Characterization of B cell responses in relation to organ transplantation

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Chapter 7

Monitoring of indirect allorecognition: wishful thinking or solid data?

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ABSTRACT

Monitoring of T cells involved in an alloimmune response requires the presence of *in vitro* assays, which can detect T cells with direct as well as indirect allospecificity. While generally accepted assays exist to measure helper and cytotoxic T cells involved in direct allorecognition, consensus about an assay for monitoring indirect T cell allorecognition in clinical transplantation is lacking. Many studies claim a relationship between the reactivity of T cells with indirect allospecificity and allograft rejection, but different approaches are used and often, essential controls are lacking. In this review, the disadvantages and pitfalls of the different approaches used so far are discussed and supported by our own *in vitro* experiments. We conclude that an international workshop is necessary to establish and validate a uniform, robust and reliable assay for the monitoring of transplant recipients and to study the actual role of indirect allorecognition in acute and chronic rejection.

INTRODUCTION

Transplantation, blood transfusion or pregnancy may result in activation of alloreactive T cells leading to rejection, graft versus host disease and/or induction of IgG alloantibodies. These alloreactive T lymphocytes recognize non-self antigens, mostly derived from the highly polymorphic major histocompatibility complex (MHC) on allogeneic cells or tissue. The way by which T lymphocytes recognize alloantigens has been the focus of many studies and can occur by two distinct, not mutually exclusive pathways: the direct and indirect pathway.

Direct allorecognition refers to the recognition of intact allo-MHC molecules expressed on the surface of donor antigen-presenting cells (APCs). Allorecognition in this way results in a vigorous immune response, due to the high precursor frequency of T cells involved in this pathway (*1*). Two hypotheses have been proposed for this T cell activation, referred to as the 'high determinant density model' and the 'multiple binary complex model'. In the high determinant density model it is presumed that every MHC molecule on the cell surface, irrespective of the bound peptide, can serve as a ligand for host alloreactive T cells. The high antigen density may thus account for the vigorous immune response. In the multiple binary complex model, the peptide bound in the groove of the allo-MHC molecule is the decisive entity. Here, each peptide-allo-MHC complex is recognized by a unique host alloreactive T cell, also leading to a strong immune response. It is obvious that direct allorecognition only occurs after transfer of tissues or cells between genetically different individuals. In contrast, the mechanism of indirect allorecognition is basically not different from MHC restricted recognition of virally encoded antigens.

Indirect allorecognition is the stimulation of recipient T lymphocytes by processed donor antigens presented as peptides in the context of self-MHC. Evidence for this alternative pathway of T cell allorecognition came from observations that graft rejection still occurred in the absence of immunogenic donor-derived passenger cells in the graft (*2*). Alloantigens shed from the graft are internalized and processed in the same way as exogenous antigens and presented by recipient APCs as peptides in self-MHC class II molecules to $CD4^+$ T cells. The frequency of T cells with indirect allospecificity is two orders of magnitude lower than T cells directly recognizing alloantigens, and the maximal response in the indirect pathway developes later (*3*).

To gain more insight into these alloimmune responses and eventually control them, it is essential to develop reliable *in vitro* assays to monitor the magnitude and specificity of a T cell alloimmune response. Although assays such as the mixed lymphocyte culture (MLC), the cytotoxic T cell precursor (CTLp) assays and HLA antibody screening have shown to

be useful for monitoring transplant recipients, we question in this review the reliability of assays used so far for the detection of indirect allorecognition in clinical transplantation.

ALLORECOGNITION AND ALLOGRAFT REJECTION

Both the direct and indirect pathway of allorecognition can lead to graft rejection. Direct recognition of alloantigens predominates in the first weeks to months after transplantation and is generated by donor-derived antigen presenting cells bearing allogeneic MHC class II molecules. $CD4^+$ T cells with exclusively direct allospecificity can mediate allograft rejection (*4*).

With elapsing time after transplantation, donor APCs fade away and the indirect pathway becomes more important. Evidence that the indirect pathway is sufficient to mediate graft rejection was provided by Auchincloss *et al.* (*5*), who used MHC class II deficient mice as donors in a skin allograft model. Indirect allorecognition of donor MHC class I antigens by host $CD4^+$ T cells could initiate rapid skin rejection. Moreover, allospecific $CD4^+$ T cells induced by the indirect pathway were involved in the generation of cytotoxic T cells against donor MHC class I and the induction of IgG alloantibody production (*5, 6*). The availability of knock out mice and depletion of specific cell subsets enabled the study of individual cell types involved in indirect allorecognition. Combined with results from other experimental studies (*2, 7-13*), this provides circumstantial evidence that T cells exclusively activated by the indirect pathway are able to mediate acute and chronic allograft rejection.

Many clinical studies in humans support the notion that an increased frequency or reactivity of T cells with indirect anti-donor allospecificity is associated with chronic graft dysfunction or rejection.

INDIRECT ALLORECOGNITION AND TRANSPLANTATION TOLERANCE

Indirect allorecognition can occur throughout the lifetime of a graft, since recipient APCs continuously migrate through the graft and encounter donor-derived peptides. Graft rejection can be caused by indirect allorecognition. However, a protective role of T cells with indirect allospecificity is also likely, as the presence of regulatory T cells with indirect allospecificity is associated with transplantation tolerance. Immunological tolerance involves central and peripheral mechanisms (*14*). An example of the role of indirect allorecognition in inducing a state of central tolerance is the prolongation of graft survival after intrathy-

mic administration of donor MHC peptides in rodents (*15, 16*). In the periphery, tolerance can be achieved by various mechanisms, including deletion and regulation of effector cells. Several studies described a state of peripheral tolerance caused by indirect allorecognition in presence of suboptimal stimulation (*17-19*), negative feedback (*20*) or regulation (*21-24*). It is also believed that the beneficial effect of pre-transplant blood transfusions (*25-28*) is mediated by regulatory T cells with indirect allospecificity (*29, 30*).
Considering the central role of indirect recognition in graft rejection and tolerance, the availability of a reliable *in vitro* assay for monitoring indirect allorecognition is crucial.

