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Modulation der T-Zell-Reaktivität durch MHC-Klasse-I Peptide und ihre Varianten: Perspektiven für eine antigen-spezifische Therapie in der Transplantation

Modulation of the T cell response with MHC class I peptides and their analogues: perspectives for an antigen-specific therapy in transplantation

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ABBREVIATIONS

APCs: antigen presenting cells

APLs: altered peptide ligands

DCs: dendritic cells

EDTA: ethylen-diamine-tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FACS: flow cytometric cell analysis

GILs: graft-infiltrating lymphocytes

IFN: interferon

IL: interleukin

LEW: Lewis rat

mAbs: monoclonal antibodies

MHC: major histocompatibility complex

PBS: phosphate buffered saline

RT-PCR: reverse transcriptase-polymerase chain reaction

TCR: T cell receptor

WF: Wistar-Furth rat

1. INTRODUCTION

Transplantation of vascularized organs is now the therapy of choice for end-stage organ failure. The major improvements in the field of surgical techniques, MHC matching and immunosuppressive drugs, are able to prolong the one-year survival of most solid organ grafts to over 90%. Since the transplant recipients require life-long immunosuppression with severe side-effects, in order to avoid tissue damage and disruption of their function, the major aim of transplantation immunology remains the downregulation of the allograft-induced immune response. The development of antigen-specific strategies should greatly facilitate the allograft-restricted suppression without the general compromise of the recipient's defense system (Gorantla *et al.* 2000).

1.1 Alloimmune response: immunologic basis for allograft rejection

After transplantation of MHC-incompatible tissues, the immune system is activated, leading finally to graft rejection. This is the major obstacle to successful transplantation. The principal targets of the immune response to an allogeneic tissue are the donor MHC molecules present on the grafted cells (Rogers *et al.* 2001). The term "allorecognition" describes the T cell recognition of genetically encoded MHC polymorphism between members of the same species.

Lechler and Batchelor were the first to suggest that allorecognition occurs in two possible ways, through the direct or indirect pathway, respectively (Lechler *et al.* 1982) (Figure 1). After allotransplantation, recipient T cells (CD4+ and/or CD8+) recognize intact donor MHC molecules displayed on the surface of donor grafted cells, so called "passenger leukocytes" (*direct allorecognition*), which are believed to be specialized APCs that provide all of the necessary stimulatory signals required for the activation of T cells. Direct allorecognition represents a special mechanism that occurs only after transplantation and can be explained by the cross-reactivity of T cells. The frequency of peripheral T cells that are able to recognize alloantigens is 1-10% of the peripheral T cell population (Suchin *et al.* 2001). A few days after transplantation, donor MHC molecules are shed from the graft, taken up and processed into small fragments by self-APCs, followed by presentation in the groove of self-MHC class II molecules on the surface of self-APCs to CD4+ T cells (*indirect*

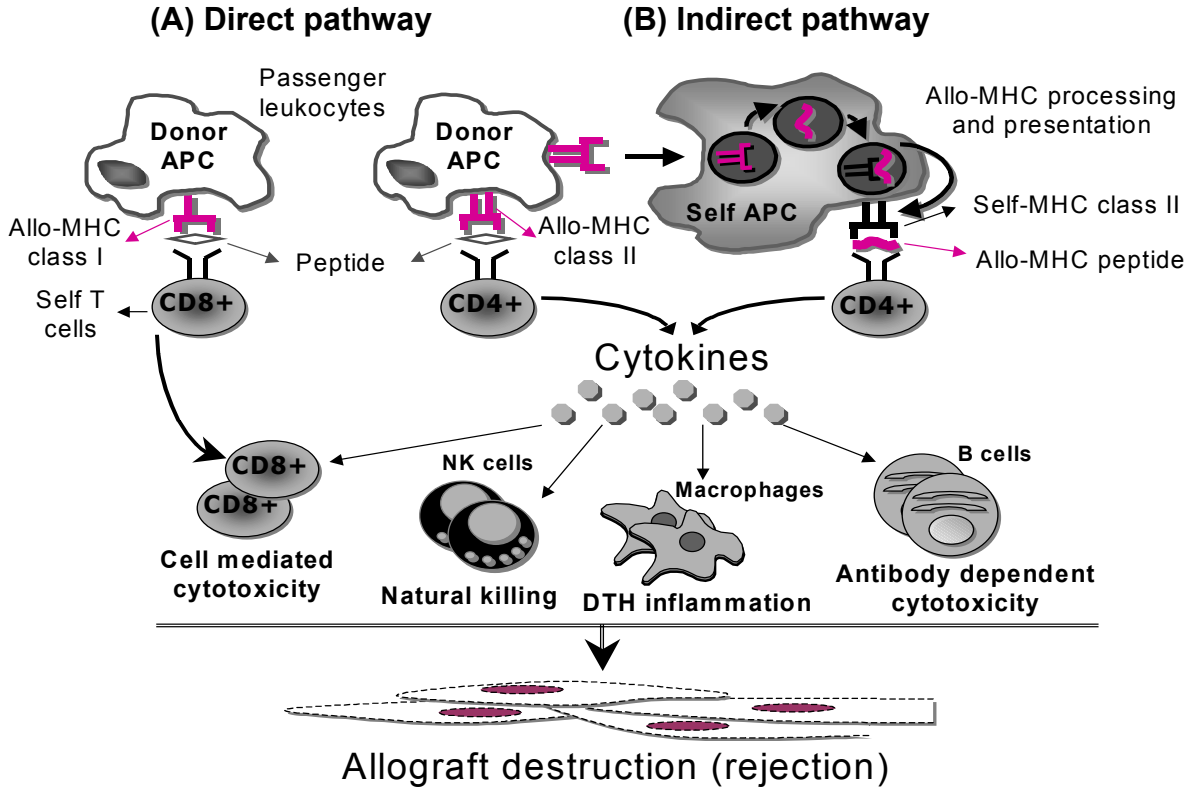


FIGURE 1. Two pathways are involved in the recognition of alloantigen by recipient T cells: the direct (A) and the indirect pathway (B).

allorecognition) (Auchincloss *et al.* 1993). The indirect pathway represents the physiological reaction against foreign antigens and depends on the continuous supply of alloantigens from the graft. It has been recently shown that CD4+ T cells play the central role in the allograft rejection through the indirect pathway (Auchincloss *et al.* 1996). Activated CD4+ T cells provide further help for the other effector cells to orchestrate the cellular and molecular mechanisms that finally lead to rejection.

1.2 Current and new strategies to suppress the alloimmune response

Transplantation between genetically different individuals evokes a quick and destructive alloimmune response that, in the absence of immunosuppression, leads to graft destruction. Since T cells are the central players in the alloimmune response, most of the current immunosuppressive drugs target the T cell activation and cytokine production, the clonal expansion, or both (Denton *et al.* 1999).

The immunosuppressive drugs developed in the past two decades have decreased the rate of acute graft rejection and improved the 1-year graft survival, but almost all the transplanted patients still require life-long immunosuppression. Moreover, these agents lack specificity and are associated with severe problems: (1) reduced immunity to infections and (2) malignant diseases, and (3) other drug-related adverse effects such as nephrotoxicity, hypertension, diabetes and hyperlipidaemia.

The newly developed strategies block the interactions between T cells and APCs, inducing the inactivation rather than elimination of alloreactive T cells. The experimental protocols include monoclonal antibodies against CD4, CD154, LFA1, ICAM1, LFA3, CD2, CD45, and CTLA4-Ig (Yu *et al.* 2001). However, the use of such antibodies in some primate and human experiments has been associated with excessive side effects, thus proving to be insufficient and ineffective (Waldmann 2001).

As a consequence of the systemic immunosuppressive effects of the currently used protocols, the objective of transplantation is to develop antigen-specific strategies with minimal secondary effects. Thus, by suppressing only the anti-donor immune response it is possible to achieve immunological unresponsiveness to the graft, in the presence of a fully competent immune system.

1.3 MHC peptides as modulators of the alloimmune response

Since the donor MHC peptides represent the principal antigens in the initiation and maintenance of the alloimmune response, it is not surprising that most experimental studies are focused on the modulation of the alloimmune response using MHC allopeptides. It has been shown that some allopeptides have potential immunomodulatory capacities when administered intrathymically (Sayegh *et al.* 1994), orally (Zavazava *et al.* 2000), or as peptide-pulsed recipient DCs (Ali *et al.* 2001). Since these studies have been performed in animal models and the responsible mechanisms need to be further investigated and elucidated, their clinical applicability remains questionable.

A more favorable approach is to alter the alloreactive T cell response by subtle changes in the sequence of the alloantigens. It has been demonstrated that there is only one optimal peptide ligand for each MHC-TCR complex; this ligand has both

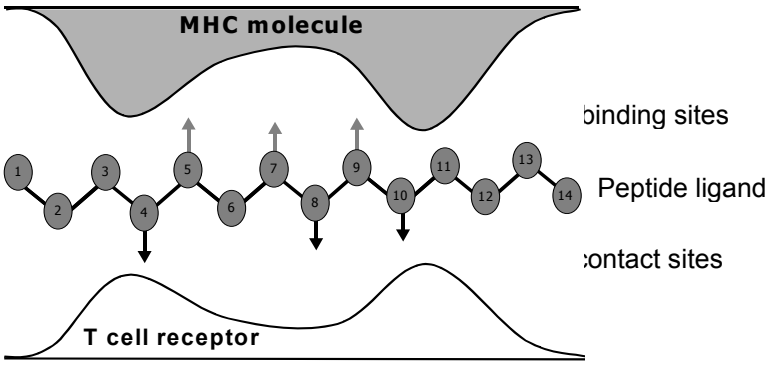


FIGURE 2. The optimal peptide ligand has the highest affinity for one MHC-TCR pair because of its TCR contact sites (black arrows) and MHC binding sites (gray arrows), which allow it to fill out the MHC and TCR binding grooves.

MHC and TCR contact residues and fits well in the binding grooves (Figure 2). Peptide ligands with amino acid substitution in the MHC anchor positions are referred to as MHC anchor-modified ligands. In addition, peptides with modifications on the TCR contact sites, generally termed altered peptide ligands (APLs), are able to alter the TCR signaling (Bielekova *et al.* 2001). However, the definition of APLs has become enlarged and includes all the peptide analogues, which contain one or more substitutions at any residue (Anderton 2001). Table 1 shows the classification of APLs based on their outcome on the antigen-specific T cell response as compared to the natural peptide ligand.

TABLE 1. Classification of altered peptide ligands according to their effects on the wild-type (wt) antigen-specific T cells (after Anderton 2001).

Up-regulation of the immune response		Down-regulation of the immune response	
Agonist	Induces response at equivalent doses to wild-type (wt) antigen	**Partial agonist**	Qualitatively different activation: cytokine production or target lysis in absence of proliferation
Weak agonist	Can stimulate T cell activation with higher doses as the wt antigen	**TCR antagonist**	Inhibits T cell activation when presented on the same APCs with the wt antigen
Agonist	Stimulates T cell activation at low doses		

APLs seem to influence the type and/or effectiveness of the T cell response by modulating the intracellular signal transduction pathways and phosphorylation of TCR-linked proteins involved in T cell activation (Abrams *et al.* 2000). APLs that act either as TCR antagonists or immune deviators have been studied in the murine model of multiple sclerosis and experimental autoimmune encephalomyelitis (EAE).

The dominant antigen epitopes for these autoimmune diseases are quite well characterized. The use of APLs derived from the immunodominant epitopes has highlighted the possibility to induce a variety of biologic activities, such as cytokine production without proliferation, changes in cytokine profile, and anergy induction. Although the therapeutic APLs have been well studied in the experimental autoimmune diseases, there are still very few data available about the APLs application in the transplantation situation.

Therefore, the aim of the study was to explore the possibility of modulating the alloimmune response using peptide analogues generated from the immunodominant MHC class I peptide. For this purpose, different Wistar-Furth MHC class I peptides (WF, RT1.A^u), covering all the MHC different amino acids between WF ($RT1^u$) as donors and LEW ($RT1^l$) as recipients, were tested for their immunogenicity. Our results show the restriction of the alloimmune response to the immunodominant peptide P1 (residues 1-19). Since the disparities between P1 and the corresponding LEW sequence are located only on positions 5, 9, and 10, we changed these allogeneic amino acids step-by-step in order to generate peptide analogues. The “recipient-adapted” peptide analogues served as a useful tool to investigate the MHC-allopeptide-TCR interactions and, secondly, to modulate P1-induced T cell activation. One of the analogues, with one allogeneic position remaining, was able to reduce the P1-induced T cell proliferation and to inhibit the effect of P1 on the allograft survival time. The present results indicate that it is possible to generate analogues from the immunodominant allopeptide by changing the allogeneic amino acids with the corresponding syngeneic or self amino acids. Our findings underline the value of such analogues in analysing the fine specificity of the dominant allopeptide as well as their potential in the modulation of alloimmune responses. This could greatly facilitate the development of the particularly required antigen-specific strategies in transplantation.

2. AIMS OF THE STUDY

1. Analyses of the immunogenicity of different MHC class I allopeptides from Wistar-Furth (WF, RT1.A^u) in Lewis (LEW, RT1.A^l) recipients. Since the alloimmune response is induced by the MHC differences between donor and recipient, we analyzed whether there is a correlation between the MHC differences and their capacity to activate T cell alloimmune response resulting in allograft rejection.

2. Generation of “recipient-adapted” peptide analogues from the immunodominant allopeptide by changing the allogeneic amino acids with the corresponding syngeneic amino acids. The analogues were tested for their ability to modulate the wild type peptide-induced alloimmune response.

3. Analyses of the relationship between the modulatory ability of “recipient-adapted” peptide analogues and a specific TCR subpopulation.

3. MATERIALS AND METHODS

3.1 Peptides

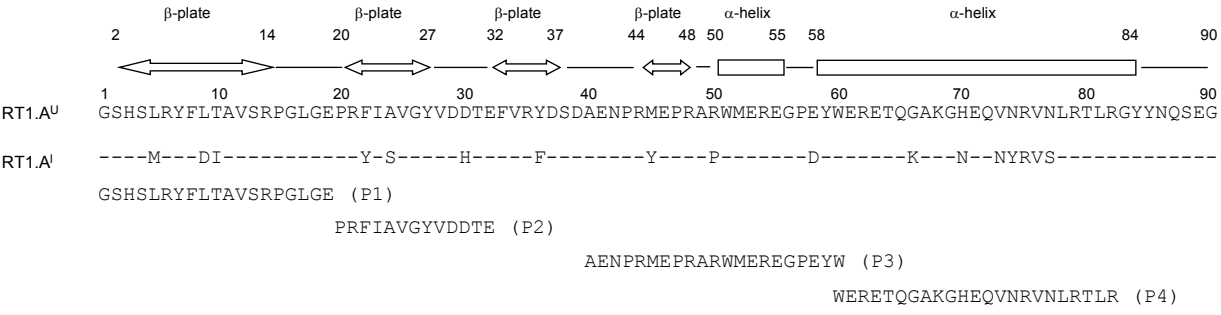
Seven Wistar-Furth (WF) MHC class I (RT1.A^u) peptides (P1-P7) were tested in the present study. The peptides were synthesized by MWG AG Biotech (München, Germany) and Jerini AG (Berlin, Germany) based on published sequences (Joly *et al.* 1995; Chowdhury *et al.* 1998). An additional 25-mer peptide (Pc, residues 110-124) was used as a control peptide. Pc comprises a region of the MHC class I molecule in which both sequences are identical. The amino acid sequences are listed in a one-letter code in Figure 3. The peptides have a purity of 75 – 95% and bear an amide group at the C-terminal end. The lyophilized peptides were dissolved in sterile PBS at 1.0 mg/ml and stored at –20°C.

FIGURE 3. Amino acid sequence alignment of WF (RT1.A^u) and LEW (RT1.A^l) MHC class I molecules. For the RT1.A^l, only the different amino acids are shown, dashes denoting identical sequences. The sequences of the tested peptides are given below the alignment. The locations on α-helical and β-plate regions are according to Bjorkman (Bjorkman *et al.* 1987).

