion during the childhood as they are only a few percent in neonatal umbilical cord blood (48). The antigens for Vγ9/Vδ2 T cells are non-peptidic molecules naturally occurring in bacterial lysates or culture supernatants (49) and certain edible plants (50, 51). These compounds are small aliphatic molecules linked to pyrophosphate moiety (PAgs) (49) or to primary amine (AAgs) (51). Virtually all PAg reactive Vγ9/Vδ2 T cells can also recognise an undefined ligand on the Burkitt's lymphoma (BL) Daudi. All PAgs and Daudi-reactive Vγ9/Vδ2 T cells bear marked junctional variability of the γ- and δ-chains of their TCRs. However, despite their heterogeneity, almost all V2J-δ sequences, derived from peripheral Vγ9/Vδ2 clones were shown to carry a distinct motif at a conserved position of the CDR3-δ region (52). Furthermore, Vγ9/Vδ2 T cells express usually a unique Vγ9-JP chain (52) and uses the C1 γ constant region, therefore a disulphide-linked TCR. In contrast, the Vγ9/Vδ2 B18 clone expressed a non-disulphide linked TCR consisting of a Vγ9-JP2 chain and the C2 γ constant region. Moreover, its CDR3-δ region did not bear any characteristic of PAgs reactive Vγ9/Vδ2 T cells (*chapter* 4).

The TCR of the Vγ9/Vδ2 B18 clone was much more similar to the VγI/Vδ1 TCRs than the classical Vγ9/Vδ2 TCR. VγI/Vδ1 usually express a non-disulphide-linked γδ TCR (53). This subset of γδ T cells seems to focus on recognition of cell surface molecules related in structure to the classical MHC molecules. Spada et al have recently demonstrated that some members of this VγI/Vδ1 T cell subset are specific for the CD1c molecule (54). VγI/Vδ1 T cells have been shown to kill target expressing stress-induced MICA and MICB MHC-encoded structures (55), with an unclear contribution of the TCR (56). In addition, several

VγI/Vδ1 T cell clones were shown to recognise classical HLA molecules. Most of them were reactive against foreign HLA molecules (57-62), (63). γδ T cells reacting in a peptide-specific manner on autologous HLA class II molecules were also reported (64, 65).
Therefore, all the reported γδ T cell clones specific for HLA or MHC-like molecules, including the Vγ9/Vδ2 B18 clone, used non-disulphide-linked TCRs. These results suggested that the particular conformation adapted by the non-disulphide-linked human γδ TCRs is more prone to react with MHC or MHC-like molecules.
Experiments asking if the recognition of the HLA-B*58 molecule by the Vγ9/Vδ2 B18 clone was peptide-specific were not really conclusive so far. However, acid washed target cells and the generation of TAP-deficient HLA-B*58 expressing cells (with ICP47 from the Herpes simplex virus (66)) demonstrated that the HLA-B*58 recognition was dependent on peptides bound to the HLA molecule. In addition, the broad Vγ9/Vδ2 B18 clone alloreactivity against tumour cells expressing HLA-B*58 suggested that the recognition might be peptide-dependent but not peptide-specific. Furthermore, Vγ9/Vδ2 B18 specificity for both HLA-B*5801 and -B*5802 alleles that bind a different array of peptides tended to exclude peptide specificity (31). However, specificity for an ubiquitous peptide presented by both HLA-B*5801 and -B*5802 molecules was not thoroughly ruled out.

We provided evidence that γδ T cells expressing a non-disulphide-linked TCR might compose a γδ T cell subset specific for MHC or MHC-like molecules. Informations on the disulphide-linked and non-disulphide-linked γδ TCR structures might provide explanations for the broad antigen recognition by γδ T cells. The fact that γδ T cells recognise such a large variety of ligands from non-peptidic PAgs to MHC molecules remains indeed a puzzling question in immunology.

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List of abbreviations

AAg	primary alkyl-amine antigen
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen presenting cell
β2m	β2-microglobulin
BMT	bone marrow transplantation
CDR3	complementary-determining region 3
CTL	cytotoxic T lymphocyte
Cx	calnexin
D	diversity region of the TCR
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	fluorescent activating cell sorting
FCS	fetal calf serum
GM-CSF	granular monocyte colony stimulating factor
GvHD	graft-versus-host disease
GvI	graft-versus-infection effect
GvL	graft-versus-leukaemia effect
HLA	human major histocompatibility complex
HPV	human papillomavirus
HSC	hematopoietic stem cell
IEL	intraepithelial T lymphocyte
Ig	immunoglobulin
IL-	interleukin-
J	joining region of the TCR
KAR	killer activatory receptor
KIR	killer inhibitory receptor
LCL	Epstein-Barr virus-transformed B lymphoblastoid cell line
MHC	major histocompatibility complex
MLR	mix lymphocyte reaction
MP	*Influenza* matrix protein
mRNA	messager ribonucleic acid
NK	natural killer cell
NKR	natural killer cell receptor
PBL	peripheral blood lymphocyte
PBS	phosphate-buffer saline
PCR	polymerase chain reaction
PHA	phytohemaglutinin
PAg	phosphoantigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
T_H	T helper lymphocytes
TNF-α	tumour necrosis factor-α
V	variable region of the TCR
VSV	vesicular stomatitis virus

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