IN VITRO ASSAYS TO MONITOR INDIRECT ALLORECOGNITION

Studies that have established key factors for measuring indirect allorecognition *in vitro* are listed in Table 1. They show that *in vitro* allogeneic T cell responses require the presence of APCs, and that $CD4^+$ cells recognize an allogeneic peptide in the context of a self-HLA class II molecule (*31, 32*). T cells described to respond to allogeneic peptides via the indirect pathway recognized synthetic HLA peptides, as well as endogenous peptides (*33*). Moreover, autologous dendritic cells (DCs) pulsed with cellular fragments were able to trigger T cells with indirect allospecificity (*34, 35*). Dominant epitopes on common HLA molecules were not limited to the hypervariable region of the HLA molecule, but could also be derived from the α_3 and the transmembrane domains (*36*). Quantitative analysis showed that T cells with indirect allospecificity have a significantly lower frequency than cells involved in the direct allorecognition pathway (*3*) and could react by both proliferation and cytolysis (*37*).
As chronic allograft rejection is thought to be mediated mainly by recipient T cells with indirect allospecificity, most *in vitro* assays aiming to detect indirect allorecognition have been performed after solid organ transplantation. As depicted in Table 2, recipient T cell reactivity to donor-like or donor-derived antigens was associated with acute as well as chronic rejection in numerous studies, except for some (*38-43*). However, in the following sections it will become evident that test conditions vary considerably among studies, while often essential controls are lacking. The different problems will be discussed, supported by our own *in vitro* experiments in order to underline the need to develop a robust and reproducible test system.

Table 1. *Publications that have established key factors in indirect allorecognition in vitro.*

Goal	Priming *in vitro*	Responders	Alloantigens	Read out	Outcome
Recognition of HLA class II peptides by Th cells (*33*)	7 day coculture of HLA-DP3$^+$ PBMC with HLA-DR3$^+$ synthetic peptides (U6)	TCL/TCC	HLA-DR3$^+$ synthetic peptides; HLA-DR3$^{+/-}$,HLA- DP3$^{+/-}$ allogeneic PBMC	3 day proliferation in presence of autologous PBMC	Recognition of synthetic HLA-DR3 as well as endogenous, denatured HLA-DR3 in context of HLA-DP3 by TCC
T cell - APC interaction involved in IAR (*31*)	No	PBMC	Allogeneic PBMC depleted and non depleted of APCs	Presence of IL-2 in supernatant after 7 day proliferation	Allogeneic T cell responses require presence of APCs; only CD4$^+$ T cells can respond in a self-restricted fashion
Ability of T cells to recognize allogeneic HLA-DR peptides (*32*)	14 day coculture of HLA-DR11$^+$/12$^+$ PBMC with HLA-DR1$^+$ synthetic peptides	T cells	HLA-DR1$^+$ synthetic peptides	7 day LDA: proliferation in presence of autologous PBMC	Allogeneic HLA-DR peptides are recognized as nominal antigens by CD4$^+$ T cells; response is self-HLA-DR restricted
		TCL/TCC	HLA-DR1$^+$ synthetic peptides	3 day proliferation in presence of autologous PBMC	
Contribution of IAR and DAR to alloreactivity (*3*)	11 day coculture of DR11$^+$/12$^+$ PBMC with allogeneic HLA-DR1$^+$ PBMC	T cells	HLA-DR1$^+$ synthetic peptides HLA-DR1$^{+/-}$ allogeneic PBMC	7 day LDA: proliferation in presence of autologous PBMC	Frequency of T cells involved in IAR is 100-fold lower than in DAR; dominant epitope: residue 21-42 of HLA-DR1, restricted by HLA-DR12
		TCL/TCC	HLA-DR1$^+$ synthetic peptides	3 day proliferation in presence of autologous PBMC	
Role of IAR in B cell stimulation (*73, 74*)	Coculture of HLA-DR7$^+$/11$^+$ PBMC with HLA-DR4$^+$ protein or synthetic peptides	TCL	sHLA-DR4 protein; HLA-DR4$^+$ synthetic peptides	3 day proliferation in presence of autologous HLA-DR11$^+$ cells; alloantibody production by autologous B cells in RIA	Dominant epitope: residue 69-88 of HLA-DR4; high concentrations of peptide can suppress IAR response; TCL provides specific B cell help
Ability of T cells to mediate IAR (*37*)	7 day coculture of HLA-DR1$^+$/DR4$^+$ PBMC with HLA-A1$^+$ and HLA-B8$^+$ synthetic peptides	TCL/TCC	HLA-A1$^+$ and HLA-B8$^+$ synthetic peptides	3 day proliferation in presence of autologous PBMC; 5hr ^{51}Cr-release CTL assay	T cells with IAR specificity are both proliferative and cytolytic

Table 1. *Continued.*

Goal	Priming *in vitro*	Responders	Alloantigens	Read out	Outcome
Use of DCs in monitoring IAR (*35*)	Coculture of T cells with alloantigen pulsed DCs	T cells	HLA-DR1$^+$, HLA-DR13$^+$ synthetic peptides; DCs pulsed with necrotic cells	6 day proliferation; 24 h IFN-γ ELISPOT	DCs can be used to monitor IAR T cells with IAR specific-ity secrete predominantly Th1 cytokines
Optimal kinetic conditions for IAR (*34*)	No	T cells	Apoptotic cells; necrotic cells; sonicated cells	5 day MLC: proliferation in presence of autologous DCs; 48 h IFN-γ ELISPOT	It takes 16-20 h for processing, intracellular routing and peptide presentation by DC sonicated cells are more potent than apoptotic cells

APCs: antigen presenting cells, CTL: cytotoxic T lymphocyte, DAR: direct allorecognition, DC: dendritic cell, ELISPOT: enzyme-linked immunosorbent spot, HLA; human leukocyte antigen, IAR: indirect allorecognition, IFN-γ: interferon-γ, IL-2: interleukin-2, LDA: limiting dilution analysis, MLC: mixed lymphocyte culture, PBMC: peripheral blood mononuclear cells, RIA: radioimmunoassay, TCC: T cell clone, TCL: T cell line, Th: T helper.

SOURCE OF ALLOANTIGEN

Indirect allorecognition refers to the recognition of a foreign peptide in the context of an HLA class II molecule on self APCs by recipient $CD4^+$ cells. The shedding of soluble HLA molecules during homeostasis or attack on graft tissue during inflammatory processes or other pathological conditions *in vivo* will lead to presence of allogeneic cells or cellular fragments for presentation to the recipients' immune system. They may end up in APCs, transported to the lymph nodes and presented to recipient T cells via the indirect pathway. The strength of this type of alloimmune response is dependent on the amount and source of antigen as well as on the strength and duration of the interaction between recipient APC and T cell. To ensure proper investigation of indirect alloimmune responses, the selection of alloantigens is crucial. Alloantigen can be derived from any polymorphic protein present in the donor and not in the recipient, but in the transplantation setting donor HLA molecules, which can be presented via the direct and indirect way, account for the vigorous alloimmune response. As alloantigen source, one can use donor cells or cellular fragments with the advantage that all possible alloantigens are available for indirect presentation. Alternatively, peptides from alloantigens can be generated synthetically for use in *in vitro* assays. The advantages and disadvantages of different sources of alloantigens will be discussed in more detail.