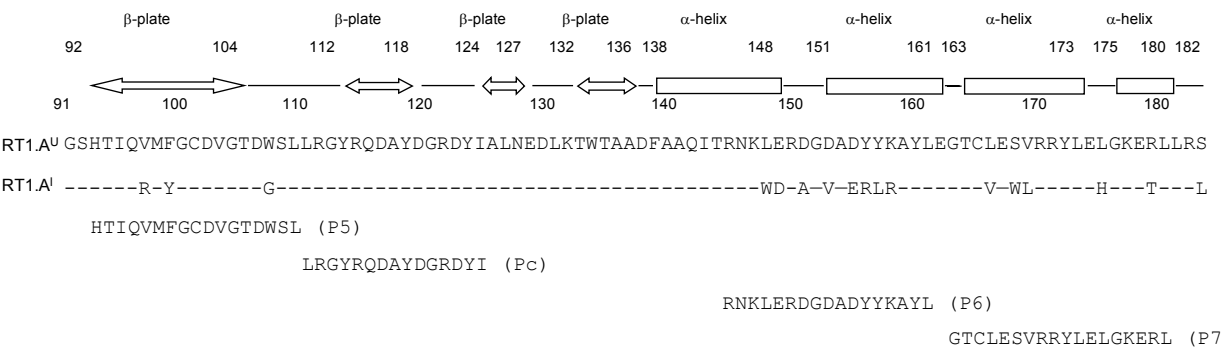
Wistar-Furth MHC class I - RT1.A^u (Donor)

Lewis MHC class I - RT1.A^l (Recipient)

α1 domain



α2 domain



The peptides are located in both the β-plate and the α-helix of either the α1 or α2 domain of the RT1.A^u molecule (Table 2) and cover almost all the MHC disparities between RT1.A^u and RT1.A^l molecules (29 from a total of 34 different amino acids).

TABLE 2. Locations of the WF allopeptides (RT1.A^u) on the MHC class I molecule

Peptide	Residues	Location
P1	1-19	β-plate, α1-domain
P2	20-32	β-plate, α1-domain
P3	40-60	β-plate, α1-domain
P4	60-82	α-helix, α1-domain
P5	93-109	β-plate, α2-domain
P6	144-160	α-helix, α2-domain
P7	166-179	α-helix, α2-domain
Pc	110-124	β-plate, α2-domain

The second set of experiments was performed using a series of 19-mer peptides, designed by amino acid substitution in the sequence of the peptide P1 (RT1.A^u, residues 1-19). Peptide analogue Ac was used as the control peptide, having the identical amino acids as those of LEW (residues 1-19). The substitutions were made in positions 5, 9, and 10, in which the two MHC class I sequences are different. We generated the "recipient-adapted" peptide analogues (A1.1-A1.6) by changing the allogeneic RT1.A^u amino acids with the corresponding syngeneic RT1.A^l amino acids. The amino acid sequences of the peptide analogues are listed in Table 3.

TABLE 3. Amino acid sequences of peptide analogues A1.1-A1.6 in one-letter code. For analogues, only the allogeneic RT1.A^u amino acids (positions 5, 9, and 10) in comparison to the syngeneic RT1.A^l amino acids (residues 1-19) are shown (bold). The syngeneic amino acids are represented for control Ac. The underlined amino acids represent the MHC class II anchor residues as described by Rammensee *et al.* 1995 and extended by Wauben *et al.* 1997.

Peptide	Amino acid sequence																		
	1	2	3	4	**5**	6	7	8	**9**	**10**	11	12	13	14	15	16	17	18	19
P1 (RT1.A^u)	G	S	H	S	**L**	R	Y	F	**L**	**T**	A	V	S	R	P	G	L	G	E
A1.1	–	–	–	–	–	–	–	–	**L**	**T**	–	–	–	–	–	–	–	–	–
A1.2	–	–	–	–	**L**	–	–	–	–	**T**	–	–	–	–	–	–	–	–	–
A1.3	–	–	–	–	**L**	–	–	–	**L**	–	–	–	–	–	–	–	–	–	–
A1.4	–	–	–	–	–	–	–	–	–	**T**	–	–	–	–	–	–	–	–	–
A1.5	–	–	–	–	**L**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A1.6	–	–	–	–	–	–	–	–	**L**	–	–	–	–	–	–	–	–	–	–
Ac	–	–	–	–	**M**	–	–	–	**D**	**I**	–	–	–	–	–	–	–	–	–
RT1.A^l	–	–	–	–	**M**	–	–	–	**D**	**I**	–	–	–	–	–	–	–	–	–

3.2 Animals

Inbred male Lewis (LEW, $RT1^{l}$) and Wistar-Furth (WF, $RT1^{u}$) rats, 8-12 weeks old, weighing 150-200 g, were purchased from Charles River Germany GmbH (Sulzfeld, Germany).

3.3 Immunization

Lewis rats were subcutaneously immunized with 100 µg of the relevant MHC class I peptide (P1-P7) or the peptide analogue (A1.1-A1.6) emulsified with an equal volume of adjuvant (TiterMax, Alexis GmbH, Grünberg, Germany) into each hind footpad. For immunization, the animals were anaesthetized shortly with Isofluran (Abbott GmbH, Wiesbaden, Germany).

3.4 Culture medium and buffers

3.4.1 Culture medium

The standard culture medium consists of RPMI 1640 medium supplemented with 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 x 10^{-5} M 2–mercaptoethanol, 1 % non-essential amino acids and 10 % fetal calf serum (FCS) (Life Technologies GmbH, Karlsruhe, Germany) as final concentrations.

3.4.2 Lyse buffer (10-fold concentrated)

The lyse-buffer used for the erythrolysis contains 1.68 M ammonium chloride, 99.88 mM potassium hydrogen carbonate and 12.6 mM EDTA. As the buffer was concentrated 10-fold, it was diluted 1:10 with sterile aqua injectabila.

3.4.3 Phosphate buffered saline (PBS)

The phosphate buffered saline contains 140 mM sodium chloride, 2.7 mM potassium chloride, 7.2 mM sodium dihydrogen phosphate, 1.47 mM potassium dihydrogen phosphate and has a pH 7.2.

3.5 Proliferation assay

3.5.1 Isolation of lymph node cells

Popliteal lymph node cells were harvested 7 days after immunization of LEW rats with individual WF MHC class I peptides. The popliteal lymph nodes were prepared mechanically and the cells were washed twice and resuspended into 1 ml culture medium.

3.5.2 Dendritic cells preparation

Naïve LEW splenic dendritic cells (DCs) were used as self-APCs. Lewis rat spleens were aseptically removed into RPMI 1640 and the spleen cells were prepared mechanically. The resulting cell suspension was washed with culture medium. The erythrocytes were removed by resuspending the pellet into 10 ml 10-fold diluted lyse-buffer, and the fluid fraction was washed with culture medium. The cell-pellet was incubated in petri dishes overnight in culture medium at 37 °C in a 5 % humidified CO_2 atmosphere. The non-adherent cells were collected and DCs were isolated by centrifugation over a 14.5 % metrizamide gradient (Linaris Biologische Produkte GmbH, Wertheim, Germany) at 4 °C and 1823 xg for 13 minutes. The mononuclear cells were gently collected from the interface, washed with culture medium and resuspended in 1 ml culture medium. The prepared syngeneic DCs were irradiated with 20 Gray before using them as APCs.

3.5.3 T cell proliferation assay

A standard T cell proliferation assay was performed in 96-well rounded-bottom plates in order to investigate peptide immunogenicity. Primed T cells (10^5 cells/well) were cultured with irradiated syngeneic DCs (10^4 cells/well) and 5 µg/well peptide, into a final volume of 150 µl/well. Control wells were set up with control peptide Pc. Plates were incubated for 3 days at 37 °C in a 5 % humidified CO_2 atmosphere and pulsed with 0.5 µCi/well [^{3}H]-thymidine for the last 6 h of culture. The [^{3}H]-thymidine incorporation was measured by using the standard liquid scintillation procedure. Experiments were set up in 6 replicates and results expressed in counts per minute (cpm) ± standard deviation (SD).

3.5.4 MHC competition assay

Since the peptide analogues were generated to modulate the immune response, we performed a MHC competition assay, in order to test the ability of analogues to compete with the wild-type peptide P1 for the MHC binding sites. Therefore, syngeneic DCs (10^4 cells/well) were incubated with an optimal concentration of peptide P1 (5 µg/well) with a final volume of 100 µl/well. After 2 h, the P1-pulsed DCs were washed with culture medium to remove unbound peptides. Various concentrations of peptide analogues (1 - 100 µg/ml) were added and incubated for another 2 h into the same volume of 100 µl/well. The cells were washed again with culture medium and the P1-primed popliteal lymph node cells were added to the culture into a final volume of 150 µl/well. As controls, various concentrations of either P1 or Ac were added to P1-pulsed syngeneic DCs. The proliferation was assessed as previously described for a standard proliferation assay.

3.5.5 TCR modulation assay

Furthermore, in order to analyze whether the analogues are able to induce anergy or non-responsiveness in P1-specific T cells, P1-primed cells were incubated with different concentrations of peptide analogues (1 - 100 µg/ml) in the presence of syngeneic DCs into a final volume of 150 µl/well. After 2 h, cells were washed with culture medium and rechallenged with P1-pulsed syngeneic DCs. As controls, peptides P1 or Ac were added in the first step to P1-primed T cells and syngeneic DCs. The proliferation was measured as described for a standard proliferation assay.

3.6 Measurement of cytokine production

Culture supernatants of *in vitro* restimulated peptide-primed lymph node cells were collected after 72 h, and the levels of IL-2, IFN-γ, IL-4, IL-10 and IL-13 cytokines were assessed by ELISA method, using BioSource Cytoscreen kits (BioSource International, Camarillo, California, USA). The samples were added to the wells coated with a cytokine-specific antibody, followed by the addition of a biotinylated second antibody and, subsequently, the streptavidin-peroxidase. A tetramethylbenzidine solution was added to produce the color reaction, and its intensity was measured as optical density (OD) at 450 nm using an automated

ELISA-reader. The cytokine concentrations in the supernatant were expressed as pg/ml.

3.7 Flow cytometry analysis

5 x 10^5 cells/sample were incubated for 15 min at 4 °C with the optimal concentrations of mAbs in 100 µl of PBS. The following mouse anti-rat antibodies, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrine (PE) were used for the analysis of cell surface molecules: W3/25 (anti-CD4), OX39 (anti-CD25), OX33 (anti-CD45RA, B cells), OX22 (anti-CD45RC) and R73 (anti-TCR $\alpha\beta$), and OX8 (anti-CD8α) (van den Berg *et al.* 2001). All antibodies were purchased by Linaris Biologische Produkte GmbH (Wertheim, Germany). The nonviable cells were identified with 7-amino actinomycin D (7-AAD) (Becton Dickinson, Mountain View, California, USA). Cell surface immunophenotypic analysis was performed by two-color staining using the FACScan (Becton Dickinson, Mountain View, California, USA) with 10,000 gated cells.

3.8 Heterotopic cardiac transplantation

Lewis rats (LEW, RT1.A^l) served as recipients and WF (RT1.A^u) rats as donors. Heterotopic heart transplantation was performed according to the method of Ono and Lindsey (Ono *et al.* 1969) using standard microsurgical techniques. Briefly, the donor-ascending aorta and the pulmonary trunk were anastomosed end-to-side to the recipient's infrarenal abdominal aorta and inferior vena cava, respectively. The allograft function was monitored by daily transabdominal palpation of cardiac contractions. The rejection was considered complete at the time of cessation of palpable ventricular contractions.

3.9 RT-PCR amplification of TCR Vß mRNA

Total cellular RNA from 10^6 T cells, derived from popliteal lymph nodes or allotransplanted heart tissues, was extracted with 1 ml Trizol Reagent (Life Technologies GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations. The air-dried RNA was dissolved in 40 µl nuclease free water (Promega Corporation, Madison, WI, USA) and stored at –80° C. Graft-infiltrating lymphocytes (GILs) were isolated after perfusion of the cardiac grafts with cold saline

and subsequently mechanically minced. The cells were resuspended in culture medium; tissue debris was removed using a 100-µm strainer. For cDNA synthesis, 30 µl RNA was reverse transcribed with MuLV reverse-transcriptase (2.5 U/µl) and Oligo $d(T)_{16}$ primer (2.5 µM) using GeneAmp RNA PCR Kit (Applied Biosystems GmbH, Weiterstadt, Germany). Five µl cDNA was amplified using a digoxigenin (DIG)-labeled TCR Cß1 primer (derived from the ß chain of the rat TCR constant region) as the downstream primer, and each one of the 22 different TCR Vß primers as the upstream primer into a final volume of 50 µl. All rat-specific primer sequences (TCR Cß1 and TCR Vß, respectively) were described by Shirwan (Shirwan *et al.* 1993) and are listed in Table 4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, was used as control. The amplification products were separated on 2 % agarose gel to control the PCR reaction. The primers were synthesized by MWG Biotech AG (Ebersberg, Germany).

TABLE 4. Oligonucleotide primers used for the analysis of TCR Vβ-chain genes[a]

Primer	Sequence (5' → 3')
Cß1	AAG GGC CCA GCT CAG CTC CAC ATG GTC AAG
Vß1	CCT GTC TTG TGG AAA CCG TAC
Vß2	CTG GGG ACA AAG AGG TCA AAT C
Vß3.3	GCT AGC AGG CTC TTT GGA TTG CC
Vß4	TCT GGT GGC AGT CAC AGG GAG TG
Vß5.2	GTG TGG CCT GGT ACC AAC AGA CTC
Vß6	TCT CCT TGC AGT AGG AAC CAC AC
Vß7	GAC CCA GAT CTG GGG CTA CGG
Vß8.1	AGA GGT GAC ATT GAG CTG TCG
Vß8.2	AAA GGT GAC ATT GAG TTG TAA G
Vß8.3	AAA TGT GAC GTT CAA CTG TCA C
Vß9	GGT TAT GCA GAA CCC AAG ATA
Vß10	ATG TTT AGC TAC AAC AAT AAG C
Vß11	ACA AAT GCT GGT GTC ATC CA
Vß12	AGG GCC ACA ATG ATC TTT TC
Vß13	GCA ATC ATA CAG GAA AGT CAG AC
Vß14	TGG CAG GCT CCA GGA GGG ACC C
Vß15	TCA CAC TGA GGG TGT TCA GGC C
Vß16	GCA GGA CAC ACA GGA CCC AAC
Vß17	GTA ACC CAG ACT CCA AGA TAC
Vß18	TGT TGT TGA TAG TCA AGT TGC
Vß19	AAG AAG CTG GGA GAA GAC CTC
Vß20	TTT ACT TTC AGA ATC AAC GGC C

[a] All the primers were published by Shirwan (Shirwan *et al.* 1993).

3.10 ELISA-based quantification of PCR products

PCR amplicons were quantified using an ELISA-based method as described (VanderBorght *et al.* 1999). Briefly, 40 µl of PCR amplicons were denatured and hybridized with 0.5 mM biotinylated TCR Cß1 sonde (5'-TGG GTG GAG TCA CCG TTT TCA G-3') for 30 min at 55 °C in a PCR cycler. Subsequently, 1 µl of the DNA hybrids was diluted with 50 µl hybridization buffer and transferred to a streptavidin-coated nuclease-free microtiter plate. Finally, the captured amplicons were incubated with anti-DIG peroxidase conjugate and then detected with peroxidase substrate solution. The peroxidase reaction was stopped with 100 µl H_2SO_4. All reagents for the PCR-ELISA were purchased from Roche Diagnostics GmbH (Mannheim, Germany). The ELISA was carried out in duplicate and the reaction intensity was measured at 450 nm (A_{450}) using an automated ELISA-reader. The expression level of each Vß gene was calculated as the percentage of total Vß gene expression using the formula $\%V\beta_x = (A_{450}\ (V\beta_x) \times 100 / \Sigma\ A_{450}\ (V\beta_n))$. Three animals per group were tested.

4. RESULTS

4.1 Investigation of regional lymph nodes after immunization

Seven days after subcutaneous immunization with the immunogenic peptide P1 (see below), the popliteal lymph nodes were enlarged and the cell number was increased ($12.3 \pm 2.7 \times 10^7$ cells), in comparison to naïve LEW ($3.3 \pm 1.4 \times 10^7$ cells) (Figure 4). The immunization was performed with TiterMax as immune adjuvant; taken alone it was not able to increase the number of lymph node cells ($2.5 \pm 0.8 \times 10^7$ cells).

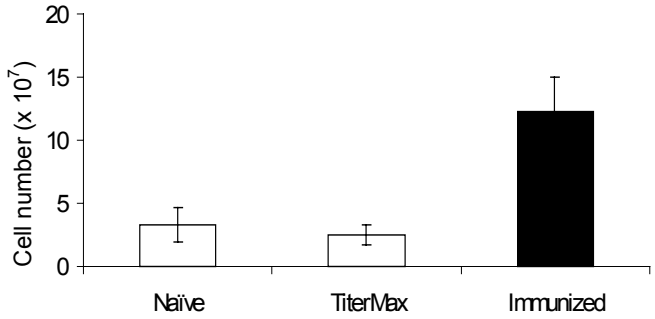


FIGURE 4. The number of lymph node cells increased after immunization with the immunogenic peptide P1. The immune adjuvant TiterMax had no influence. Bars represent the mean (± SD) of three animals per group.