Allogeneic cells depleted of APCs

Theoretically, depletion of allogeneic APCs will prevent the response of recipient $CD4^+$ T cells towards foreign HLA class II molecules, referred to as the direct way of allorecognition. As shown in Table 2a, the stimulation of recipient $CD4^+$ T cells with APC-depleted allogeneic peripheral blood mononuclear cells (PBMC) correlated with acute rejection in two studies (*44, 45*), whereas other studies found no correlation between indirect allorecognition and acute allograft rejection (*39, 41, 46*). However, it cannot be ruled out that direct allorecognition still interferes in the immune reaction as small numbers of residual donor APCs may remain present. Therefore, the use of cellular fragments lacking intact HLA molecules seems to be a more safe way of alloantigen delivery for indirect recognition.

Cellular fragments

A big advantage of the use of fragments derived from cells of the organ donor is that theoretically the full repertoire of alloantigens is covered. After natural processing, peptides derived from the HLA class I and class II molecules but also from other (minor) transplan-

tation antigens are presented by the recipients' antigen presenting cells. A disadvantage is that the specificity of the alloreactive T cells is not known. Many different techniques have been used for the fragmentation of cells, which makes it difficult to compare results obtained by different studies. Nevertheless, all studies listed in Table 2b found a positive association between patient response to donor derived cellular fragments and acute or chronic allograft rejection (*47-52*). However, it should be noted that not all studies included proper negative controls such as third party cellular fragments.

Fragmentation of cells or cell membranes has some pitfalls. First, without further analysis, the content of a suspension after fragmentation is unknown with respect to the size of the particles. The suspension may contain whole membrane-derived HLA molecules or much smaller components similar in size to the synthetic peptides used in other experimental designs. Few studies performed Western blot analysis or ELISA to demonstrate the presence of HLA molecules after fragmentation, but even then it is unknown whether the HLA molecules need to be intact in order to be processed and presented by an APC (*48,51*). When membrane fragments still contain intact HLA molecules and costimulatory molecules, these can cause allorecognition via the direct pathway by patient responder cells. Recipient APCs may also acquire and integrate intact donor-derived HLA molecules, a process called trogocytosis (*53*). Thus, the absence of cells in the preparation does not warrant absence of direct allorecognition. Second, the concentration of relevant constituents is unknown. A wide range of cellular fragments was used (equivalents of 5×10^4 to 2×10^6 cells), compared to the final concentration of synthetic peptides (10-20 μg/ml per synthetic peptide) used in most studies. The concentration of relevant peptides in a fragmented cell preparation is not known and may be much lower compared to the concentrations usually applied for synthetic peptides.

The availability of many different protocols for fragmentation of cells to obtain donor-derived antigens, underscores the lack of consensus on a robust and reliable test system. To further illustrate this, we tested the reactivity of two $CD4^+$ T cell clones against APCs with the specific restriction element after incubation with different preparations of the cellular fragments. Clone 4.1 recognizes an HLA-A2 peptide in the context of HLA-DR1. Clone 2014, derived from the ThoU6 cell line (*33*), recognizes an HLA-DR3 peptide in the context of HLA-DP3. Fragmentation of $HLA\text{-}A2^+$ and $HLA\text{-}DR3^+$ cells was performed using methods described by various studies (overview in Table 3). The reactivity of the clones towards natural ligand, synthetic peptides and cellular fragments was determined in proliferation and ELISPOT assays. Both clone 4.1 and clone 2014 recognized the natural ligand and the specific synthetic peptide but none of the cellular fragments (Figure 1).

Table 2. *In vitro assays measuring indirect allorecognition after solid organ transplantation*[a].

Goal	Patients	Responders	Alloantigens	Read out	Outcome
(a) Removal of allogeneic APCs					
In vitro assay predictive for kidney graft rejection (*44*)	With (n=23) and without (n=19) acute rejection	PBMC	APC-depleted allogeneic PBMC	MLC	Rejection only occurs in patients who retain their self-restricted pathway of alloreactivity (IAR)
Role of IAR after kidney transplantation (*45*)	With (n=10) and without (n=29) rejection	PBMC	APC-depleted allogeneic PBMC	IL-2 in supernatant after 7 day MLC	Activation of IAR pathway correlates with risk of acute rejection
Role of IAR in heart transplantation (*39*)	Before and after transplantation	PBMC	APC-depleted donor spleen cells	6 day MLC; IL-2 in supernatant after 3 day proliferation in LDA	No IAR of alloantigens; MLC and LDA are not usable to measure IAR
Role of IAR after kidney transplantation (*46*)	With (n=2) and without (n=11) chronic rejection	PBMC	APC-depleted allogeneic PBMCs	5 day MLC	IAR of alloantigens is present in patients with and without chronic rejection
Role of IAR after kidney transplantation (*41*)	With (n=2) and without (n=6) chronic rejection	PBMC	APC-depleted donor or 3P PBMCs	5 day / 9 day MLC	IAR is present in transplanted patients irrespective of rejection
(b) Cellular fragments					
Most effective way of alloantigen delivery for IAR (*47*)	No	TCC (EL26)	HLA-A2^{+} synthetic peptides (residues 92-120, final conc: 10 µg/ml); HLA-A2$^{+/-}$ frozen/thawed PBMC (equivalent of 5×10^{4} cells/well)	3 day proliferation in presence of HLA-DR15^{+} APCs	Frozen/thawed donor cells are most efficient for alloantigen delivery
Role of IAR in chronic rejection after heart transplantation (*47*)	With (n=7) and without (n=4) chronic rejection	T cells	Frozen/thawed donor spleen cells; Frozen/thawed 3P antigens; equivalent of 5×10^{4} cells/well	3 day LDA: proliferation in presence autologous APCs	Elevated frequencies of donor specific T cells with IAR in patients with chronic rejection