In order to investigate T cell activation after immunization, lymph node cells were analyzed for the expression of CD25 (IL-2Rα) molecules as an activation marker using flow cytometric analysis (FACS). As shown in Figure 5 (A), the proportion of CD4+ CD25+ T cells increased to only 5.5 % after immunization with allopeptide P1

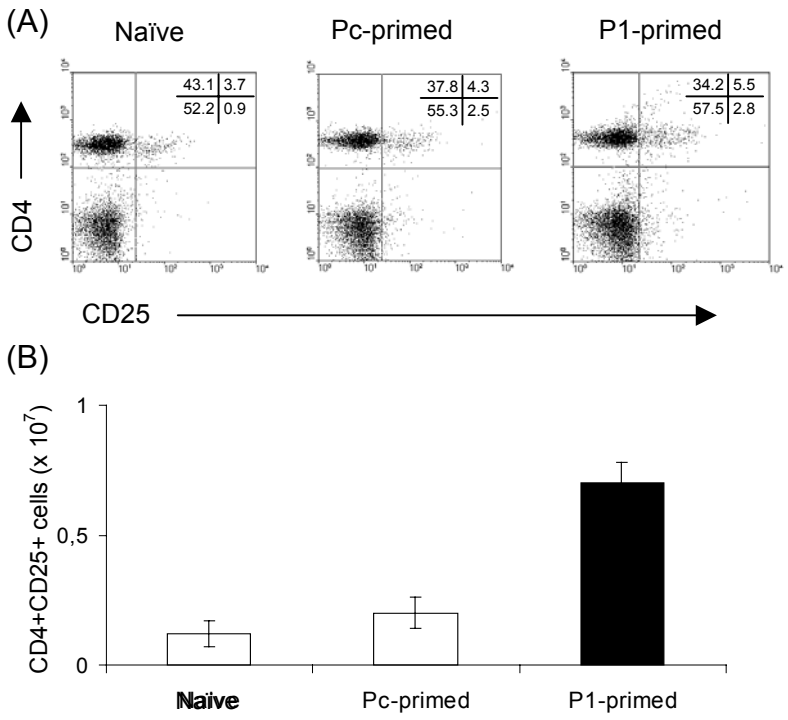


FIGURE 5. Cells were double-stained with anti-CD4 (W3/25) and anti-CD25 (OX39) mAbs and analyzed by flow cytometry. (A) The proportion of activated CD4+ T cells in the regional lymph nodes increased moderately, while (B) the total number of activated T cells increased markedly after immunization with the immunogenic peptide P1.

in comparison to naïve LEW cells (3.8 %). The total number of cells in the lymph nodes of P1-immunized animals increased about 3-fold in comparison to naïve cells, consequently the number of activated CD4+ T cells increased about 6-fold in this situation (Figure 5 (B)). It is noteworthy that immunization with peptide Pc had no influence on the activated T cell population when compared to naïve cells. These results indicate that the subcutaneous immunization with an immunogenic allopeptide is able to activate the immune system of recipients as demonstrated by the enlarged lymph nodes with elevated total cell-numbers as well as activated CD4+ T cells.

Next, the allopeptide-primed lymph node cells were analyzed for the distribution of different populations. As shown in Figure 6, the fraction of T cells slightly decreased after immunization (63.7 % to 54.4 %), while the CD4+ / CD8+ T cell ratio was not affected by the immunization (range 2.6 – 2.9). In contrast, the percentage of B cells increased after immunization, from 27.8 % in naïve lymph node cells to 40.3 % in the allopeptide-immunized animals. These results indicate that both T cells, as shown by the increased number of CD25+ CD4+ T cells and B cells, as shown by the increased cell number, are activated as a response to immunization. This indicates that immunization seems also to activate the humoral response.

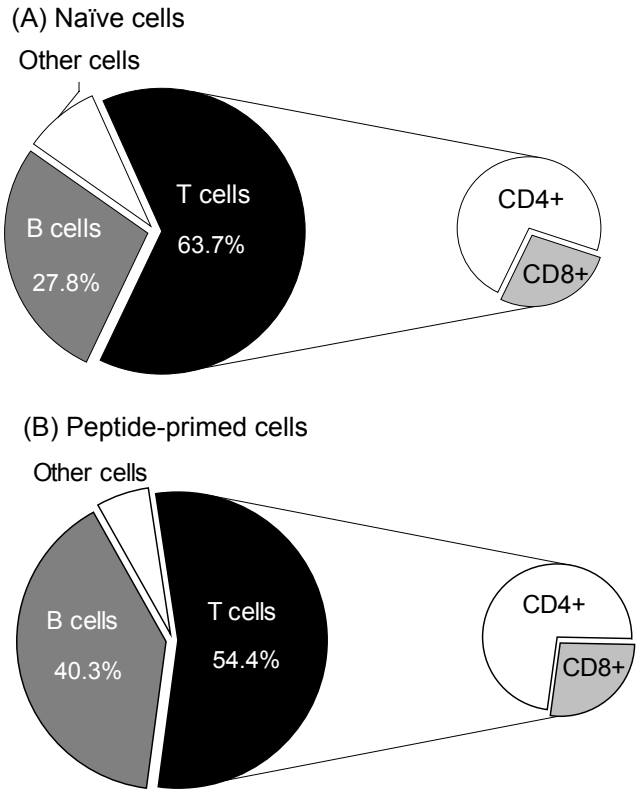
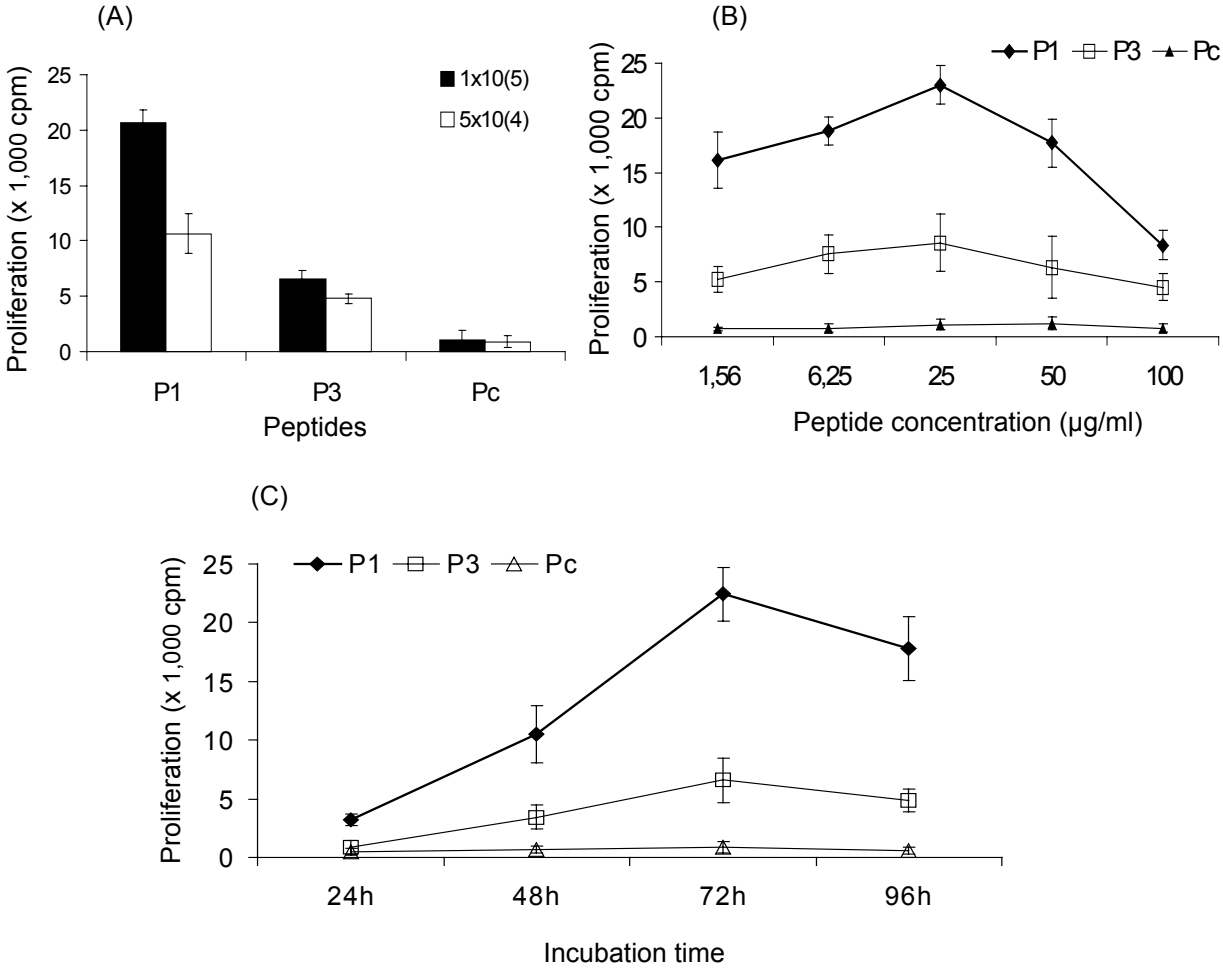


FIGURE 6. Flow-cytometric analysis (FACS) of the effect of immunization with allopeptides on the distribution of the different lymph node cell populations. (A) Naïve lymph node cells and (B) peptide-primed cells were stained with anti-TCR αβ (R73), anti-CD45RA (OX33), anti-CD4 (W3/25), and anti-CD8 (OX8) mAbs. After immunization, the proportion of B cells increased, In addition, the CD4+ / CD8+ T cell ratio was unmodified after immunization.

4.2 Determination of the optimal conditions for the T cell proliferation assay

First, we addressed the following questions: what are the parameters of choice for the T cell proliferation assay in regard to incubation time, necessity of primed T cells, and optimal antigen concentration used for restimulation. To establish this, different amounts of peptide-primed T cells isolated 7 days after immunization were incubated *in vitro* with syngeneic DCs and different concentrations of peptides. The cells were pulsed with [^{3}H]-thymidine after 24 h, 48 h, 72 h, and 96 h, respectively. As shown in Figure 7 (A), peptide P1 induced the highest proliferation when 10^5 P1-primed T cells were restimulated (21,300 ± 2,500 cpm). This effect was not evident in the control peptide Pc and peptide P3, both having low proliferation capacity (see below). Furthermore, the maximum proliferation was induced by 25 µg/ml of peptide (23,500

FIGURE 7. Selection of the optimal conditions for the proliferation assay. (A) The highest T cell proliferation was obtained with 10^5 T cells. (B) The maximum proliferation was induced by 25 µg/ml antigen and (C) after 72 h incubation. Results represent the mean (± SD) of 6 replicates from three animals per group.



± 2,100 cpm for P1 and 8,570 ± 1,800 cpm for P3). The proliferative response decreased when peptides were added at high concentration (100 µg/ml) to 8,350 ± 1,400 cpm for P1 and 4,500 ± 1,100 cpm for P3 (Figure 7 (B)). The next parameter for testing was the incubation time. After 72 h of incubation, the proliferation showed the highest values for peptides P1 (22,450 ± 2,300 cpm) and P3 (6,700 ± 1,600 cpm), respectively, while Pc as control was not influenced (Figure 7 (C)). To summarize, the T cell proliferation assay established in our laboratory requires 10^5 peptide-primed T cells, which should be restimulated with 25 µg/ml peptide for 72 h. These conditions represent the standard parameters for the next experiments.

4.3 Immunogenicity of donor MHC class I allopeptides

Next, we addressed the question of whether the MHC differences between WF as donors and LEW as recipients are reflected by the immunogenicity of allopeptides. Seven WF MHC class I allopeptides (RT1.A^u, P1-P7), covering 29 from a total of 34 allogeneic amino acids (Figure 3) were tested in LEW (RT1^l) rats using the T cell proliferation assay. T cell proliferation to the individual peptides was considered positive, and the respective peptide as immunogenic, when the [^{3}H]-thymidine incorporation was higher than 2,000 cpm, corresponding to the 2-fold mean value of the Pc-induced proliferation (Figure 8). The peptide-primed T cells showed a hierarchical distribution of specific proliferation with high values for the peptides

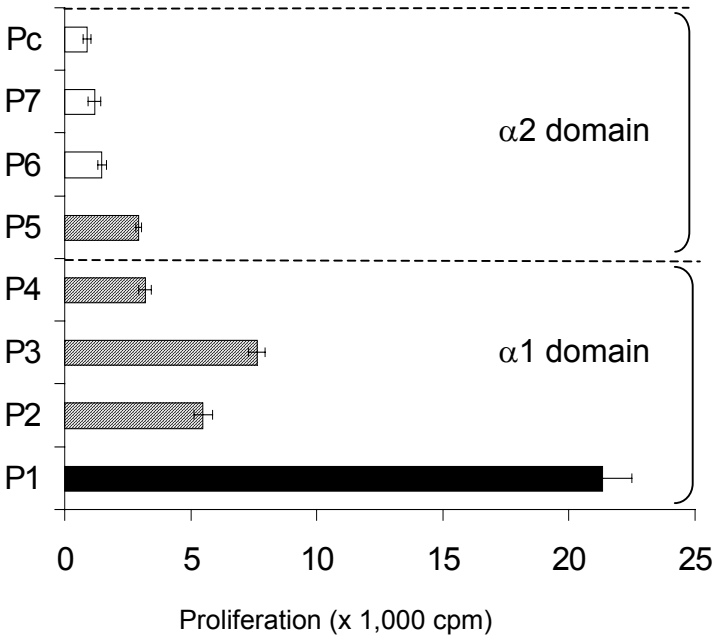


FIGURE 8. Hierarchical distribution of the proliferative responses to individual WF MHC class I peptides (RT1.A^u). Lewis rats were immunized with individual WF MHC class I peptides. Peptide P1 induced the strongest proliferation and represents the dominant peptide. P2-P5, inducing lower proliferation, are considered subdominant peptides, while P6 and P7 are non-immunogenic in LEW rats. Results represent the mean (± SD) of 6 replicates from 3 animals per group.

located in the α1 domain (P1-P4) and lower for those derived from the α2 domain (P5-P7), although the number of allogeneic amino acids is equally distributed between the two domains (Figure 3). Thus, five of the seven WF MHC class I peptides were immunogenic after immunization of LEW rats (P1-P5). In addition, peptide P1, which covers 3 allogeneic amino acids, induced the strongest T cell proliferation (21,500 ± 1,137 cpm). Peptides P2 and P3, each comprised of three allogeneic positions, induced a lower proliferative response (5,500 ± 400 cpm, and 7,600 ± 300 cpm). The other immunogenic peptides, P4 and P5, induced a limited proliferation of the primed T cells (3,200 ± 200 cpm and 2,900 ± 230 cpm), although peptide P4 contains 7 different amino acids in comparison with the LEW sequence (Figure 3). Moreover, peptides P6 and P7, with 8 and 5 allogeneic positions, were not able to induce specific proliferation in LEW rats. These results indicate that the immunogenicity is not influenced by the number of MHC disparities, but possibly by their quality. While in the WF-LEW rat strain combination four (P1-P4) of the five immunogenic peptides are located on the α1-domain, it seems that this domain is more important for the induction of the alloimmune response in our model. In conclusion, the WF MHC class I peptide P1 represents the dominant allopeptide for the LEW alloreactive T cells, peptides P2-P5 are subdominant, and P6 and P7, are non-immunogenic peptides (Table 5).

TABLE 5. The immunogenicity of MHC class I peptides does not correlate with the number of allogeneic amino acids. Four (P1-P4) out of the five immunogenic peptides are located on the α1-domain .

Peptides	MHC location	Allogeneic amino acids	Immunogenicity
P1	α1-domain	3	Dominant
P2	α1-domain	3	Subdominant
P3	α1-domain	3	Subdominant
P4	α2-domain	7	Subdominant
P5	α2-domain	3	Subdominant
P6	α2-domain	8	Non-immunogenic
P7	α2-domain	5	Non-immunogenic
Pc	α2-domain	0	Control

Next, we examined whether the induction of specific T cell proliferation correlates with the cytokine production. The supernatants of peptide-primed T cell cultures were collected after 72 h of incubation. The dominant peptide P1 induced markedly higher levels of IL-2 (11-fold increase), IFN-γ (139-fold increase), IL-10 (2.4-fold increase) and IL-13 (15-fold increase) in comparison to the control peptide Pc (Table 6). The immunogenic peptides P2-P5 (see also Figure 8) induced increased levels of IFN-γ, while the IL-2 production was limited (56-76 pg/ml). Interestingly, peptide P4 with a low proliferation capacity induced high levels of IFN-γ (253 pg/ml). Since peptides P6 and P7 lack proliferation, the cytokine production induced by these peptides were considered as background. P1 with only 3 allogeneic amino acids, represents the dominant peptide, inducing the strongest T cell proliferation with the highest cytokine levels.

TABLE 6. Cytokine production induced by the individual WF MHC class I peptides after *in vivo* immunization and specific *in vitro* restimulation of the primed T cells. Results represent the mean (± SD) of duplicates from two animals per group.

Cytokines (pg/ml)	P1	P2	P3	P4	P5	P6	P7	Pc
IL-2	278±19	76±4	62±20	74±16	56±3	36±12	33±1	18±1
IFN-γ	1,802±269	160±4	77±17	253±10	61±6	37±1	32±2	13±4
IL-4	6±1	3±1	6±1	6±1	16±3	2±1	3±1	1±1
IL-10	97±8	41±5	91±5	87±5	36±2	25±1	14±1	40±4
IL-13	192±6	15±2	87±3	91±3	65±1	30±4	40±5	13±1

4.4 Evidence for immunodominance between MHC class I allopeptides

In order to confirm the immunodominance of peptide P1, LEW rats were immunized with a mixture of 5 allopeptides having different immunogenicities: 3 immunogenic (P1, P2 and P3), 1 non-immunogenic (P6), and the control peptide Pc (Figure 9). The primed lymph node T cells were restimulated *in vitro* with the individual peptides in the presence of syngeneic DCs. Peptide P1 was able to induce the strongest proliferation of mixture-primed T cells (22,350 ± 1,800 cpm). Peptides P2 and P3, which also consist of 3 allogeneic positions, had only a minimal proliferation effect (2,400 ± 500 cpm and 2,900 ± 500 cpm). There was no proliferative response when cells were restimulated with the non-immunogenic peptide P6 (1,400 ± 200 cpm) or

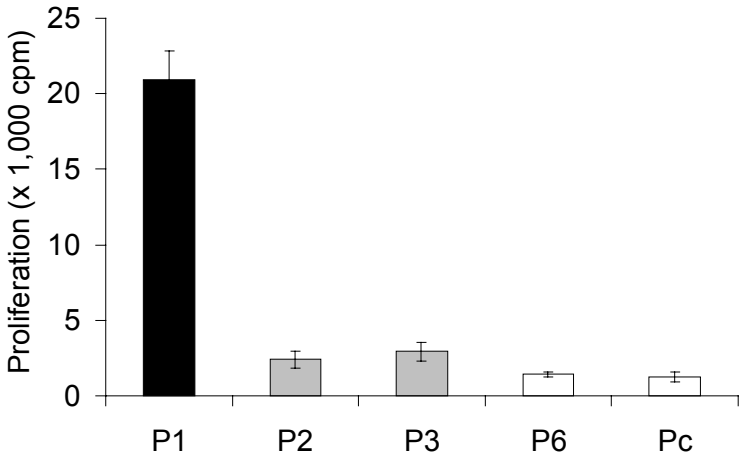


FIGURE 9. T cell proliferation after immunization with a mixture of P1, P2, P3, P6 and Pc. The primed T cells proliferated stronger against P1, while P2 and P3 induced a limited proliferation, confirming the dominance of P1. Bars represent the mean (± SD) of 6 replicates from 2 animals per group.

the control peptide Pc (1,200 ± 300 cpm). Since P1 was able to induce the strongest T cell proliferation of individual peptide-primed T cells as well as of peptide mixture-primed T cells, the results confirm the dominance of peptide P1 in LEW responders.

4.5 Specificity of P1-primed T cells

In order to test the specificity of P1-primed T cells for the other MHC class I peptides, we analyzed the proliferation of P1-primed T cells in the presence of each of the seven synthetic peptides (P1-P7). As shown in Figure 10, only the immunogenic peptides P2 and P3 were able to induce a weak proliferation of P1-primed T cells (4,200 ± 890 cpm and 6,700 ± 1,200 cpm). Noteworthy is that these two peptides also induced a strong proliferation of specific T cells (Figure 8). Peptides P4-P7 failed to induce a proliferative response of the P1-primed T cells (< 2,000 cpm). These results show that there is less cross-reactivity between the P1-primed T cells and other allopeptides. This is unconstrained by the number of allogeneic positions covered by the peptides, but eventually by their specific immunogenicity. This finding

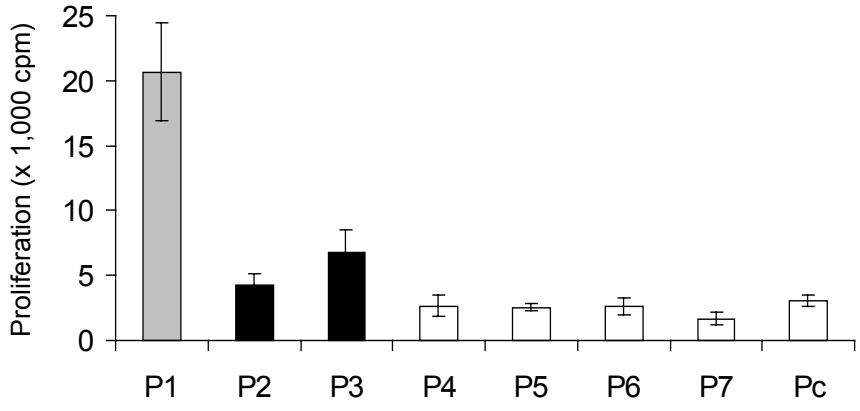


FIGURE 10. Specificity of P1-primed T cells for MHC allopeptides. Only P2 and P3 were able to induce proliferation of P1-primed T cells. The other MHC class I peptides failed to activate such T cells.

indicates the specificity of the cells primed during immunization with the immunodominant peptide P1 in our system.

4.6 Recognition of donor MHC class I peptides is MHC class II-restricted

In order to investigate the importance of MHC class I or class II molecules for the presentation of synthetic MHC class I peptide in the *in vitro* proliferation assay, we performed an antibody blocking assay. P1-primed T cells were incubated with syngeneic DCs and peptide P1 in the presence of different antibodies. As shown in Figure 11, the proliferation of P1-primed T cells was markedly reduced in the presence of anti-MHC class II (OX6, anti-RT1.B) (8,680 ± 1,200 cpm) as well as anti-CD4 (W3/25) (6,150 ± 2,200 cpm) antibodies, whereas anti-MHC class I (OX18, anti-RT1.A) and anti-CD8 (OX8) antibodies did not influence the T cell proliferation.

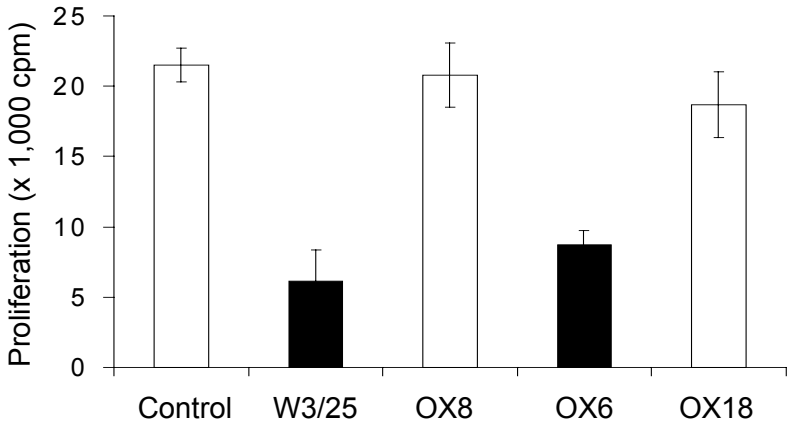


FIGURE 11. Antibody blocking assay for P1-primed T cells. P1-primed T cells failed to proliferate in the presence of anti-CD4 (10 µg/ml) and anti-MHC class II (12.5 µg/ml) mAbs in the *in vitro* proliferation assay. Control wells were set up with 12.5 µg/ml of irrelevant IgG antibody.

4.7 Effect of immunization with MHC class I allopeptides on allograft survival

To test whether the *in vitro* findings are of importance in the *in vivo* transplantation situation, the effect of an immunization with donor MHC class I peptides on the allograft survival time was analyzed in a heterotopic cardiac transplantation model between WF rats ($RT1^u$) as donors, and LEW rats ($RT1^l$) as recipients. The animals were divided into the following groups: control group, composed of either non-immunized or WF skin-transplanted LEW recipients, the P1 group, composed of P1-immunized LEW recipients, and 3 additional groups consisting of P3, P7, and Pc immunized LEW recipients (Figure12 (A)). Another group consisted of LEW recipients, which received P1-primed lymph node cells 7 days before transplantation

(Figure 12 (B)). None of the transplanted animals received immunosuppressive therapy before or after transplantation. The non-immunized animals rejected their allografts within 8 days after transplantation, while those animals that received WF skin grafts (the strongest sensitization procedure), induced an accelerated rejection of heart allografts within 48 h. Immunizing with the control peptide Pc or non-immunogenic peptide P7 had no influence on the allograft survival time. Surprisingly, peptide P3 with a low T cell proliferation and cytokine production was able to significantly reduce the cardiac allograft survival time to 5.5 ± 0.5 days, comparable to the dominant peptide P1 (4.5 ± 0.5 days). Furthermore, the transfer of P1-primed T cells into LEW recipients induced the same effect on the allograft (5.3 ± 0.5 days), confirming the *in vitro* dominance of these cells. In conclusion, immunization with WF peptide P1, covering only 3 allogeneic amino acids, seems to be able to activate the alloimmune system of LEW recipients as confirmed by *in vitro* proliferation and cytokine production of P1-primed T cells as well as by the reduction of allograft survival time.

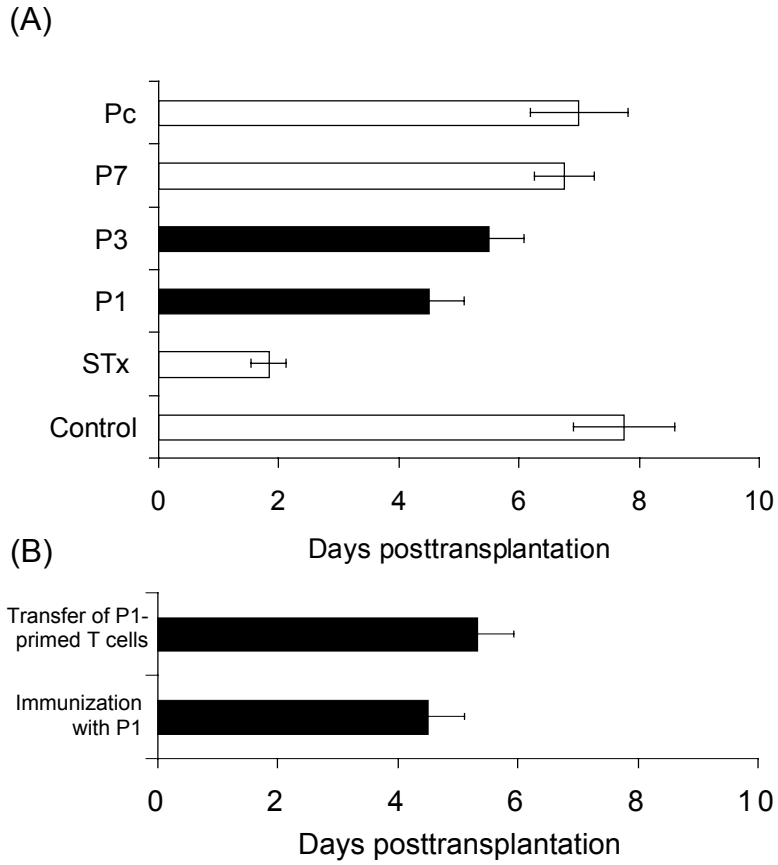


FIGURE 12. (A) Immunization with the immunogenic peptides, P1 or P3 reduced the allograft survival time. The WF skin-transplanted (STx) recipients rejected their grafts within the first 48 h after transplantation. The non-immunized control animals, and the animals immunized with P7 or Pc, rejected their grafts within 8 days. (B) The transfer of P1-primed T cells into naïve LEW recipients induced the rejection of allografted hearts within 5 days, comparable to the immunization with P1.
(n = 4 animals per group)

Summary 4.1 – 4.7: Not all the tested WF MHC class I peptides (RT1.A^u) were immunogenic in the LEW (RT1.A^l) recipients. There was a hierarchical distribution of their immunogenic abilities, as shown by the *in vitro* proliferation assay. Almost all of the immunogenic peptides are located on the α1 domain of a MHC class I molecule, but the immunogenicity was unconstrained by the number of covered allogeneic amino acids. In addition, peptide P1, comprised of only 3 allogeneic amino acids, induced the strongest T cell proliferation and produced high levels of cytokines, especially IL-2 and IFN-γ. Furthermore, the immunodominance of peptide P1 was confirmed by the significant reduction of the allograft survival time in the heterotopic heart transplantation model.

Introduction 4.8 – 4.13: Since the first set of experiments demonstrated the central role of the immunodominant MHC class I allopeptide P1 in the induction of an alloimmune response, the next step was to design “recipient-adapted” peptide analogues derived from peptide P1. The analogues were designed by changing step by step the allogeneic RT1.A^u (non-self) amino acids with the corresponding syngeneic RT1.A^l (self) amino acids on the three different positions 5, 9, and 10. The six analogues were further analyzed for their ability to modulate the alloimmune response

4.8 Kinetic and dose-dependent proliferation induced by peptide analogues

First, we tested whether the proliferation responses induced by the peptide analogues follow the optimal conditions established for the MHC class I peptides. The analogue-induced proliferation was assessed after different incubation times (24, 48, 72, 96, and 120 h). After 72 h of *in vitro* restimulation, almost all the analogues (A1.1-A1.4, and A1.6) showed a maximum proliferation, whereas the analogue A1.5 induced the maximum proliferation after 96 h (44,290 $\pm$ 4,200 cpm) (Figure 13 (A)). In addition, the proliferative responses induced by the analogues A1.1 and A1.5 were dose-dependent (Figure 13 (B)). As a control analogue, Ac was not able to induce a proliferative response (4,500 $\pm$ 420 cpm) and its values were not influenced by the incubation time or the peptide doses used for the restimulation, as it was recognized as a self-peptide.

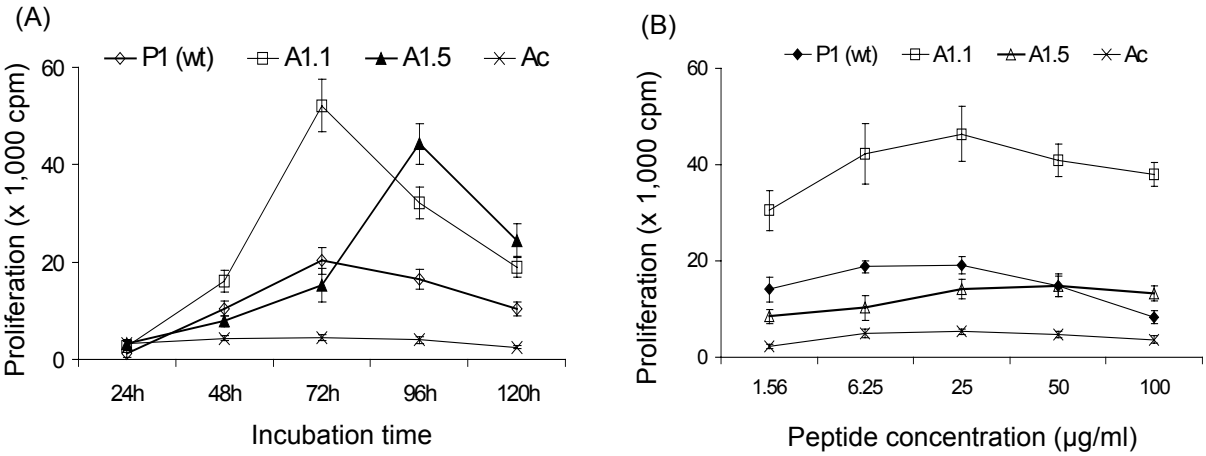


FIGURE 13. (A) Kinetic analysis of T cell proliferation. Analogue A1.1 and peptide P1 induced the maximum proliferation after 72 h of incubation, while analogue A1.5 reached its maximum after 96 hours. (B) The analogues A1.1 and A1.5 induced a dose-dependent specific T cell proliferation.