Table 2. *Continued.*

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of DAR and IAR in chronic rejection after kidney transplantation (*48, 52*)	With (n=9) and without (n=13) chronic rejection	PBMC	Frozen/thawed donor PBMC	5 day proliferation	Higher T cell reactivity to donor antigens in patients with chronic rejection
Role of DAR and IAR in chronic rejection after lung transplantation (*49*)	With (n=8) and without (n=11) BOS	T cells	Frozen/thawed donor spleen cells; frozen/thawed 3P antigens; equivalent of 5×10^4 cells/well	Presence of IL-2 in supernatant after 3 day LDA proliferation in presence of autologous APCs	Higher T cell reactivity to donor antigens in patients with BOS
Role of DAR and IAR in acute rejection after heart transplantation (*50*)	Before, during and after acute rejection (total: n=13)	PBMC	Frozen/thawed donor spleen cells; equivalent of 2×10^5 cells/well	40 h IFN-γ ELISPOT	Higher T cell reactivity to donor antigens during acute rejection ELISPOT: sensitive method to measure DAR and IAR
A non-invasive, immune monitoring tool to measure DAR and IAR after kidney transplantation (*51*)	With (n=9) and without (n=10) acute rejection	PBMC	Frozen/thawed donor PBMC or spleen cells; frozen/thawed 3P antigens; equivalent of 10^6 cells/well	15 h IFN-γ FCCS in presence of autologous APCs	Higher T cell reactivity to donor antigens in patients with acute rejection; FCCS: clinically useful method to measure DAR and IAR
(c) Synthetic peptides					
Role of IAR in allograft rejection after heart transplantation (*54-59*)	With and without acute or chronic rejection	PBMC	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 μM of each peptide	3 day proliferation (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor allopeptides correlates with acute and chronic rejection; frequency of T cells involved in IAR is 10-50 fold higher in graft than in blood; epitope spreading exists in patients with chronic rejection
		Graft T cells	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 μM of each peptide	3 day blastogenesis assay after 7 day expansion in presence of autologous APCs	

Table 2. *Continued.*

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of IAR in acute or chronic rejection after heart/lung transplantation (*38*)	With acute rejection (n=12) and chronic rejection (n=3)	PBMC	Corresponding to donor and 3P; HLA class I derived; 15-mer peptides; final concentration: 20 µg/ml	4 day proliferation	No response to incompatible donor HLA class I peptides or syngeneic peptides
Role of IAR in acute and chronic rejection after liver transplantation (*60-62*)	With and without rejection	PBMC	Corresponding to donor; 32 HLA-DR alleles; residues 1-19, 21-39, 62-80; 1 µM of each peptide	3 day proliferation (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor allo-peptides correlates with acute and chronic rejection; activation of IAR occurs early after trans-plantation
A clinically useful assay to study IAR in chronic rejection after kidney transplantation (*63*)	With (n=16) and without (n=28) chronic rejection	PBMC	Corresponding to donor and 3P; HLA-DR1, -DR15, -DR3; 20-mer, β-chain hypervariable regions; final concentration: 3.125-100 µg/ml	7 day proliferation; prolif-eration (after 7 day LDA) in presence of autologous PBMC	T cell reactivity to donor peptides in patients with chronic rejection; epitope switching occurs in some patients
Quantitate and character-ize the IAR in chronic rejection after kidney transplantation (*64,65*)	One HLA-A2- patient with chronic rejection of HLA-A2⁺ kidney allograft	PBMC TCC (EL26)	HLA-A2; residues 57-84, 92-120, 138-170; final conc: 10 µg/ml HLA-A2; residues 57-84, 92-120, 138-170; HLA-A2 analogues with single aa sub-stitutions; final concentration: 10 µg/ml	Presence of IL-2 in supernatant after 3 day proliferation in LDA 3 day proliferation in pres-ence of B-LCLs	T cells with indirect anti-HLA-A2 specificity are present *in vivo* Recipient APCs are responsible for maintaining these cells *in vivo* Dominant epitope: residue 92-120 of HLA-A2, restricted by HLA-DR15; altered peptides induce tolerance in T cells with IAR specificity
Role of IAR of HLA class I derived peptides in lung transplantation (*66*)	With (n=5) and without (n=4) BOS Healthy individuals (n=3)	PBMC	Corresponding to donor and 3P; cocktail of HLA-A1, -A2, -B8, -B44; residue 60-84 (α_1 domain); final concentration: 50 µg/ml	7 day proliferation; 3 day proliferation (after 7 day LDA) in presence of autologous PBMC	Higher proliferative response and precursor frequency of T cells towards donor HLA class I peptides in patients with BOS

Table 2. *Continued.*

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Role of IAR after kidney transplantation (*42*)	Patients with stable graft function (n=10)	T cells	Corresponding to donor; HLA-A2; overlapping 5-15 mer; final concentration: 2.5-10 µg/ml	5 day proliferation in presence of autologous APCs Presence of IL-2 in supernatant	T cell reactivity to donor alloantigen in patients without clinical signs of rejection
Role of IAR of HLA class II derived peptides in lung transplantation (*67*)	With (n=9) and without (n=9) BOS	PBMC	Corresponding to donor and 3P; cocktail of HLA-DR1, -DR3, -DR15; β-chain hypervariable region; final concentration: 6.25 to 100 µg/ml	7 day proliferation; 3 day proliferation (after 7 day LDA) in presence of autologous PBMC	Higher proliferative response and precursor frequency of T cells towards donor HLA class II peptides in patients with BOS
Role of IAR of HLA-DR peptides before and after kidney transplantation (*40*)	With (n=18) and without (n=10) acute rejection Healthy individuals (n=20)	PBMC	Corresponding to donor and 3P; HLA-DR; 14-21 mer; final concentration: 50 µg/ml	7 day proliferation Cytokine ELISA of culture supernatant	No association between proliferation and acute rejection; also IAR in healthy individuals; predominant IL-10 production in patients with IAR
A sensitive method to measure IAR in patients after kidney transplantation (*68*)	Stable (n=12) and high risk (at least one acute rejection episode) patients (n=15)	PBMC	Corresponding to donor and 3P; HLA-DR; final concentration: 10 µg/ml	48 h IL-5, IL-10 or IFN-γ ELISPOT	More IFN-γ producing T cells upon peptide stimulation in high risk patients compared to stable patients; ELISPOT assay is useful for monitoring IAR
Alloreactivity after kidney transplantation (*43*)	With and without chronic rejection	PBMC	Corresponding to donor; HLA-A, -B, -DR alleles; final concentration: 1-30 µg/ml	24 h IFN-γ ELISPOT	No association between indirect alloreactivity and chronic rejection
Antigenic properties of HLA-A2 derived peptides (*36*)	Patients on dialysis, awaiting for a kidney transplant	PBMC	HLA-A2; overlapping; 15- or 16-mer; final concentration: 4 or 10 µg/ml	HLA-DR peptide binding assay; 48 h IFN-γ ELISPOT assay	Dominant epitopes: also in α_3 and transmembrane domain Positive anti-HLA-A2 antibody history associated with response to A2 peptides; Also response to peptides identical to self

Table 2. *Continued.*

Goal	Patients	Responders	Alloantigens	Read out	Outcome
Activity of cells through IAR after kidney transplantation (69)	With (n=8) and without (n=3) acute rejection	PBMC	Corresponding to donor; HLA-DR; 14- to 21-mer; final concentration: 10-50 µg/ml	7 day proliferation	No association between proliferation and acute rejection
		TCL	Corresponding to donor; HLA-DR; 14- to 21-mer; 10 µg/ml	Proliferation inhibition ELISA/CBA of culture supernatants; Foxp3 flow cytometric analysis and real-time PCR	Indirect alloreactive TCL produces inflammatory and regulatory cytokines; $CD4^{+}CD25^{+}Foxp3^{+}$ TCL can suppress both IAR and DAR

[a]The listed references in Table 2 are in vitro studies measuring indirect allorecognition in transplanted human individuals corresponding to the following MeSH terms: indirect allorecognition, indirect presentation and alloantigens, indirect recognition and alloantigens, indirect presentation and allogeneic, indirect and alloantigen and rejection, synthetic peptides and rejection, T helper and allogeneic and rejection.