4.9 Characterization of peptide analogue-primed T cells

First, the peptide analogues A1.1–A1.6 were tested whether single or double substitutions have any influence on the proliferative response. Analogue Ac, corresponding to LEW sequence (residues 1-19) was used as a control. The individual analogues were analyzed in a standard proliferation assay of analogue-primed lymph node T cells. When compared to the wt peptide P1-induced T cell proliferation, the analogues can be categorized into three groups (Figure 14): (a) The analogue A1.1 with 2 allogeneic amino acids (L9, T10) and a substituted amino acid (L5 with M5), which induced a stronger T cell proliferation (45,098 $\pm$ 2,600 cpm) than P1. (b) The analogue A1.5, containing the residue L5 as the only allogeneic amino acid, which had a weaker specific proliferation capacity (11,450 $\pm$ 1,400 cpm) in comparison to P1; and (c) the other analogues (A1.2-A1.4, and A1.6), which were able to induce a specific proliferation comparable with peptide P1. These results show that the analogues with given substitutions are able to induce a specific proliferative response and these proliferations depend on the position where the substitution was made.

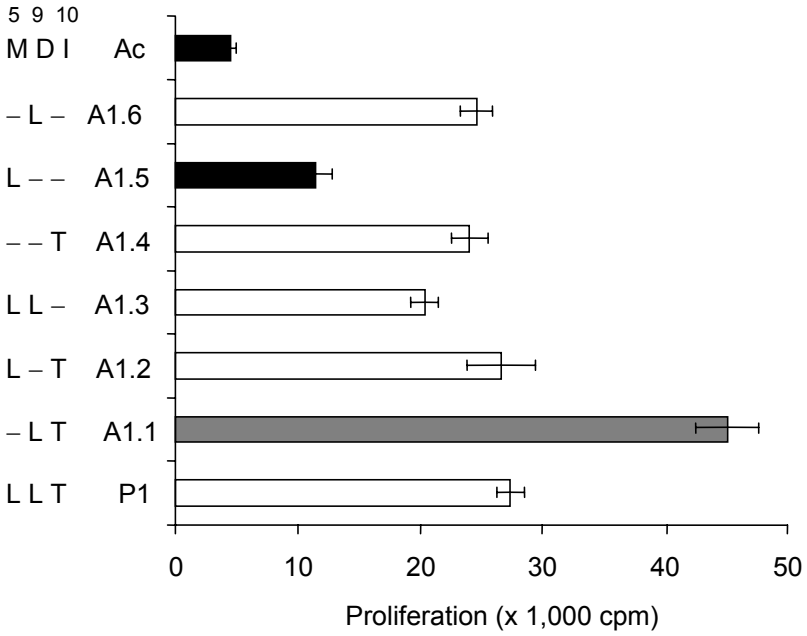


FIGURE 14. Peptide analogues have different specific proliferative capacity. The allogeneic amino acids are shown on the left side. A1.1 (two allogeneic amino acids) induced a stronger proliferation, whereas A1.5 (one different amino acid) induced a weaker proliferative response in comparison to P1. Bars represent the mean (± SD) of 6 replicates from 3 animals per group.

In order to further characterize the activation capacity of the analogues, the cytokine production was measured in the supernatant of 72 h-cultures of analogue-primed T cells (Table 7).

TABLE 7. Cytokine production by peptide analogue-primed T cells. Cytokine levels were measured by ELISA in the 3-day culture supernatants. Data are mean values (± SD) of duplicates from 3 animals per group.

Peptide	Cytokine levels (pg/ml)				
	IL-2	IFN-γ	IL-4	IL-10	IL-13
P1	278±19	1,802±269	6±1	97±8	192±6
A1.1	2,172±28	9,270±170	4±0,3	221±48	161±31
A1.2	755±36	877±63	18±5	41±12	80±21
A1.3	558±59	553±49	10±1	75±36	306±36
A1.4	163±16	535±69	279±25	80±20	261±45
A1.5	485±42	1404±56	344±68	172±13	49±3
A1.6	422±21	390±25	1±0.1	138±25	26±9
Ac	40±5	60±6	1±0.1	36±8	22±4

All analogues except A1.4 induced higher levels of IL-2 in comparison to the wt peptide P1, indicating that there is no correlation between the specific proliferation and IL-2 production. Analogue A1.1 with the L9, T10 allogeneic amino acids induced the highest levels of IL-2, IFN-γ, IL-10 and IL-13. Interestingly, analogues with one allogeneic position, which induce the same specific T cell proliferation as P1, are able

to produce an altered cytokine profile: e.g. A1.4 (T10) induced lower levels of IL-2 and IFN-γ (163 ± 16 pg/ml, and 535 ± 69 pg/ml) but elevated levels of IL-4 and IL-13 (279 ± 25 pg/ml, and 261 ± 45 pg/ml). In contrast, the analogue A1.5, with weaker specific proliferation ability, induced a high level of IFN-γ (1,404 ± 56 pg/ml) similar to P1, associated with increased IL-4 and IL-10 secretions. These data underline the implication of each allogeneic position in the activation of the specific T cells.

Altogether, the generated analogues are able to induce a specific immune response characterized by specific proliferation and cytokine production (Table 8).

TABLE 8. Relationship between the specific proliferation and cytokine production and the allogenicity of the peptide analogues.

Peptide	Allogenicity[a]		Specific proliferation[b]	Specific cytokines[c]
P1[d]	L L T	3		
A1.1	– L T	2	>	IL-2, IFN-γ, IL-10
A1.2	L – T	2	=	IL-2, IL-4
A1.3	L L –	2	=	IL-2, IL-4, IL-13
A1.4	– – T	1	=	IL-4, IL-10, IL-13
A1.5	L – –	1	<	IL-2, IFN-γ, IL-4, IL-10
A1.6	– L –	1	=	IL-2, IL-10
Ac[e]	M D I	0	No	No

[a]Allogenicity corresponds to the number of allogeneic amino acids for each analogue. Only the three different positions (5, 9, and 10) are shown.

[b]The specific proliferative capacities are qualitatively expressed as high (> P1), low (< P1), or equal (= P1) in comparison to the wt peptide P1 (21,500 ± 1,137 cpm).

[c]Each cytokines level is compared to the level produced by P1: IL-2 > 278±19 pg/ml, IFN-γ > 1,802±269 pg/ml, IL-4 > 6±1 pg/ml, IL-10 > 97±8 pg/ml, and IL-13 > 192±6 pg/ml. Only the cytokines that fulfill these values are shown.

[d]P1 is the wt peptide with three allogeneic amino acids.

[e]Ac is the peptide with syngeneic sequence.

As shown in Table 8, the number of allogeneic amino acids does not correlate with the intensity of the proliferative response, whereas the substitutions made on these positions give us some indication of their importance in the MHC-peptide-TCR interaction. Thus, when L5 is not substituted and one of the amino acids on position 9 or 10 is modified (A1.2, A1.3), the resulting peptides have the same proliferation

ability as P1, whereas A1.1 with modified L5 induced a higher proliferation than P1. These data correlate with the finding described by Rammensee (Rammensee *et al.* 1995) and extended by Wauben (Wauben *et al.* 1997) that L5 represents a possible MHC anchor residue. The substitution of L5 with M5 induced a higher proliferation (A1.1), probably caused by the higher MHC binding affinity of methionine. Furthermore, analogue A1.5 with only one allogeneic amino acid (L5) induced a lower proliferation in comparison to P1. As a whole, these results indicate that position 5 is important for the MHC contact, while the other two positions (9 and 10) could be important for TCR contact.

4.10 Specificity of peptide P1-primed T cells for its peptide analogues

Since the analogue-primed T cells showed a different proliferative capacity, the next question was whether the specificity of P1-primed T cells is influenced by these substitutions. Therefore, P1-primed T cells were restimulated *in vitro* with peptide analogues based on the standard proliferation assay. As shown in Figure 15, P1-primed T cells were able to proliferate in the presence of the analogues A1.1 (L9, T10) and A1.6 (L9) (36,200 ± 2,400 cpm and 27,900 ± 3,100 cpm, respectively). Analogues A1.2, A1.3, and A1.4 induced a lower proliferation of the P1-primed T cells in comparison to P1 (9,350 – 16,100 cpm), indicating that single changes on positions 9 or 10 still allow the activation of the P1-primed T cells, but at a lower level. In addition, the P1-primed T cells failed to proliferate in the presence of A1.5 (3,800 ± 1,500 cpm), comparable to the syngeneic peptide Ac (3,250 ± 1,200 cpm). This finding indicates that the double substituted analogue A1.5, with unmodified MHC

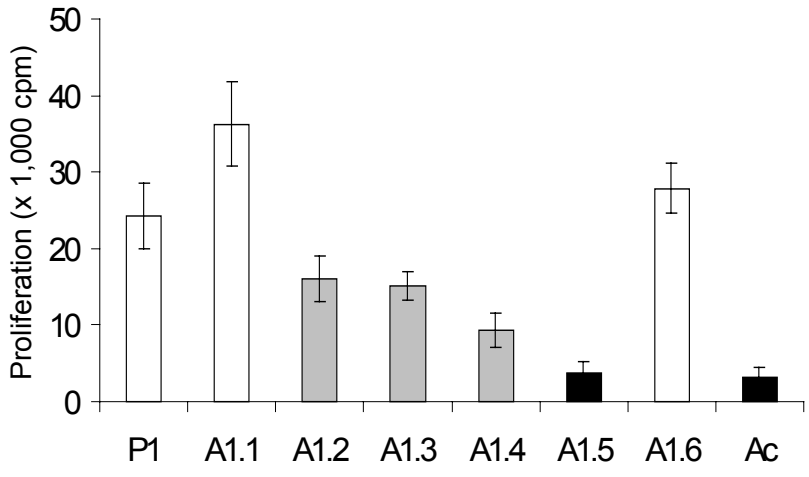


FIGURE 15. Specificity of P1-primed T cells for the analogues. P1-primed T cells were restimulated *in vitro* with the peptide analogues and syngeneic DCs. A1.5 and the control Ac failed to induce proliferation of P1-primed T cells. Bars represent the mean (± SD) of 6 replicates from 3 animals.

contact (L5), either down-regulated P1-induced activation or the peptide was not recognized by P1-primed T cells. Following the classification described by Anderton (Anderton 2001) (Table 1), the analogues could be categorized as follows: A1.1 as agonist, A1.6 as agonist and A1.2, A1.3, and A1.4 as weak agonist peptides. Analogue A1.5 was able to down-regulate the proliferation of P1-primed T cells.

4.11 TCR modulation ability

Next, we performed a TCR modulation assay in order to investigate the mechanism responsible for the lack of proliferation of P1-primed T cells induced by analogue A1.5. The schematic presentation of the assay is shown in Figure 16.

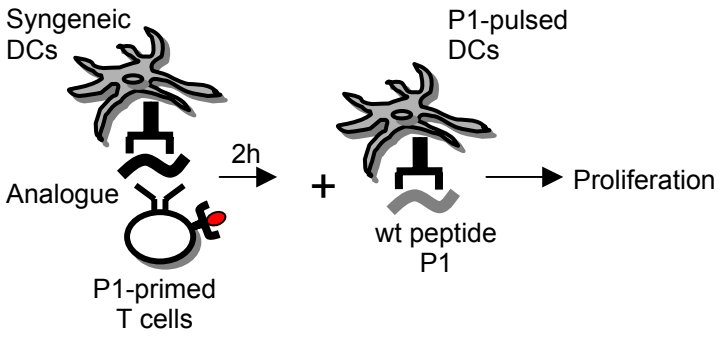


FIGURE 16. TCR modulation assay. P1-primed T cells were incubated for 2 h with various concentrations of analogues and syngeneic DCs. After 2 h, P1-pulsed DCs were added and the proliferation of P1-primed T cells was measured as described for the standard proliferation assay.

Figure 17 shows that the P1-primed T cells, pre-incubated with either the control peptide Ac (33,900 ± 2,700 cpm for 10 µg/ml) or the wt peptide P1 (30,600 ± 2,900 cpm), were able to proliferate after the second *in vitro* rechallenge with P1. Pre-incubation of P1-primed T cells with analogue A1.5 did not influence the proliferation after the restimulation (29,500 ± 2,200 cpm for A1.5 vs. 30,600 ± 2,900 cpm when

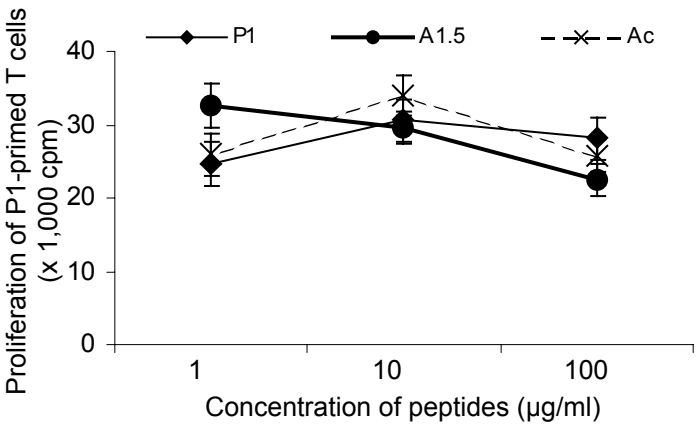


FIGURE 17. TCR modulation assay. P1-primed T cells pre-incubated with A1.5 proliferated after rechallenge with P1, indicating that A1.5 did not induce anergy or other permanent negative signals in P1-primed T cells. Results represent the mean (± SD) of 6 replicates from 2 animals per group.

cells were pre-incubated with 10 µg/ml peptide). This finding indicates that A1.5 did not induce a state of non-responsiveness in P1-primed T cells through the T cell receptor (Figure 15).

4.12 Analogue A1.5 acts as a MHC competitor

The next question we addressed was whether A1.5 is able to compete with the wt peptide P1 for the MHC binding. For this purpose we performed a MHC competition assay as presented in Figure 18.

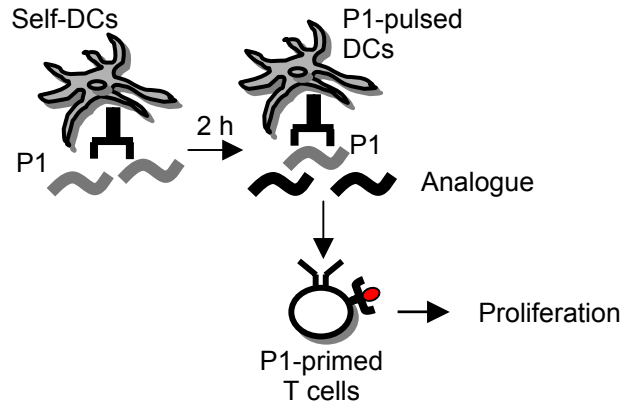


FIGURE 18. Schematic presentation of the MHC competition assay. Syngeneic DCs were pulsed with P1 and after 2 h various concentrations of analogues were added in order to compete for the MHC binding. After 2 h, proliferation of P1-primed T cells was measured as described for the standard proliferation assay.

Control peptide Ac did not influence the proliferation of P1-primed T cells, showing a similar dose-dependent proliferation to P1 (Figure 19). While at a concentration of 100 µg/ml the proliferation of P1-primed T cells seems to show a high-dose inhibition, only the lower concentrations were considered significant. The analogue A1.5 was able to reduce the proliferation when added at 1 and 10 µg/ml (14,461 ± 2,100 cpm vs. 30,200 ± 2,900 cpm for P1). These results indicate that A1.5 with only one allogeneic position acts as a MHC competitor.

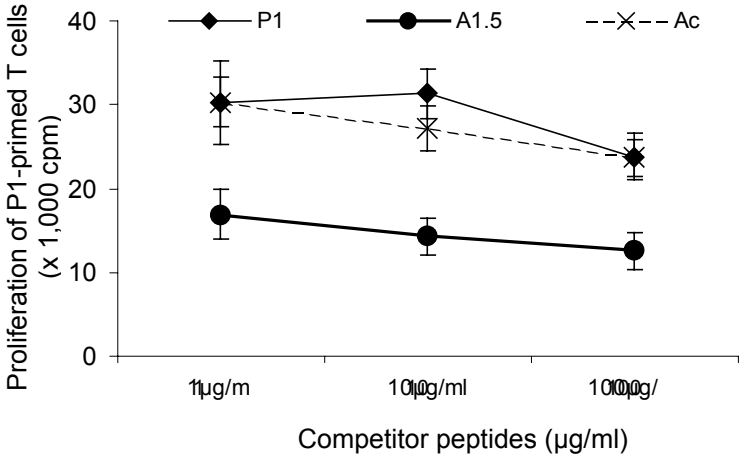


FIGURE 19. MHC competition assay. A1.5 reduced the proliferation of P1-primed T cells when presented on the same DCs. The data indicate the competition of A1.5 with the wt peptide P1 for MHC binding. Results represent the mean (± SD) of 6 replicates from 2 animals per group.