3P: third party, aa: amino acid, APCs: antigen presenting cells, B-LCL: B lymphoblastoid cell line, BOS: bronchiolitis obliterans syndrome, CBA: cytometric bead array, DAR: direct allorecognition, ELISA: enzyme-linked immunosorbent assay, ELISPOT: enzyme-linked immunosorbent spot, FCCS: flow cytometry cytokine secretion assay, HLA: human leukocyte antigen, IAR: indirect allorecognition, IFN-γ: interferon-γ, IL-2: interleukin-2, LDA: limiting dilution analysis, MLC: mixed lymphocyte culture, PBMC: peripheral blood mononuclear cells, PCR: polymerase chain reaction, TCC: T cell clones, TCL: T cell lines.

To exclude that the lack of proliferation of the T cell clones was due to fragmentation induced toxicity, T cell clones and the specific allopeptide were cultured with (supernatants of) cellular fragments. Fragmentation did not result in inhibitory or toxic substances and the response to the specific peptide remained intact (data not shown).

Synthetic peptides

The studies presented in Table 2c used overlapping, synthetic peptides as source of donor antigen (*36, 38, 40, 42, 43, 54-69*). Both HLA class I and class II peptides corresponding to the donor phenotype, were used to stimulate patient cells. However, it can be assumed that donor-HLA class I-derived peptides are the major inducers of recipient T cell activation leading to chronic allograft rejection, since the expression of HLA class I molecules in the graft is far more abundant than that of HLA class II, especially after donor passenger cells bearing HLA class II molecules have disappeared. In that perspective, it is odd that several studies aim at the detection of a T cell response to synthetic HLA-DR peptides rather than to HLA class I peptides (*54-63, 67*).

The advantage of using synthetic peptides is the exact knowledge of the antigen (amino acid length and concentration), resulting in a reproducible assay. By varying the amino acid length, immunodominant epitopes were identified for several HLA antigens. However, the indiscriminate dissection of a protein into synthetic peptides has disadvantages, one of them being the creation of new epitopes (neo-epitopes). Peptides may be synthesized which do not occur *in vivo*, due to absence of natural splicing sites at relevant positions on the full protein. On the other hand, when working from the encoded sequence, one does not take into account that several posttranslational modification mechanisms can occur *in vivo*. This includes glycosylation of proteins to improve protein folding and stability of the peptide-HLA complex (*70*). By exogenously offering 15- to 30-mer synthetic peptides to recipient APCs, they may end up directly in an HLA molecule in unglycosylated form. In addition, posttranslational splicing of peptides has been shown to occur (*71*). In peptide synthesis that is purely based on amino acid sequence of the full protein the latter two possibilities are simply not considered. Apart from this, and more importantly, a major flaw in many studies is the lack of appropriate peptide controls. To support the results of indirect T cell activation studies, inclusion of control peptides based on self-HLA sequences, seems to be a logical corollary, but is rarely performed.

Half of the studies used only peptides corresponding to the donor phenotype and based their conclusive remarks on the response to the specific peptide. These studies should be considered non-conclusive as responses to synthetic peptides corresponding to self-HLA molecules do occur frequently. Since the adaptive antigen-specific immune system distin-

Table 3. *Overview of different methods to fragment cells.*

	PBMC				
Fragmentation method	1 (*47,50*)	2	3 (*48,51*)	4[a]	SALs
Cells	20×10^6 PBMC	20×10^6 PBMC	PBMC	PBMC	20×10^6 SALs
Lysis	3× N_2/37°C[b] RPMI	3× N_2/37°C RPMI	3× N_2/37°C Tris-EDTA-based buffer; 1/5000 NP-40	3× N_2/37°C in Tris-EDTA-based buffer; 1/8000 NP-40	4× N_2/37°C RPMI
Protease inhibitors	–	–	0.1 mM PMSF	0.1 mM PMSF	–
	–	–	1/200 mixture	1/800 mixture	–
	–	–	5 ng/ml SBTI	5 μg/ml SBTI	–
1st centrifugation step	–	20 min, 16,000 *g*, RT	2 min, 1000 *g*, 4°C	2 min, 2000 *g*, 4°C	–
Sample	Whole solution	Supernatant	Supernatant	Supernatant	Whole solution[c]
2nd centrifugation step	–	–	45 min 14,000 *g*, RT	2 min, 3000 *g*, 4°C	–
Sample	–	–	Pellet	Supernatant	–
3rd centrifugation step	–	–	–	60 min, 100.000 *g*, 4°C	–
Sample	–	–	–	Pellet	–

[a]*Personal communication Dr. Maria Hernandez-Fuentes, King's College London, UK.*
[b]*Freezing/thawing cycle in liquid nitrogen and waterbath, respectively.*
[c]*Before use, cell suspension was filtered to remove cloths.*
EDTA: ethylenediaminetetraacetic acid, PBMC: peripheral blood mononuclear cells, PMSF: phenylmethylsulphonyl fluoride, RT: room temperature, SALs: single antigen cell lines, SBTI: soybean trypsin inhibitor.

guishes self from non-self, starting with deletion of T cells in the thymus with a high affinity for self-antigens, it is not likely that individuals recognize a natural self peptide presented in the context of their own HLA class II. Figure 2 shows that reactivity was observed to synthetic peptides based on self-HLA molecules using cells from a healthy non-primed individual or umbilical cord blood (UCB). The peptides used here probably differ from naturally processed peptides and are seen as neo-epitopes by the autologous T cells. A logical consequence is that in some cases the reactivity towards synthetic allopeptides is interpreted incorrectly. This type of reactivity is not restricted to T cell responses to synthetic HLA peptides but also accounts for responses towards synthetic RhD peptides in RhD-positive individuals, as tested in our laboratory (data not shown). Our observations are in agreement with others. Hanvesakul *et al.* showed recognition of peptides with a sequence identical to self in patients awaiting a renal transplant, as determined in IFN-γ ELISPOT assay (*36*). Barker *et al.* described proliferation of naïve T cells of RhD-positive individuals to synthetic RhD peptides and concluded that this was presumably due to cor-

respondence of the peptides to cryptic epitopes normally not presented *in vivo* (*72*). Two other studies also demonstrated that non-primed T cells are able to respond to allogeneic synthetic peptides (*40, 42*). Two possibilities arise for the response to self: absence of regulatory mechanisms or induction of neo-epitopes. Natural regulatory mechanisms, which prevent occurrence of an autoimmune response *in vivo*, may be absent in *in vitro* test systems. A more plausible explanation lies in the synthetic character of the peptides. A synthetic peptide does not use the normal route of processing and may end up as a new amino acid sequence in an HLA molecule of an APC. Responder T cells will recognize such

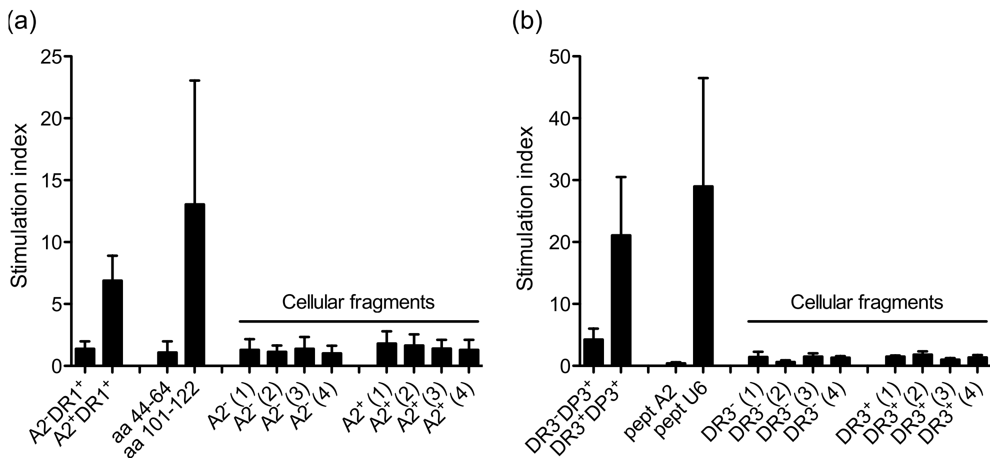

Figure 1. *Response of Clone 4.1 (panel a) and Clone 2014 (panel b) towards natural ligand, synthetic peptides and cellular fragments. Clone 4.1 (recognizes HLA-A2 in HLA-DR1) proliferated upon stimulation with natural ligand ($A2^+DR1^+$ cell) or specific $A2^+$ peptide (aa 101-122), but not upon stimulation with $A2^+$ or $A2^-$ cellular fragments, produced by method 1, 2, 3 or 4 (n=3). Clone 2014 (recognizes HLA-DR in HLA-DP3) produced IFN-γ as measured in ELISPOT upon stimulation with natural ligand ($DR3^+DP3^+$ cell) or specific $DR3^+$ peptide (U6), but not upon stimulation with $DR3^+$ or $DR3^-$ cellular fragments, produced by method 1, 2, 3 or 4 (n=2). Stimulation index panel a: counts per minute (CPM) of stimulated clone divided by CPM of unstimulated clone (medium control). Stimulation index panel b: IFN-γ production of stimulated clone divided by IFN-γ production of unstimulated clone (medium control).*

a new epitope (neo-epitope) in the context of self HLA class II; however this type of recognition is not necessarily related to indirect recognition of a naturally processed allopeptide. To our opinion, positive tests obtained with synthetic peptides should be confirmed by reactivity against the natural ligand.

EXPERIMENTAL SET UP

Besides the antigenic stimulus, the responder cell population, as well as the experimental setup of an *in vitro* assay, are crucial. The optimal location to detect recipient T cells with indirect allospecificity would be in or near the graft, since T cells migrate towards the graft after they have been triggered by recipient APCs presenting a donor derived peptide. In the graft they are supposed to exert their effector functions, which eventually may cause graft rejection. Although the frequency of T cells with indirect allospecificity during rejection may be 10-50 fold higher in the graft than in the periphery (*54*), for practical reasons most studies have been performed with PBMC.

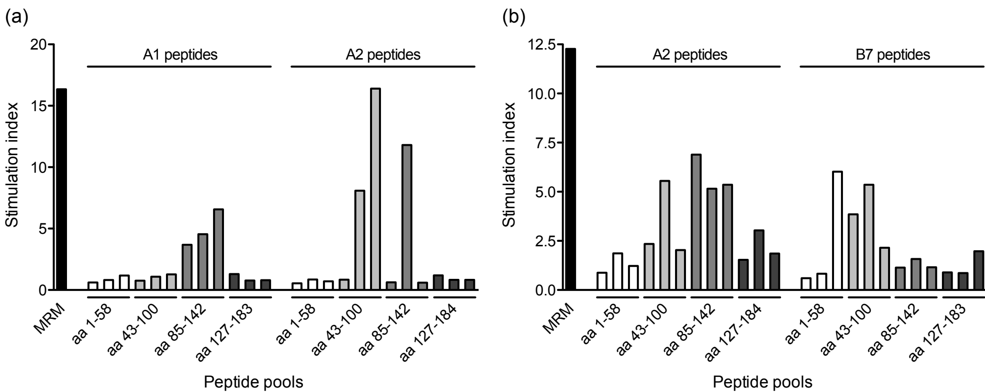

Figure 2. *Proliferative response (in triplicate) of healthy control PBMC (panel a) and umbilical cord blood (UCB; panel b) towards foreign synthetic peptides (HLA-A1 and HLA-A2 respectively) and peptides corresponding to self (HLA-A2 and HLA-B7 respectively). HLA type of healthy control: A2, A25, B7, B18, Cw4, Cw12, DR1, DR15, DQ5, DQ6. HLA type of UCB: A3, A32, B7, B35, Cw4, Cw7, DR11, DR15, DQ3, DQ6. Peptides were added in pools of 3 overlapping 30-mer peptides. Proliferation was measured at day 7. MRM: memory recall mix. Stimulation index: counts per minute (CPM) of stimulated cells divided by CPM of unstimulated cells (medium control).*

In some studies donor antigens were added to PBMC, by which the encounter of antigen, processing and presentation by autologous APCs occured in the same environment as presentation to T cells. Only a few studies used a more sophisticated system, in which autologous APCs were purified by T cell depletion, incubation with antigen and finally added to responder T cells (*42, 47, 49*). To refine the antigen presenting cell population, we performed costaining experiments of fragments of single antigens lines (SALs) and different cell populations (Figure 3). Only $CD14^+$ cells (monocytes) showed costaining of PKH26

labeled SAL fragments and may be able to internalize the fragments, process them and finally present them in the context of HLA class II. This allows for selection of the proper APC population and control the ratio between CD14⁺ APCs and T cells. In order to develop and validate a reliable *in vitro* system, standardization and quality controls within the different phases leading to indirect allorecognition are inevitable.