4.13 Influence of peptide analogues on the allograft survival time

Since the previous experimental results demonstrated that in comparison to P1, analogue A1.5 had weak proliferation ability and A1.1 stronger proliferation ability, these two analogues were further investigated for their *in vivo* effect in the allograft situation (Figure 20). Lewis rats were immunized with individual peptide analogues 7 days before they received a heterotopically transplanted WF heart. A1.1-immunized animals rejected their allografts within 5 days (5.2 $\pm$ 0.5 days) similar to the wt peptide P1-immunized recipients (4.5 $\pm$ 0.5 days). In concordance with the low T cell proliferation induced by the analogue A1.5, this peptide had no influence on the allograft survival time when compared to the control group, non-immunized recipients (8.8 $\pm$ 0.5 days). Furthermore, when LEW recipients were immunized with a mixture composed of equal amounts of P1 and A1.5, the allograft was rejected after 7.7 $\pm$ 0.6 days. These results can be explained by the MHC competition between A1.5 and P1, as shown by the MHC competition assay (Figure 19), i.e. A1.5 was able to diminish the negative effect of P1 on the allograft survival.

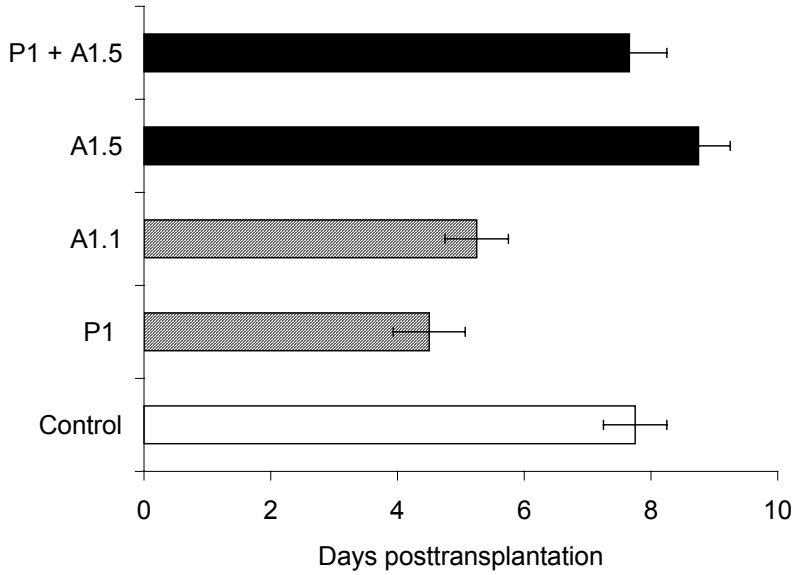


FIGURE 20. Allograft survival time induced by the immunization with peptide analogues (n = 4 animals per group). A1.1 reduced the allograft survival time similar to peptide P1, whereas A1.5 had no influence. Immunization with a mixture of P1 and A1.5 prevented the P1-induced reduction of allograft survival time.

Summary 4.8 – 4.13: The “recipient-adapted” peptide analogues were designed by changing the allogeneic RT1.A^u amino acids one by one with the syngeneic RT1.A^l amino acids, from the RT1.A^u immunodominant peptide P1 as wild-type peptide. The six analogues containing such substitutions were tested in a standard proliferation assay for their ability to induce a specific immune response. Our results indicate that position 5 may be MHC anchor, while the other two allogeneic positions (L9, T10) are important for the TCR contact (Table 8, Figure 15). Next, the analogues were tested for their capacity to activate the wt peptide P1-primed T cells. Following the classification described by Anderton (Anderton 2001), analogues A1.1 and A1.6 are agonists, and A1.2, A1.3, and A1.4 weak agonist peptides, while A1.5 could be classified as a partial agonist or TCR antagonist. Further experiments demonstrated that A1.5 acts as a MHC competitor. The *in vitro* characteristics of A1.5 were confirmed by the *in vivo* experiments. Immunization with A1.5 does not influence the allograft survival time but does diminish the negative effect of the wt peptide, probably by competing for the MHC binding as showed in the MHC competition assay. In conclusion, “recipient-adapted” peptide analogues, such as A1.5, are able to down-regulate the alloimmune response induced by the dominant allopeptide P1 by a MHC competition mechanism.

Introduction 4.14 – 4.17: The next set of experiments was performed in order to characterize the TCR Vß repertoire of the peptide-primed T cells after immunization, *in vitro* restimulation and allotransplantation. Since the allopeptide in the MHC-peptide-TCR complex exclusively contacts the TCR β chain (Qadri *et al.* 2001), the TCR Vß usage was analyzed using RT-PCR ELISA-based method.

4.14 TCR Vß repertoire of MHC class I allopeptides-primed T cells

First, we addressed the question whether the immunogenicity of the tested allopeptides is associated with a defined TCR Vß repertoire after immunization. The percentage expression of TCR Vß genes in the Pc-primed cells was used as control. The TCR Vß repertoire of freshly isolated T cells primed after immunization with self-peptide Pc showed minimal differences from the naïve T cell population (data not shown).

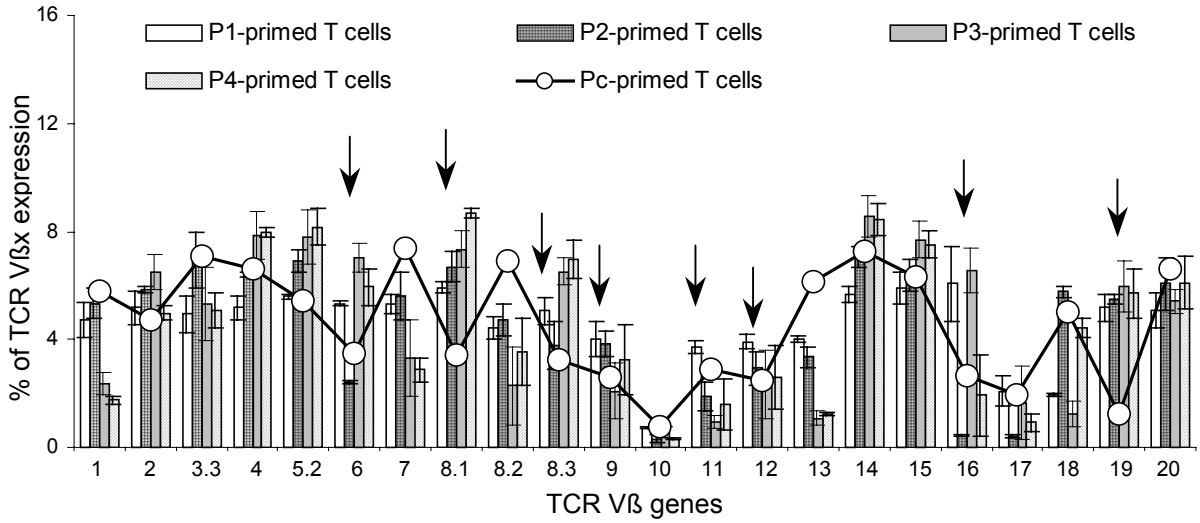


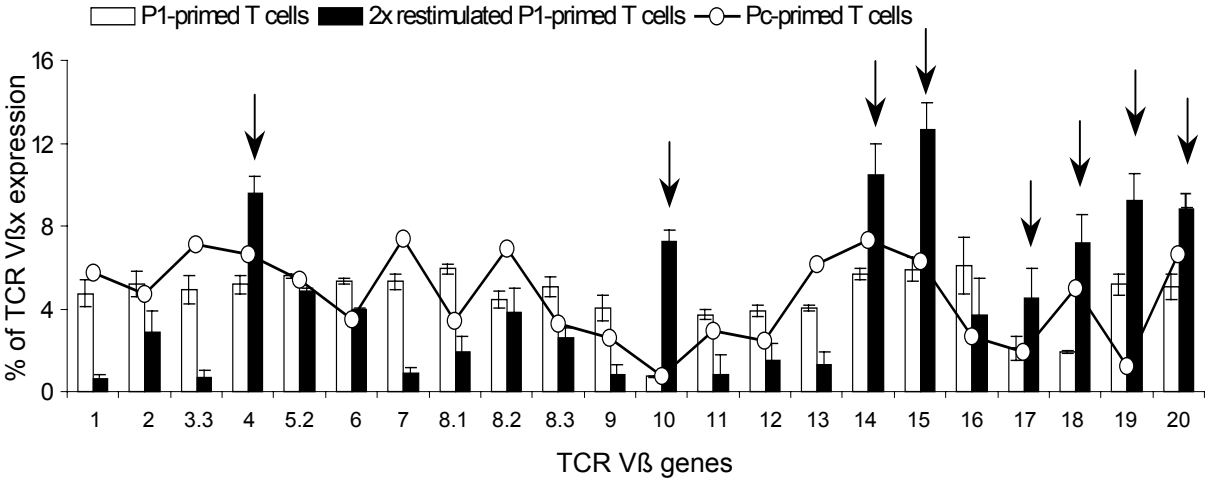
FIGURE 21. TCR Vß repertoire of MHC class I peptide-primed T cells after *in vivo* immunization. Bars represent mean (± SD) of duplicates from 2 animals per group. TCR Vß repertoire in Pc-primed T cells was used as control.

In comparison to the Pc-primed T cells, immunization with the immunogenic peptides P1-P4 activated characteristic TCR Vß subpopulations, which slightly differ from the control Pc (Figure 21). For example, P1-primed T cells overexpressed Vß 6, 8.1, 8.3, 9, 11, 12, 16, 19 genes. These results indicate that immunization with such allopeptides modify the TCR repertoire of naïve T cells.

4.15 TCR Vß repertoire of P1-primed T cells after *in vitro* restimulation

The next question was whether repetitive *in vitro* restimulation gives rise to a restriction or alteration in the TCR Vß repertoire. The TCR Vß repertoire of Pc-primed

FIGURE 22. TCR Vß repertoire after serial *in vitro* restimulation of P1-primed T cells. Arrows indicate the overexpressed TCR Vß genes after 2-fold *in vitro* restimulation. Bars represent mean (± SD) of duplicates from 2 animals per group. TCR Vß in Pc-primed T cells was used as control.

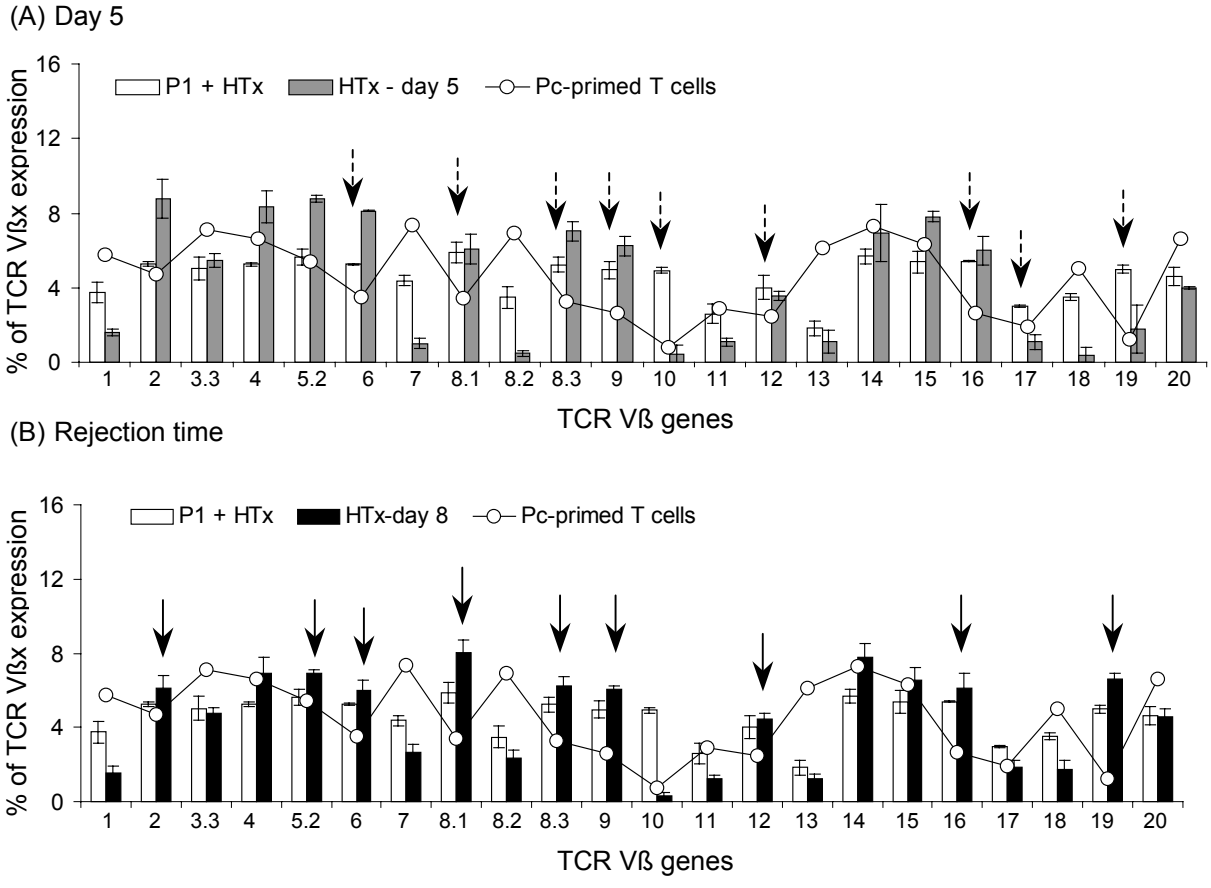


T cells was used as control. As described above (Figure 21), T cells isolated after immunization with peptide P1 have an oligoclonal TCR Vß repertoire (Vß 6, 8.1, 8.3, 9, 11, 12, 16, 19), whereas after the second cycle of *in vitro* restimulation (14 days), the TCR Vß repertoire changed to Vß 4, 10, 14, 15, 17, 18, 19, 20 genes (Figure 22). These results show that repetitive *in vitro* restimulation induces a different TCR Vß repertoire in P1-primed T cells compared to those T cells that were freshly isolated after 7 days post-immunization. Therefore, further investigations are necessary to analyze the effect of the dominant peptide P1 in the induction and maintenance of the alloimmune response *in vitro* as well as *in vivo*.

4.16 TCR Vß repertoire of graft-infiltrating lymphocytes after allotransplantation

The next step was to investigate whether the same TCR subpopulations, characterized above after 2-fold *in vitro* restimulation, are involved in the rejection of

FIGURE 23. Comparative presentation of TCR Vß repertoires in GILs harvested on day 5 (A) and at rejection time (B), from P1-immunized (P1 + HTx) and non-immunized (HTx) recipients. TCR Vß repertoire in Pc-primed T cells was used as control. Bars represent mean (± SD) of duplicates from 2 animals per group. Arrows indicate the overexpressed Vß in GILs from P1-immunized (dashed) and non-immunized (black) recipients at the time of rejection.



WF allografts in LEW recipients. Therefore, graft-infiltrating lymphocytes (GILs) were harvested from P1-immunized LEW recipients on day 5, the day of complete rejection. GILs were analyzed from the non-immunized recipients on day 5 (non-rejected grafts) and day 8, the day of complete rejection in these animals. As illustrated in Figure 23 (B), GILs isolated at the time of rejection from both P1-immunized (day 5) and non-immunized (day 8) recipients are characterized by almost the same TCR Vß subpopulations Vß 6, 8.1, 8.3, 9, 12, 16, and 19, in comparison to Pc-primed T cells as control (Figure 23 (A)). The TCR Vß repertoire in GILs from rejected hearts in non-immunized recipients slightly differs from that identified on day 5 (non-rejected grafts). Interestingly, the TCR Vß repertoire in the rejected grafts correlates with the profile observed after *in vitro* restimulation of P1-primed T cells P1, suggesting a particular role of these TCR Vß subpopulations in the activation of alloreactive T cells after immunization and/or transplantation.

4.17 TCR Vß usage of P1-derived peptide analogue-primed T cells

The analogues (A1.1-A1.6) derived from the immunodominant peptide P1 were analyzed to determine whether the substitutions of the allogeneic positions in P1 influence the TCR Vß repertoire.

TABLE 9. TCR Vß repertoire in peptide analogue-primed T cells in comparison to the control peptide Pc-primed T cells.

Peptide analogue	Overexpressed TCR Vß genes
P1	Vß **6**, 8.1, **8.3**, 9, 11, 12, **16**, **19**
A1.1 (L9 T10)	Vß 2, 5.2, **6**, 8.1, **8.3**, 9, 12, 15, **16**, **19**
A1.2 (L5 T10)	Vß 2, 5.2, **6**, 8.1, **8.3**, 14, 15, **16**, **19**
A1.3 (L9 T10)	Vß 5.2, **6**, **8.3**, 9, 15, **16**, 17, **19**
A1.4 (T10)	Vß 5.2, **6**, 8.1, **8.3**, 9, 12, 15, **16**, 17, **19**
A1.5 (L5)	Vß 2, 4, 5.2, **6**, 8.1, **8.3**, 9, 14, 15, **16**, **19**
A1.6 (L9)	Vß 2, 4, 5.2, **6**, 8.1, **8.3**, 9, 14, 15, **16**, **19**

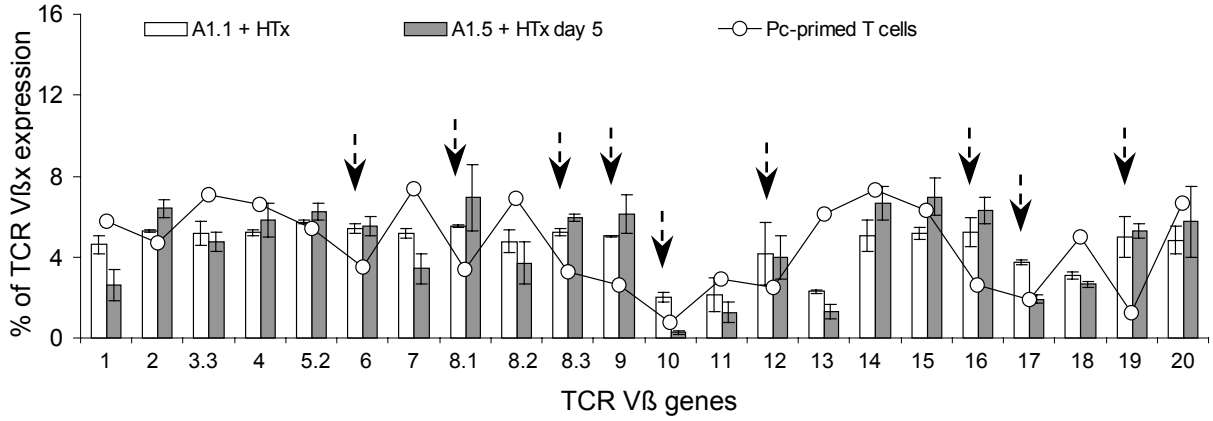
As shown in Table 9, the overexpression of TCR Vß 6, 8.3, 16, and 19 genes obtained in P1-primed T cells was also found for all the P1-derived analogues (see Appendix). These results show that the substitutions in the allogeneic position are still able to activate some wt peptide P1-specific TCR subpopulations. In addition, each

analogue induces, after immunization, the activation of some other specific TCR Vß genes.

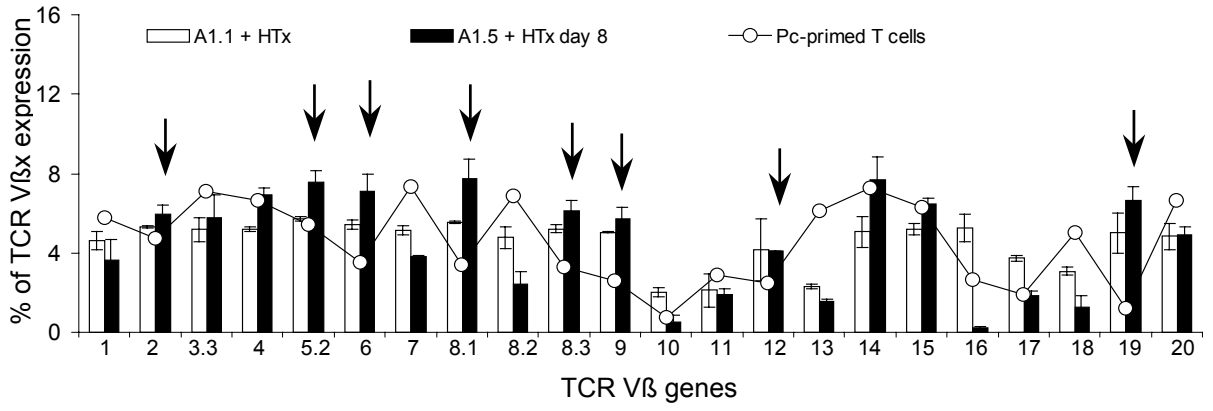
The next question to address was whether the TCR subpopulations involved in the allograft rejection are also seen in the graft-infiltrating lymphocytes (GILs) isolated from the analogue-immunized recipient. As shown in Figure 24 (A), TCR Vß 6, 8.1, 8.3, 9, 12, 16, 17 and 19 represented most of the Vß expressions in GILs of rejected grafts, when recipients were immunized with the agonist A1.1 (day 5) or analogue A1.5 (day 8) comparable to P1-immunized recipients. There were no differences between the TCR Vß usage of GILs isolated from A1.5-immunized animals on day 5 (non-rejected grafts) and day 8 (rejected grafts) (Figure 24 (B)). Thus, one may conclude that the immunmodulatory ability of the MHC competitor A1.5 does not involve the activation of a specific TCR subpopulation.

FIGURE 24. Comparative presentation of TCR Vß repertoires in GILs harvested on day 5 (A) and at rejection time (B). TCR Vß repertoire in the Pc-primed T cells was used as control. Bars represent mean (± SD) of duplicates from 2 animals per group. Arrows indicate the overexpressed TCR Vß in GILs from A1.1 (dashed) and A1.5-immunized (black) recipients at the time of rejection.

(A) Day 5



(B) Rejection time



Summary 4.14 – 4.17: The four immunogenic allopeptides activate characteristic TCR Vß subpopulations after *in vivo* immunization. In addition, the overexpression of some TCR Vß subpopulations in the GILs, isolated from the rejected grafts of P1 or A1.5-immunized recipients, correlates with the profile observed after repetitive specific *in vitro* stimulation of P1-primed T cells. Our finding correlates with data from some groups that described a diverse TCR Vß usage (Kato *et al.* 1996), in contrast to others, which report a restricted one (Shirwan *et al.* 1993). In addition, we found no correlation between the modulation of the alloimmune response through analogues such as A1.5 and a specific TCR Vß subpopulation.

5. DISCUSSION

The accessibility of sequence data for the mouse, rat, and human major histocompatibility complex (MHC) provides the opportunity to study the role of self-restricted T cell allorecognition in allograft rejection using synthetic MHC-derived peptides. In this study, we present evidence that the MHC class I allopeptides are important for the indirect pathway of allorecognition involved in allograft rejection.

5.1 The importance of donor MHC class I peptides in the induction of a T cell alloimmune response

The indirect pathway of allorecognition is dependent on the ability of recipient APCs to process and to present the donor MHC peptides that can be recognized as immunogenic by recipient T cells. Our finding that the synthetic MHC class I allopeptides are recognized by the $CD4^+$ T cells in the context of MHC class II molecules is consistent with the data from human and rodent experiments (Shirwan *et al.* 1995; Stegmann *et al.* 2000). We were able to demonstrate the central role attributed to CD4+ T cells by the resulting increase in the number of CD4+ CD25+ T cells in the regional lymph nodes after immunization with immunogenic peptide and secondly, by the lack of proliferation of peptide-primed T cells in the presence of anti-MHC class II and anti-CD4 antibodies. In addition, it has been recently demonstrated that the majority of alloreactive CD4+ T cells recognize MHC-bound peptides, thus, underlying the dependency of the alloimmune response on peptides (Mendiratta *et al.* 1999).

5.1.1 Hierarchical distribution of the immunogenicity of MHC class I allopeptides

An important feature of the indirect pathway is that the T cell response to an allopeptide may be limited to one or only a few dominant antigenic determinants, thus, focusing the T cell response to a selected number of allopeptides (Sercarz *et al.* 1993). The dominant determinants from the donor MHC molecules are efficiently processed and presented, and afterwards activate the alloreactive T cells during allograft rejection. While it is difficult to predict which particular donor MHC-derived peptide induces an alloreactive T cell response, the aim of this study was to screen

the WF MHC class I molecule (RT1.A^u) in order to ascertain the immunogenicity distribution within it. The seven synthetic RT1.A^u peptides (P1-P7) that span the α1 and α2 domains of the MHC class I molecule cover 29 of the 34 disparate amino acids (85% of the disparities). Five (P1-P5) of the 7 peptides tested were found to induce a specific T cell proliferation in LEW responders. In addition, it is a hierarchical distribution of the immunogenicity of allopeptides: peptide P1 is immunodominant, while P2, P3, P4, and P5 are the subdominant peptides, and P6 and P7 are the non-immunogenic peptides (Sitaru 2002). The presence of such dominant allopeptides is comparable to other studies using a series of overlapping MHC peptides in other rat strain combinations (Lovegrove *et al.* 2001) and in mice (Benichou *et al.* 1994). Furthermore, the immunodominance of P1 was confirmed by the induction of a strong proliferation of those T cells that were primed after immunization with a mixture of five peptides and by the high levels of cytokines (IL-2, IFN-γ) produced upon *in vitro* restimulation.

The hierarchical distribution of allopeptide immunogenicity within the RT1.A^u molecule shows a direct correlation with their location. The highly immunogenic peptides (P1, P2, and P3) are located on the α1 domain, covering the β-pleated sheet region, whereas the non-immunogenic peptides are located on the α2 domain. In the WF-ACI strain combination, the dominant RT1.A^u peptide is localized at the end of a α-helical region of the α2 domain (Chowdhury *et al.* 1998), while in the ACI-WF combination, the location of the dominant RT1.A^a peptide corresponds to the α-helical region of α1 domain. These data indicate the specificity of the immunodominant peptide for a certain donor-recipient MHC combination, but the location is indisputable within the hypervariable regions of donor MHC class I molecules.

In addition, it is well known that the MHC disparities between donor and recipient are responsible for the induction of the alloimmune response. Surprisingly, the immunogenicity appeared to be unconstrained by the number of differences between donor and recipient amino acid sequences. The peptides P4 and P6, in which 7 (of 22) and, respectively, 8 (of 24) amino acids are different, were the least immunogenic of the 7 peptides, as demonstrated by *in vitro* proliferation and cytokine production. In contrast, the high immunogenic allopeptides P1, P2, and P3 consist of only 3

allogeneic amino acids each. Therefore, the number of donor-recipient differences appears to play a negligible role in immunogenicity, although the type of these amino acids (the structure and the special characteristics of each amino acid) may be of great significance for the subsequent T cell recognition and activation.

5.1.2 Influences of MHC class I allopeptides on the allograft survival time

Since there is increasing evidence indicating the importance of allopeptides in mediating graft rejection, the immunogenicity of such peptides was further tested in a heterotopic heart transplantation model. Our finding that LEW (RT1.A^l) recipients immunized with immunogenic RT1.A^u allopeptides (P1, P3, respectively) 9 after the transfer of P1-primed T cells reject their allografts more rapidly compared to non-immunized animals is supported by studies in rodent transplantation models (Fangmann *et al.* 1992; van Denderen *et al.* 2001). Interestingly, immunization with the subdominant peptide P3 resulted in an accelerated heart graft rejection comparable to the dominant peptide P1 (see Figure 12); it is possible to suppose that in the allograft situation the subdominant determinants are also sufficient to activate the T cell alloimmune response and subsequently, to initiate the allograft rejection. In order to explain this observation, further investigations are required.

5.1.3 Influence of dominant allopeptide on the TCR Vß repertoire of alloreactive T cells

It has been shown that immunodominance leads to limitations in the TCR repertoire of alloreactive CD4+ T cells (reviewed by Douillard *et al.* 1999). Fasso demonstrated the early selection of TCR repertoire resulting in a relatively homogenous T cell population, a phenomenon known as clonal selection in the CD4+ compartment (Fasso *et al.* 2000). Different groups have studied the TCR Vß usage of T cells activated during *in vivo* and *in vitro* alloresponses in both animal models (Douillard *et al.* 1998; Guillet *et al.* 2002) and humans (Datema *et al.* 1994). We found almost the same TCR Vß profile after serial *in vitro* restimulation of P1-primed T cells and in freshly isolated GILs from rejected hearts. Since our *in vitro* conditions allow the indirect pathway of recognition to occur, the data demonstrate the implication of this pathway in the induction of acute rejection.

In summary, the WF MHC class I (RT1.A^u) peptide P1 is immunodominant due to the activation of recipient's alloimmune system, as confirmed by *in vitro* proliferation and

cytokine production of P1-primed T cells as well as by the reduction of allograft survival time in P1-immunized and/or P1-primed T cells transferred to LEW recipients ($RT1^l$).

5.2 Peptide analogues from the dominant allopeptide as a strategy to modulate the alloimmune response

As a step toward developing more efficient tools for antigen-specific suppression of the alloimmune response, it has been demonstrated that the activation of the alloreactive T cells induced by the dominant allopeptide can be altered by fine changes in the amino acid sequence. Data are available concerning the modulation of the T cell alloimmune response as a strategy to develop antigen-specific therapy, through the use of altered peptide ligands (APLs) or peptide analogues of an immunodominant antigen. Therapeutic APLs have been studied in more detail in experimental autoimmune diseases such as multiple sclerosis, and rheumatoid arthritis (Anderton 2001), and type 1 diabetes (Alleva *et al.* 2002), in which APLs are derived from the dominant epitopes of the autoantigens. These APLs were able to competitively inhibit the activation of native peptide-specific T cell clones. Based on the experimental data of EAE, there were initiated phase II clinical trials for the treatment of multiple sclerosis in humans (Bielekova *et al.* 2000).

In the previous set of experiments we have demonstrated that $RT1.A^u$ peptide P1 with three allogeneic positions (5, 9, and 10) is dominant in LEW ($RT1.A^l$) recipients. The next step was to design and characterize “recipient-adapted” analogues from P1. The analogues (A1.1-A1.6) were generated from P1 by changing the allogeneic $RT1.A^u$ amino acids (L5, L9, and T10) one by one with the corresponding syngeneic $RT1.A^l$ amino acids (M5, D9, and I10). Our results demonstrate that the analogues, consisting of either one or two allogeneic amino acids are still able to induce a specific T cell proliferative response. The proliferative capacity did not correlate well with the specific cytokine productions. For example, analogue A1.5 with only one allogeneic amino acid (L5) had a weaker specific proliferative capacity associated with higher levels of cytokines (IL-2, IFN-γ, IL-4, and IL-10) as compared to the wild-type peptide P1. By taking advantage of the *in vivo* model of transplantation, we have shown that the agonist A1.1 is able to strongly activate the rejection mechanisms

(like P1), while A1.5 seems to have only minimal influence (no reduction in allograft survival time).

We hypothesized that such substitutions in the dominant peptide are able to induce the activation of other TCR Vß subpopulations in comparison to the wild-type peptide. The TCR Vß repertoire analysis of T cells activated after immunization with the individual peptide analogues indicated the presence of several common TCR subpopulations (TCR Vß 6, 8.1, 8.3, 9, 16, and 19). Therefore, our initial hypothesis was not really sustained and the data indicate that such analogues maintain the ability to activate the wild-type peptide-specific TCR subpopulations.

Since the analogues show various T cell activation capacities, we then analyzed the specificity of the T cells activated after immunization with the wild-type peptide P1 for its analogues. Afterwards, the analogues were classified as follows: A1.1 and A1.6 as agonists (the same proliferation) and A1.2, A1.3, and A1.4 as weak agonist peptides (weaker proliferation). In contrast, analogue A1.5 was able to down-regulate the proliferation of P1-primed T cells.

The mechanisms responsible for this effect are still subject to investigation (Kersh *et al.* 1996). Therefore, we postulated that it is possible to induce a lasting negative signal through the TCR (anergy) or that it acts as a competitor for the MHC-binding site respectively. Since A1.5 did not influence the proliferation of P1-primed T cells when cells were pre-incubated with A1.5-pulsed DCs and then rechallenged with the P1-pulsed DCs, we conclude that it is unlikely that A1.5 induced a permanent negative signal through the TCR of P1-primed T cells. However, in an elegant study, using T cell clones from transplanted patients, it has been demonstrated that partial agonists could be used to induce unresponsiveness to the dominant MHC class I allopeptide (Frasca *et al.* 2000).