As read out, most studies focused on the capacity of CD4⁺ cells to proliferate upon stimulation, but differ in other test characteristics. Proliferative capacity is often measured upon coculture with synthetic peptides or cell fragments directly or after stimulation in limiting dilution analysis (LDA). LDA quantifies the response by measuring the frequency of alloreactive cells, but involves prolonged *in vitro* culture systems, which may affect estimations of the true *in vivo* frequencies. More recently, ELISPOT and flow cytometric approaches are used to estimate the number and phenotype of alloreactive T cells respectively. The ELISPOT is more sensitive than other techniques, such as MLC and ELISA, but represents a snapshot of the T cell response. Some of the cytokine-producing cells may not proceed

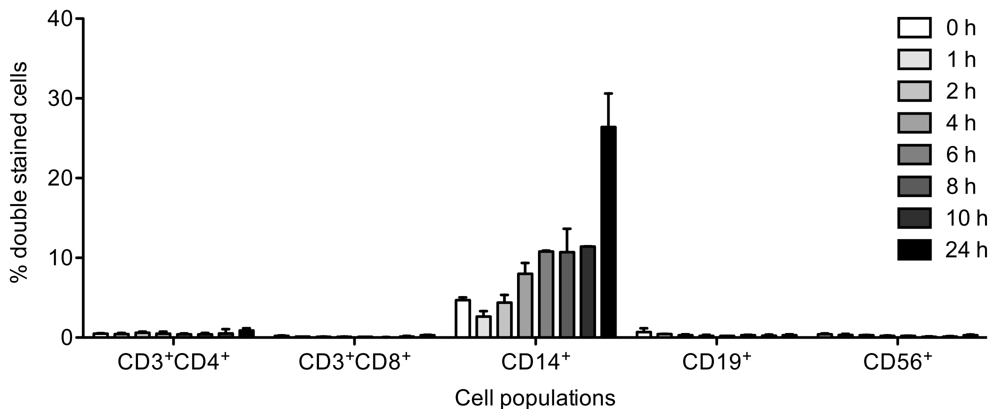

Figure 3. *Costaining of SAL fragments on different cell populations. SAL fragments (stained with PKH26) were incubated for various lenghts of time with PBMC (stained for different cell populations) (n=2). The percentage of double stained cells was determined in flowcytometric analysis.*

to proliferation or may undergo activation-induced cell death (*68*). The flow cytometric approach allows for the individual characterization of cells and the subsequent staining for cytokines to refine the categorization of specific cell subsets, rather than just surface markers. Because a major feature of T cells with indirect allospecificity is their low fre-

quency, a sensitive technique is required. In this respect, the phenotypic characterization of alloreactive T cells has an additional benefit above standard proliferation assays.
When monitoring patients, it is essential to distinguish the response of naïve cells from that of primed donor reactive T cells. To develop an *in vitro* assay able to detect T cells with primed indirect allospecificity, one should focus on the detection of memory cells. Therefore, knowledge about the kinetics of a response is crucial. In our above-described experiment using stained SAL fragments cocultured with stained PBMC, it was possible to monitor the double positive-stained cells at different time points (Figure 3). Within 24 h after coculture, 25% of the $CD14^+$ cells showed costaining of SAL fragments. This time span is in agreement with results of a recently performed study in which a time period of 16-20 h was found to be necessary for processing, intracellular routing and peptide presentation by APCs (34). Considering the use of primed responder cells and the uptake and processing of antigen within 1 day, one may question the need for a 7 day incubation period to detect memory cells as is used in most studies. Because cytokine secretion precedes proliferation upon stimulation, incubation of recipient PBMC with donor antigens in ELISPOT was usually much shorter. Nevertheless, a study focusing on the kinetics of a memory is required to shed light on this.

CONCLUDING REMARKS

Various studies claim a relationship between the reactivity of T cells with indirect allospecificity and graft rejection. However, many different approaches are used and often, essential controls are lacking. Hardly any study confirms the presence of indirect recognition using different approaches including the natural ligand. This is essential as all assays used so far clearly have disadvantages and pitfalls.
The use of donor cells for fragmentation has our preference as antigen source for *in vitro* assays because they contain the full HLA phenotype. However, no method to fragment cells has emerged as optimal, which is emphasized by the lack of consensus. Additionally, one should include control experiments when using fragmented cells to exclude recognition of alloantigens via the direct pathway. The exact knowledge of the alloantigen when using synthetic peptides is overshadowed by the ability to create neo-epitopes. This turned out to be a big obstacle in defining a specific response and requires at least the use of control peptides. Besides different antigenic sources, additional differences exist in test characteristics between studies in which most of them do not distinguish a naïve response from a memory response. To monitor the low frequency of T cells with indirect

allospecificity *in vitro*, it is essential to determine the kinetics and optimal conditions to detect a memory response.
Detailed knowledge about the indirect allorecognition is required and it would be the right time to set up an international workshop to establish and validate a proper and robust assay to measure T cells with indirect allospecificity.

MATERIALS AND METHODS

Subjects and sample collection

PBMC from heparinized peripheral blood from healthy individuals or mononuclear cells from umbilical cord blood (UCB) were isolated by standard Ficoll density-gradient centrifugation. PBMC were used immediately as responder cells in proliferation assays or were cryopreserved in RPMI 1640 (Gibco, Paisley, UK) with 20% fetal calf serum (FCS, Gibco) and 10% dimethyl sulfoxid (DMSO, Fluka, Buchs, Switzerland). Cells were frozen at -70°C and stored in liquid nitrogen until further use.

T cell clones

The T cell line ThoU6 was obtained as described before (*33*). In brief, PBMC of individual Tho (DPB1*0301$^+$, DRB1*0301$^-$) were primed in the presence of an HLA-DR3 peptide (aa 67-85: LLEQKRGRVDNYCRHNYGV) in RPMI 1640 supplemented with 15% pooled human serum and gentamycin. After 7 days, the cultures were collected and restimulated with irradiated autologous feeder cells at a ratio of 10:1 and synthetic HLA-DR3 peptide. The line was cloned by limiting dilution in the presence of T cell growth factor (TCGF, Biotest, Germany) and irradiated autologous feeder cells. The CD4$^+$ clone 2014 was kept in culture by weekly stimulation with irradiated allogeneic PBMC with IL-2 in RPMI 1640 supplemented with 10% pooled human serum.
The CD4$^+$ T cell clone 4.1 recognizes an HLA-A2 peptide (aa 101-122: CDVGSDWRFLRGYHQYAYDGKD) presented in HLA-DR1. The clone was maintained in culture by weekly stimulation with irradiated allogeneic PBMC and allogeneic B cells, PHA and IL-2 in IMDM (Gibco) with 10% pooled human serum. Both T cell clones were used in functional assays between 1 and 2 weeks after restimulation.