These results strongly support the idea that the down-regulation of the proliferation in P1-primed T cells depends on an early event of T cell activation, such as MHC presentation, rather than the induction of a general state of anergy or unresponsiveness. Thus, in a MHC competition assay, A1.5 appears to display higher MHC binding affinity than the wild-type peptide P1, inhibiting P1-primed T cell proliferation when presented on the same DCs, in equimolar amount.

Consistent with this *in vitro* ability of A1.5, we found an *in vivo* suppression of the early induction of allograft rejection when recipients were immunized with a mixture of A1.5 and P1 (allograft rejection as in non-immunized recipients). This effect could be explained by the competition for binding to the MHC molecule, which thus allows A1.5 to control the induction of allograft rejection. Similar observations have been previously reported for the experimental allergic encephalomyelitis (EAE) in a mouse model (Kumar *et al.* 1990). In this study, a doubly substituted analogue from the myelin basic protein (MBP) was described, which binds with high affinity to the MHC molecule and seems to be protective against EAE as well as effective in reversing established EAE.

In addition, our recent results indicated that recipients immunized separately in one footpad with one of the peptides P1 or A1.5 rejected their allograft similar to those immunized in both footpads with P1. In this situation, P1 had no competition for MHC binding and, therefore, induced an early graft rejection. Moreover, it has been suggested that the interaction with such altered peptide ligands can result in different phenotypes of the T cells, from selective stimulatory functions to a complete switch (immune deviation) in their function (Sloan-Lancaster *et al.* 1996). In order to acknowledge this, further investigations of the T cell functions after a rechallenge with analogues are required.

To summarize, our data show the possibility of investigating the fine specificity of dominant peptide P1 using peptide analogues. Our results indicate that the immunogenicity is dependent on the presence of the allogeneic position and furthermore, that there is no linear relationship between the number of the allogeneic positions and the immunogenic ability. Analogue A1.5, with only one allogeneic amino acid (L5) was found to be a good candidate for analyzing the modulation of P1-induced alloimmune response, because it induced the lowest proliferation associated with high cytokine production (IL-4 and IL-10) and, in addition, did not influence the allograft survival time. We demonstrated that A1.5 acts as a MHC competitor *in vitro* as well as *in vivo* and feel that further investigations are necessary to clarify the responsible mechanisms.

These observations emphasize the possibility that analogues or APLs from the dominant allopeptide represent a promising antigen-specific strategy to suppress the

alloimmune response responsible for the potential graft damages. Further studies should investigate the ability of such analogues not only to prevent the early activation of rejection mechanisms, but also to suppress the alloimmune response after transplantation resulting in a prolongation of the allograft survival time.

In conclusion, the immunosuppressive drugs will probably remain the backbone support system to suppress the alloimmune response, in combination with a more selective and specific therapy with minimal toxic side effects, such as the altered peptide ligands.

SUMMARY

Transplantation is now firmly established as a therapeutic approach to extend and improve the life of patients in the final stages of organ failure. It has been demonstrated that transplantation between genetically non-identical individuals leads to the activation of the recipient's alloimmune response as a major determinant of transplant outcome. T cell recognition of foreign MHC molecules plays a key role in initiating and sustaining allograft rejection. To prevent the risk of rejection, patients are given immunosuppressive drugs, which are non-specific and have major side-effects (infections, malignancies). It has been shown that the alloreactive T cells specifically recognize donor MHC-derived peptides. This implies that it may be possible to develop antigen-specific strategies in order to modulate the alloimmune response by peptide analogues and specifically altered peptide ligands.

The purpose of this study was to explore the potential of "recipient-adapted" analogues from the dominant MHC class I peptide to modulate the alloimmune response. Beside the significant role of donor dominant determinants in the rejection process, we tested seven 13-to-24-mer peptides from the Wistar-Furth MHC class I molecule (WF, RT1.A^u) for their possible immunogenicity in a fully MHC-mismatched WF to Lewis (LEW, RT1^l) rat strain combination. Secondly, the immunodominant allopeptide was selected to generate analogues in order to investigate their modulatory capacity. All peptides were tested *in vitro* in a standard proliferation assay and *in vivo* using a heterotopic heart transplantation model.

Our findings show that five peptides (P1-P5) were able to induce specific T cell proliferation in LEW responders. Furthermore, we found a hierarchical distribution of the determinants: peptide P1 as a good candidate for the immunodominant determinant, while P2, P3, P4, and P5 as subdominant epitopes and the other two peptides, P6 and P7, as non-immunogenic determinants of WF MHC class I molecule. Furthermore, the dominance of P1 was confirmed by the strong proliferation induced after immunization with a mixture of peptides in the presence of P1. This hierarchical distribution of the proliferative response correlated with the cytokine production. Peptide P1, comprising only 3 allogeneic amino acids (L5, L9,

and T10) induced the strongest T cell proliferation and produced high levels of cytokines, especially IL-2 and IFN-γ. In addition, the immunodominance of peptide P1 was confirmed by the significant reduction in the allograft survival time in comparison to the non-immunized control animals. Since the TCR Vß repertoire of rejected graft-infiltrating cells in rejected allografts was similar to the profile observed after *in vitro* restimulation of P1-primed T cells, we concluded that peptide P1 is able to activate the alloreactive T cell population. Our results demonstrate the particular role of the dominant peptide P1 (residues 1-19) in the allograft rejection in WF to LEW rat strain combination.

In the second set of experiments, we investigated the fine specificity of the dominant peptide P1-activated T cells using peptide analogues from P1. The "recipient-adapted" analogues were designed by changing the allogeneic RT1.A^u amino acids (L5, L9, T10) one-by-one with the correspondent syngeneic RT1.A^l amino acids (M5, D9, I10) in the sequence of peptide P1. The six peptide analogues (A1.1-A1.6) consisting of either one or two allogeneic amino acids were able to induce a specific T cell proliferative response and cytokine production. Analogue A1.5 with only one allogeneic amino acid (L5) was of particular interest because it induced a low T cell proliferation and high cytokine levels, especially IL-4 and IL-10. In addition, immunization with A1.5 did not influence the allograft survival time in comparison to the non-immunized LEW recipients. A1.5 was the only analogue able to down-regulate the proliferation of P1-primed T cells. Our results reveal that A1.5 is an MHC competitor as confirmed by the *in vitro* MHC competition assay and the inhibition of the negative effect of P1 on the allograft survival time when recipients were immunized with a mixture of P1 and A1.5.

These findings suggest that it is possible to design peptide analogues, such as A1.5, which do not stimulate the dominant peptide P1-specific T cell population and even more, are able to block its presentation in the MHC molecule.

In all, the results indicate that the specific suppression of indirect allorecognition can be achieved by using peptide analogues of the dominant allopeptide.

ZUSAMMENFASSUNG

Ursache der Transplantatabstoßung ist in erster Linie die genetische Differenz im Haupthistokompatibilitätskomplex (MHC) zwischen Transplantatspender und Empfänger. Dabei stellen die aus den Fremd-MHC-Molekülen durch empfänger-eigene antigenpräsentierende Zellen prozessierten MHC-Peptide einen wichtigen Stimulus zur Aktivierung alloreaktiver T-Lymphozyten des Transplantat empfängers dar. Für die Transplantation bedeutsam ist, dass sowohl diese, als auch synthetische MHC-Peptide, wenn sie die genetische Differenz zwischen einer bestimmten Spender-Empfänger-Kombination repräsentieren, die Alloimmunantwort induzieren und damit die Abstoßung fördern.

Das Ziel dieser Arbeit war, dass bereits in zahlreichen Experimentalmodellen für Autoimmunerkrankungen erfolgreiche Konzept der antigenspezifischen Immuntherapie mit Peptidvarianten oder *altered peptide ligands* auf die Transplantation zu übertragen. Anders als bei Autoimmunerkrankungen basiert die Alloimmunantwort aber nicht auf ein einyelnes Peptidantigen und darüber hinaus beeinflußt die jeweilige Spender-Empfänger-Kombination sehr stark dieses Peptidantigen-Repertoire.

Um die Frage zu klären, welche der potentiellen MHC-Peptidantigene in der Alloimmunaktivierung dominieren, wurden Untersuchungen im Nagermodell für die allogene Spender-Empfänger-Kombination Wistar-Furth (WF, $RT1^u$) und Lewis (LEW, $RT1^l$) durchgeführt. Für die Transplantation wird erwartet, dass solche gezielt hergestellten Peptidvarianten sowohl die Aktivierung alloreaktiver T-Lymphozyten als auch weitere Funktionen, wie z. B. die Produktion von Cytokinen, hemmen. Dieser antigenspezifische, und wahrscheinlich auch nebenwirkungsfreie Therapieansatz könnte möglicherweise zur langfristigen Erhaltung der Transplantatfunktion führen. Durch Vergleich der Sequenzen für das MHC-Klasse-I Molekül beider Ratten-Stämme wurden für die $\alpha 1$ und $\alpha 2$ Domäne - dies ist der extrazelluläre, für die Bindung von Peptiden unterschiedlichster Herkunft verantwortliche Bereich des Moleküls - insgesamt 34 Positionen identifiziert, in denen beide Stämme unterschiedliche Aminosäuren aufweisen. Diese Differenzen werden von sieben synthetischen, mit den entsprechenden Bereichen des Spender MHC-Klasse-I

Moleküls identischen MHC-Peptiden repräsentiert, welche zwischen 13 und 24 Aminosäuren lang sind.

Die immunstimulierende Wirkung dieser Peptide (P1 bis P7) wurde im Proliferationsassay und im Transplantationsmodell bestimmt. Ausschließlich das Peptid mit der Bezeichnung P1 induzierte mit über 20.000 cpm die stärkste, mit einen Th1-dominierten Cytokinmuster (IL-2 und IFN-γ) einhergehende T-Zellproliferation. Lewis-Empfänger, die vor der Transplantation mit diesem Peptid immunisiert wurden, stießen ihre von WF-Spendern stammenden heterotopen Herztransplantate beschleunigt ab. Von sieben potentiellen Peptidantigenen induzierte somit ausschließlich Peptid P1 eine dominante Alloimmunaktivierung und erscheint als Peptidantigen zur Herstellung von Peptidvarianten prädestiniert.

P1 weicht in drei Aminosäurepositionen von der entsprechenden Sequenz der Lewis-Ratte ab. Durch sequentiellen Austausch dieser drei in der WF-Sequenz befindlichen *allogenen* Aminosäuren durch die entsprechenden *syngenen* Aminosäuren in der LEW-Sequenz führte zu sechs Peptidvarianten. Diese *Empfänger-angepassten* Varianten A1.1 bis A1.6 wurden anschließend auf ihre Fähigkeit untersucht, eine peptidspezifische T-Zellproliferation zu inhibieren, die möglicherweise mit einer protektiven Wirkung auf die Transplantatfunktion einhergeht. Von diesen Peptidvarianten induzierte nur Variante A1.5, sie besitzt noch eine allogene Aminosäure an Position 5, eine reduzierte T-Zellproliferation von 11.450 cpm, die mit einem Th2-dominierten Cytokinmuster (IL-4 und IL-10) korreliert. Zusätzlich hemmte A1.5 die Proliferation der P1-spezifischen T-Lymphozyten. Im Gegensatz zum Ausgangs-peptid P1 beeinflußte A1.5 nicht die Abstoßung heterotoper Herztransplantate und konnte, wurde es in Kombination mit P1 appliziert, die P1-induzierte Transplantatabstoßung verzögern.

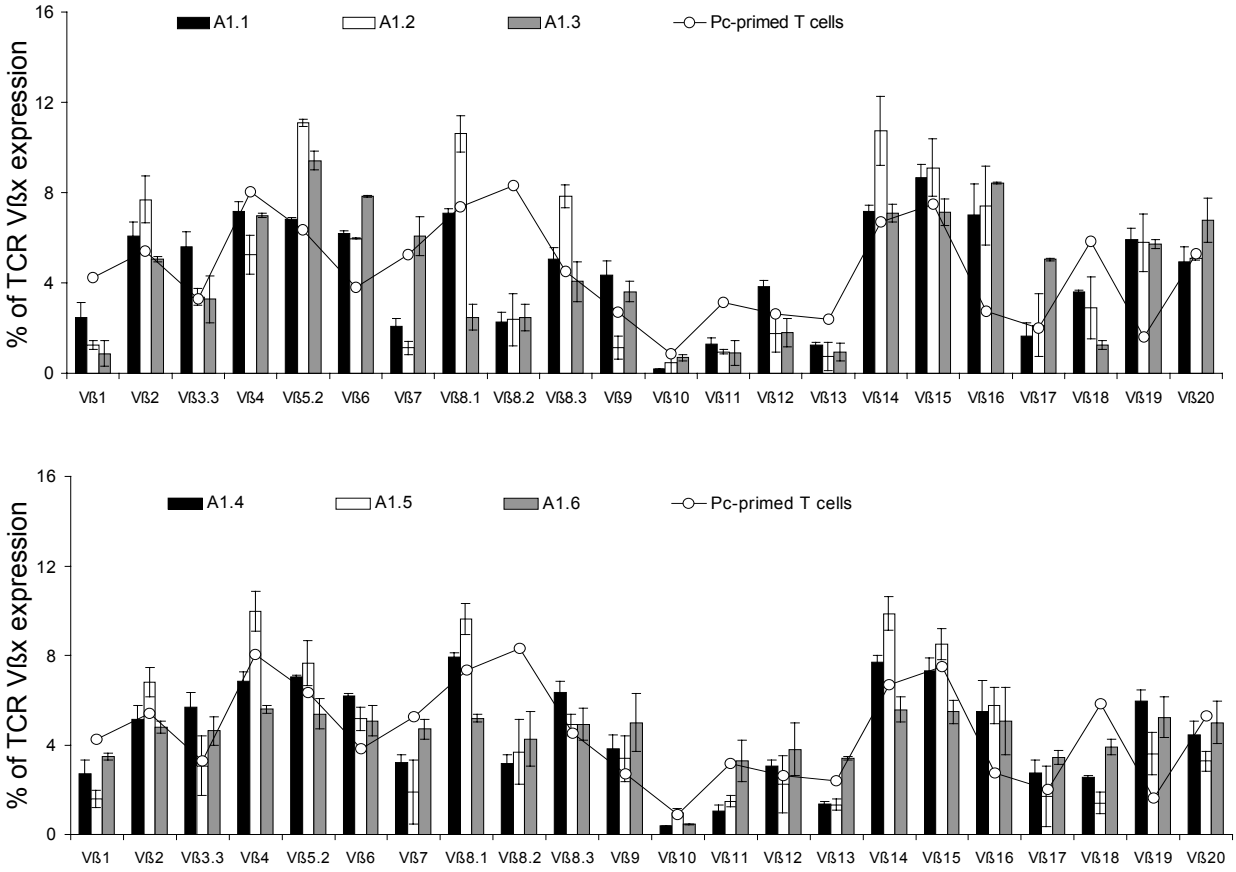
Um diese immunmodulatorische Fähigkeit der Variante A1.5 weiter zu untersuchen, wurde das Peptid in einem T-Zellrezeptor-Modulationsassay sowie in einem MHC-Kompetitionsassay getestet. Die Ergebnisse zeigten, dass A1.5 nicht die T-Zellproliferation über den T-Zellrezeptor inhibiert, sondern über die verstärkte Bindung an das MHC-Klasse-II Molekül, wodurch das Peptid P1 wahrscheinlich aus der Bindungstasche verdrängt wird. Dieses Ergebnis wurde von weiteren *in vivo* Daten unterstützt. Wurden beide Peptide getrennt und nicht im Gemisch appliziert,

konnte A1.5 die abstoßungsinduzierende Wirkung von P1 nicht mehr kompensieren, und das Transplantat wurde zum gleichen Zeitpunkt nach Transplantation abgestoßen wie in P1-immunisierten Tieren.

Die Peptidvariante A1.5 wirkt somit als MHC-Kompetitor und verhindert die P1-induzierte Aktivierung alloreaktiver T-Lymphozyten. Die Ergebnisse zeigen, dass die gezielte Herstellung von Varianten aus dem immundominanten MHC-Klasse-I Peptid für eine bestimmte Spender-Empfänger-Kombination somit eine mögliche Perspektive zur antigenspezifischen Hemmung der Transplantatabstoßung darstellt.

APPENDIX

TCR Vß repertoire of T cells primed after *in vivo* immunization with "recipient-adapted" peptide analogues (A1.1-A1.6), in comparison to Pc-primed T cells as control.



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