Synthetic peptides

Synthetic 20-mer overlapping peptides corresponding to residues 45-66 and 101-122 of α_1 and α_2 domain from HLA-A2 alleles and synthetic 30-mer overlapping peptides represent-

ing the α_1 and α_2 domain of HLA-A1, -A2, -B7 and -B8 were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II; MultiSynTech, Witten, Germany). The purity of the peptides was determined by analytical reversed-phase high performance liquid chromatography (HPLC).

Cellular fragments

Fresh or thawed PBMC or SALs were used for fragment preparation. Fragmentation of PBMC was performed using 4 different methods:

- Method 1: PBMC were lysed by three cycles of freezing (liquid N_2) and thawing (water-bath: 37°C) in RPMI 1640 complete medium at a concentration of 2×10^7 cells/ml. The absence of whole intact cells was confirmed by microscopy (*47, 50*).
- Method 2: PBMC were lysed by three cycles of freezing (liquid N_2) and thawing (waterbath: 37°C) in RPMI 1640 complete medium at a concentration of 2×10^7 cells/ml, centrifuged at 16.000 *g* for 20 min and the supernatant was collected.
- Method 3: PBMC were lysed by three cycles of freezing (liquid N_2) and thawing (waterbath: 37°C) in a Tris-EDTA-based buffer containing 1/5000 NP-40 (Fluka, 74385), 0.1mM PMSF (Sigma P7626; Buchs, Switserland), 1/200 protease inhibitor mixture (Sigma, P8340) and 5 ng/ml soybean trypsin inhibitor (Sigma, T6522), centrifuged at 1000 *g* for 2 min, and the supernatant was collected and further centrifuged at 14.000 *g* for 45 min at 4°C (*48, 51*).
- Method 4: PBMC were lysed by three cycles of freezing (liquid N_2) and thawing (waterbath: 37°C) in a Tris-EDTA-based buffer containing 1/8000 NP-40 (Fluka, 74385), 0.1mM phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma, Buchs, Switserland), 1/800 protease inhibitor mixture (Sigma, 8340) and 5 µg/ml soybean trypsin inhibitor (Sigma, T6522), centrifuged at 2000 *g* for 2 min (4°C), followed by a centrifugation step of the supernatant at 3000 *g* for 2 min (4°C). Supernatant was harvested and further centrifuged at 100.000 *g* for 60 min (4°C) in the buffer described above. The pellet was resuspended in 10-20 µl of solubilising solution: urea (Sigma, U0631), 2% CHAPS (Calbiochem, San Diego, CA, USA) and protease inhibitor cocktail (Roche 1836153; Basel, Switserland) (personal communication Dr. Maria Hernandez-Fuentes, King's College London, UK).

Fragmentation preparations were tested for protein concentrations using BCA protein assay kit (Pierce, Rockford, IL, USA) and stored at -70°C until further use.

Fragmentation of SALs was similar to method 1, but one additional round of freeze/thawing was performed. Before use, the cell suspension was filtrated through a 70 µm cell

strainer (Falcon; BD Bioscience, Bedford, MA, USA) to remove cell cloths.

Proliferation assays: healthy control and UCB

A total of 2×10^5 PBMC of healthy individuals or mononuclear UCB cells were incubated with a pool of three synthetic HLA-derived peptides at a final concentration of 10 µg/ml per peptide. As a positive control a memory recall mix (MRM) was used containing tetanus toxoid (SVM, Bilthoven, the Netherlands), mycobacterium tuberculosis sonicate (Statens Serum Institute, Copenhagen, Denmark) and Candida albicans (HAL Allergy, Haarlem, the Netherlands). The cells were plated out in round-bottom 96-well plates (Costar, Cambridge, MA, USA) in a total volume of 200 µl of RPMI 1640 complete medium supplemented with 5% autologous serum. After 6 days, wells were pulsed with 1 µCi of ^{3}H-TdR (Amersham International, Amersham, UK) and the cultures were harvested onto glass fiber filters 18 h later. Proliferation was measured by ^{3}H-TdR incorporation using a betaplate counter (Wallac, Turku, Finland).

Proliferation assay: clone 4.1

A total of 10^5 irradiated (30 Gy) $A2^-DR1^+$ PBMC were pulsed overnight with $A2^+$ synthetic peptides at a final concentration of 10 µg/ml per peptide or $A2^{+/-}$ cellular fragments at a final concentration of 1µg/well in IMDM complete medium with 5% pooled human serum. Also, intact $A2^-DR1^+$, $A2^+DR1^+$ or $A2^-DR1^-$ cells were kept overnight in Iscove´s medium. T cell clone 4.1, which was kept overnight at 37°C in Iscove's medium containing 100 U/ml IL-2, was then added at 5×10^3 cells per well. After 48 h, wells were pulsed with 1 µCi of ^{3}H-TdR and proliferation was measured as described above.

Enzyme linked immunospot (ELISPOT) assay: clone 2014

A total of 10^5 irradiated (30 Gy) $DR3^-DP3^+$ PBMC were pulsed overnight with $DR3^+$ synthetic peptides at a final concentration of 10 µg/ml per peptide or cellular fragments at a final concentration of 1 µg/well IMDM with 5% pooled human serum. T cell clone 2014, which was kept overnight at 37°C in Iscove's medium containing 20 U/ml IL-2, was then added at 5×10^3 cells per well, centrifuged for a short time to concentrate the cells and stored for 2 h at 37°C. Then, cells were harvested and transferred to an ELISPOT plate (MAHA S45 10, Millipore, Amsterdam, the Netherlands) coated with a mouse anti-human IFN-γ mAb (Mab 1-D1K; Mabtech, Nacka, Sweden) followed by blocking for 1 h at 37°C with RPMI 1640 containing 5% pooled human serum. Cells were incubated in the ELISPOT plate for 24 hr at 4°C to allow spot formation. Plates were washed with PBS supplemented with Tween20 and an IFN-γ detection antibody (Mab 7-B6-1-biotin; Mabtech, Nacka,

Sweden) was added for 2 h at room temperature (RT). The conjugate (extravidin alkalin phosphatase, Sigma E2636) was added for 1 h at RT, followed by a washing step and addition of the substrate (BCIP-NBT tablet, Sigma B5655) for 5 min at RT. The reaction was stopped with tap water. The resulting spots were counted using a Bioreader 3000 Pro (BioSys, Karben, Germany).

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